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Lipid-mediated mode of action of local anesthetics on lipid pores induced by polyenes, peptides and lipopeptides

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ABSTRACT

The effects of local anesthetics (LAs), namely, lidocaine (LDC), prilocaine (PLC), mepivacaine (MPV), bupivacaine (BPV), procaine (PC), and tetracaine (TTC), on the steady-state transmembrane conductance induced by the *cis*-side addition of the antifungal polyene macrolide antibiotic, nystatin (NYS), in planar lipid bilayers were studied. The addition of TTC to model membranes comprising DOPC and cholesterol (33 mol%) led to a nearly twenty-fold increase in the steady-state NYS-induced membrane conductance. BPV slightly enhanced the channel-forming activity of polyene. LDC, PLC, MPV, and PC did not affect the NYS-induced transmembrane current. We concluded that the effects of LAs on the channel-forming activity of NYS were in agreement with their effects on the elastic properties of model membranes. The ability of aminoamide LAs to promote calcein leakage from large unilamellar DOPC-vesicles was decreased in the following order: BPV » LDC ≈ PLC ≈ MPV. LDC, PLC, and MPV produced a graded leakage of fluorescent marker from liposomes, up to 10–13%. A initial sharp jump in fluorescence after the introduction of BPV was attributed to the solubilization of liposomes and the formation of mixed DOPC:BPV-micelles. Differential scanning microcalorimetry (DSC) of large unilamellar DPPC-vesicles showed that the main transition temperature (*Tm*) is continuously decreased upon increasing concentrations of TTC. A sharp drop in the enthalpy of the transition at higher TTC concentrations indicated a formation of anesthetic/lipid mixed micelles. In contrast to TTC, PC slightly decreased *Tm*, broadened the DSC signal and did not provoke vesicle-to-micelle transition. Both the calcein leakage and DSC data together with the results of measurements of threshold voltages that are required to cause the lipid bilayer breakdown might indicate an alteration in the curvature lipid packing stress, induced by BPV and TTC. The data presented here lend support to a lipid-mediated mode of LAs action on NYS pores via an alteration in curvature stress near the *trans*-mouth. Similar results were obtained for several lipid pores, formed by polyene amphotericin B, lipopeptide syringomycin E, and the peptides magainin and melittin. This finding further developed the concept of non-specific regulation of lipid pores by LAs. In conclusion, the combination of nystatin with LAs could be a novel treatment for efficient therapy of superficial and mucosal candidiasis.

1. Introduction

Nystatin (NYS) and amphotericin B (AMB) are the most well-known polyene macrolide antibiotics used to treat human fungal infections [1–3]. Polyenes form weakly selective ion permeable pores in target pathogen cell membranes that lead to leakage of K^+ and other small metabolites and, as a consequence, to the death of the fungus [2,4]. The high affinity of polyene macrolides to membrane sterols and their role in the antifungal activity of the antibiotics have been discussed for decades [2,5,6]. Besides increasing the membrane permeability, polyenes are able to immobilize the lipids and to promote the formation of ordered domains [7–9] that might affect a number of biochemical processes in the living cells [6]. Behavior of polyenes in the lipid bilayer might be altered by its mesoscopic organization. Lipid domains enriched with sterols plays a pivotal role in polyene functioning [10,11]. The affinity of macrolides to phosphocholine liposomes nonmonotonically changes up to 5 times in the range of 20–40 mol% of ergosterol in the membrane [12]. There is also the difference between the action of the antibiotics in cholesterol- and ergosterol-containing membranes, e.g. the higher activity of the antibiotics in ergosterol-enriched membranes vs. cholesterol-enriched ones, a different localization of polyenes in the domains including cholesterol and ergosterol, alteration in properties of ion channels induced by polyenes [2,13–16]. Polyenes ion channels are

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localized predominantly in the boundary region of the ordered lipid domains in the ergosterol-enriched membranes but not in the cholesterol-enriched ones [10]. Single-length polyene channels used to show weak cation selectivity, low conductance and may be activated by the positive voltages [17,18]. Also the channels in the ergosterol-containing membranes are more stable than in cholesterol-enriched ones [17,19].

The higher affinity of polyenes to the ergosterol compare to cholesterol determines the antibiotic activity against most of the pathogenic fungi. At the same time the interaction of the polyenes with the cholesterol-enriched mammalian membranes implies the toxic influence on the human cells [20]. Therefore, polyene pharmacological applications are limited by serious dose-dependent side effects [3,20,21]. Combined use of this polyene macrolide with low molecular weight synergists is a promising way to improve macrolides therapeutic efficacy. Recently, Chulkov and Ostroumova [22] showed that the introduction of some flavonoids strongly enhances the channel-forming activity of antibiotic. The authors have hypothesized that these flavonoids affect curvature stress near the lipid mouth of single-length polyene pores.

The lateral pressure profile of a bilayer can be modified by the addition of local anesthetics (LAs) [23,24]. This could have irreversible consequences for membrane-embedded ion channels [25,26]. Despite the long history of LA use, much remains unknown about the mechanisms behind the therapeutic efficacy of these drugs. In general, it is commonly accepted that agents of the "caine" family inhibit surges in action potentials by sodium channels in the peripheral nerves. A clear correlation between pharmacological potency and LA hydrophobicity suggests that binding to the membrane is, at the very least, an important intermediate step toward specific interactions with protein targets. It has been shown that anesthetics can fluidize the membrane [27]. Although the nature of this "fluidization" has not been clearly identified, it could be related to expansion of the membrane or to the disordering of phospholipids in the membrane bilayer. The role of the membrane matrix in regulating anesthetic activity has been intensively investigated. Cafiso showed a correlation between the activity of general anesthetics and the membrane dipole potential [28].

The lipid environment of ion channels can affect their activity, via electrical or mechanical changes in the bilayer. This is shown for gramicidin channels [29]. The packing density of the lipid bilayer enhances the electrical activity of pores created by HPA3 peptides [30]. Apetrei et al. showed that modifying the electrical and mechanical properties of membrane RH 421 styryl dye may induce the pore-forming activity of antimicrobial peptides in reconstituted planar bilayers [31]. In addition, external amphiphile agent-mediated changes in the physicochemical properties of membrane may easily affect the channel-forming activity of the alamethicin peptide [32].

Here, we provide evidence of a critical role for LA-induced changes in the elastic properties of lipid bilayers for polyene channel-forming activity. We also expand this concept to other lipid pores, formed by various peptides and lipopeptides.

2. Materials and methods

All chemicals were of reagent grade. Synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), ergosterol, and cholesterol (Chol) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Lidocaine (LDC), bupivacaine (BPV), prilocaine (PLC), mepivacaine (MPV), procaine (PC), and tetracaine (TTC) hydrochlorides, calcein, Sephadex G-75, Triton X-100 (TX-100), EDTA, NaCl, KCl, HEPES, DMSO, nystatin (NYS), amphotericin B (AMB), magainin I, melittin, and Phospholipids Assay Kit were purchased from Sigma Chemical (St. Louis, MO). Syringomycin E (SRE) was isolated and purified as described previously [33] and was kindly offered by Dr. J.Y. Takemoto (Utah State University, USA). Water was distilled twice, deionized and degassed.

2.1. Planar lipid bilayers

Virtually solvent-free planar lipid bilayers were prepared using a monolayer-opposition technique [34] on a 50-μm-diameter aperture in a 10-μm-thick Teflon film separating two (*cis* and *trans*) compartments of a Teflon chamber. Lipid bilayers were made from a mixture of 67 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 33 mol% Chol (DOPC:Chol) from stock solutions in pentane (2 mg/ml). Solutions of 2.0 M KCl were buffered using 5 mM Hepes-KOH at pH 7.4. After the membrane was completely formed and stabilized, NYS, AMB, SRE, magainin I, and melittin from stock solutions (in DMSO, ethanol or water) were added to the aqueous phase on the *cis*-side of the bilayer, to obtain final concentrations ranging from 40 to 60 μM of NYS, 20–60 μM of AMB, 5–20 μM of SRE, 1–3 μM of magainin I and 35–55 nM of melittin. The ratio of channel-forming agent to lipid was approximately 1. LAs in mM water or ethanol stock solutions were added to both sides of the membrane at a final concentration of 1 mM. Ag/AgCl electrodes with 1.5% agarose/2 M KCl bridges were used to apply the transmembrane voltage (V) and measure the transmembrane current (I). "Positive voltage" refers to the case in which the *cis*-side compartment is positive with respect to the *trans*-side. Current measurements were carried out using an Axopatch 200 B amplifier (Molecular Devices, LLC, Orlean, CA, USA) in the voltage clamp mode. Data were digitized by Digidata 1440A and analyzed using pClamp 10 (Molecular Devices, LLC, Orlean, CA, USA) and Origin 7.0 (OriginLab Corporation, Northampton, MA, USA). Data acquisition was performed with a 5 kHz sampling frequency and low-pass filtering at 200 Hz. The current tracks were processed through an 8-pole Bessel 100 kHz filter. All experiments were performed at room temperature.

The peptides melittin and magainin have easily recognizable pore-forming activity in a DOPC sterol-free membrane [31], while the presence of Chol in the bilayer is essential for pore-forming activity by polyenes [17]. The channel-forming activity of NYS, AMB, SRE, magainin I, and melittin in the absence and after the introduction of the LAs was characterized by a steady-state membrane conductance (G) under the given experimental conditions (V was equal to 50 mV for NYS and AMB, −50 mV for SRE and melittin, and −100 mV for magainin I). Mean ratios ($G_{LA}/G_{control}$) of steady-state membrane conductance induced by NYS, AMB, SRE, magainin I or melittin in the presence (G_{LA}) and in the absence of LA ($G_{control}$) were averaged from 3 to 9 bilayer samples (mean ± SD). The average amplitude of current through single-length polyene channel (I_{sc}) was roughly estimated from a stationary noise analysis of the multichannel transmembrane current as a ratio of the mean square variance of the current (ΔI) to the mean current ($<I>$): $I_{sc} = \Delta I^2/<I>$ [35]. The registration of step-like fluctuations related to single AMB channels was performed in POPC:Chol (67:33 mol%) bilayers bathed in 2 mM KCl (5 mM Hepes-KOH, pH 7.4) in the absence and presence of 1 mM PC or TTC.

The threshold voltages that cause DOPC membrane breakdown before and after adsorption of 1 mM LAs, V_{bd}, were measured using triangle-shaped ramps (±10 mV/s) in the range of 0 to $\pm V_{bd}$. No difference between positive and negative voltages was observed.

2.2. Fluorescence assay to measure dipole potential changes

The LA-induced changes in membrane dipole potential were measured using a fluorescent lipid probe, di-8-ANEPPS [36]. Large unilamellar vesicles from a mixture of 67 mol% DOPC and 33 mol%