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Selective permeabilization of lipid membranes by photodynamic action via formation of hydrophobic defects or pre-pores

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ABSTRACT

To gain insight into mechanisms of photodynamic modification of biological membranes, we studied an impact of visible light in combination with a photosensitizer on translocation of various substances across artificial (vesicular and planar) bilayer lipid membranes (BLMs). Along with induction of carboxyfluorescein leakage from liposomes, pronounced stimulation of lipid flip-flop between the two monolayers was found after photosensitization, both processes being prevented by the singlet oxygen quencher sodium azide. On the contrary, no enhancement of potassium chloride efflux from liposomes was detected by conductometry under these conditions. Illumination of planar BLMs in the presence of a photosensitizer led to a marked increase in membrane permeability to amphiphilic 2-*n*-octylmalonic acid, but practically no change in the permeability to ammonia, which agreed with selective character of the photosensitized leakage of fluorescent dyes from liposomes (Pashkovskaya et al., Langmuir, 2010). Thus, the effect on transbilayer movement of molecules elicited by the photodynamic treatment substantially depended on the kind of translocated species, in particular, on their lipophilicity. Based on similarity with results of previous electroporation studies, we hypothesized about photodynamic induction of "pre-pores" or "hydrophobic defects" permeable to amphiphilic compounds and less permeable to hydrophilic substances and inorganic ions.

1. Introduction

Photosensitized damage to cellular membranes mediated by reactive oxygen species [1] is one of the key processes leading to cell killing upon photodynamic treatment. Aimed at elucidating mechanistic details of this process, numerous attempts were made to uncover them in pure lipid membrane systems simulating biological membranes, i.e. planar bilayer lipid membranes (BLMs) and unilamellar lipid vesicles (liposomes).

Exposure of planar BLMs either to UV light solely [2] or to visible light in the presence of an added photosensitizer [3–5] caused an increase in transmembrane ionic current ultimately leading to membrane rupture which required the presence of unsaturated lipids in the membrane composition [6]. In addition, products of peroxidation of unsaturated fatty acids stimulated ion transport through planar BLMs [7,8]. Parallelly, photodynamic treatment of liposomes was shown to provoke their destabilization [9–16], manifesting itself, in particular, in liposomal leakage of glucose (or glucose-6-phosphate) [9,11,16–18] and fluorescent dyes such as carboxyfluorescein (CF) [19,20] and sulforhodamine B [21]. Beginning from the earliest studies on the subject [9–12,14], singlet oxygen has been implied to participate in photosensitized permeabilization of liposomes. According to a large body of evidence [12,17,20,22], the presence of double bonds is a prerequisite for this process to occur. Both chemically and UV-induced lipid peroxidation also resulted in glucose [23–26] and carboxyfluorescein [27–29] leakage from liposomes.

Rather contradictory data were obtained while examining changes in ionic permeability of liposomes associated with lipid peroxidation. In [11], sodium cation efflux correlated with very slow (in the scale of hours) glucose-6-phosphate leakage from liposomes observed after visible light irradiation in the presence of a photosensitizer. According to [30], chemically induced lipid peroxidation elicited slow efflux of calcium cations from liposomes. On the other hand, cation loss did not correlate with lipid peroxidation both for sodium [31] and calcium cation efflux from liposomes [32,33]. No induction of potassium cation efflux was observed upon lipid peroxidation induced by UV illumination [34].

To elucidate the nature of the photodynamic effect on lipid bilayer permeability, we employed here a series of techniques for measuring transmembrane movement of compounds differing in lipophilicity. To explain the results obtained, we addressed the ideas of formation and evolution of hydrophobic and hydrophilic pores previously put forward to account for permeability changes occurring upon electroporation and lipid phase transition [35–43]. Ultimately, a new mechanism involving

Abbreviations: BLM, bilayer lipid membrane; DPhPC, diphytanoylphosphatidylcholine; eggPC, egg yolk phosphatidylcholine; Chol, cholesterol; TTFB, tetrachlorotrifluoromethylbenzeimidazole; α,α-DC11, 2-*n*-octylmalonic acid; CF, 5(6)-carboxyfluorescein; pyPC, 1-lauroyl-2-(1′pyrenebutyroyl)-sn-glycero-3-phosphocholine; $AlPcS_3$, aluminum trisulfophthalocyanine; USL, unstirred layer

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hydrophobic pore formation was suggested to underlie the process of photodynamic membrane permeabilization.

2. Materials and methods

Egg yolk phosphatidylcholine (eggPC), diphytanoylphosphatidylcholine (DPhPC), and cholesterol (Chol) were from Avanti polar lipids (Alabaster, Alabama). 5(6)-carboxyfluorescein (CF) and calcein were from Sigma. Aluminum trisulfophthalocyanine ($AlPcS_3$) and chlorin e6 were from Porphyrin Products (Logan, UT). 1-lauroyl-2-(1′pyrenebutyroyl)-sn-glycero-3-phosphocholine (pyPC) was prepared by Dr. Sergei Kovalchuk from Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences as described in [44]. 2-*n*-octylmalonic acid (α,α-DC11) was a generous gift of Dr. D. Bondarenko from the A.N. Bakh Institute of Biochemistry, Russian Academy of Science; it was prepared by alkylation of diethyl malonate with n-undecylbromide [45]. The protonophore tetrachlorotrifluoromethylbenzeimidazole (TTFB) was a gift of Prof. Lev Yaguzhinsky (Moscow State University).

Liposomes loaded with CF or calcein were prepared from eggPC and cholesterol (10 mg and 3 mg) in 1 ml solution containing 100 mM CF or 70 mM calcein titrated with Tris-base by extrusion through 100-nm filter (Avanti Mini-Extruder). The unloaded dye was then removed by passage through a Sephadex G-50 coarse column using 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.0 as eluting buffer. To measure the rate of CF or calcein efflux, the liposomes were diluted to the final concentration of 5 μg/ml in the same buffer and the fluorescence at 520 nm (excitation at 490 nm) was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). Photosensitizers were added from 1-mM stock water solutions to liposomes diluted in the buffer solution prior to illumination. At the end of each recording, 0.1% Triton-X100 was added to complete the efflux process. Illumination of samples was performed with a xenon lamp with intensity of 0.4 W/cm^2. To prevent photobleaching of fluorescent dyes, the KS-10 red pass filter (transmission band from 600 to 2800 nm, 90% transmission at $\lambda > 600$ nm) was used.

Liposomes loaded with KCl were prepared from eggPC and cholesterol (10 mg and 3 mg) in 1 ml solution containing 500 mM KCl by extrusion through 100-nm filter (Avanti Mini-Extruder). The potassium chloride was then removed by passage through a Sephadex G-50 coarse column using a solution of 1 M urea. To measure the rate of KCl efflux, the liposomes were diluted to the final concentration of 300 μg/ml in the same solution and the conductivity was monitored with Ekspert 002 conductometer (Econics, Russia).

The measurement of lipid flip-flop was performed as described by Müller et al. [46]. To label eggPC/Chol vesicles with synthetic lipid probe pyPC (1-lauroyl-2-(1′pyrenebutyroyl)-sn-glycero-3-phosphocholine) exclusively on the outer leaflet, 5 μM pyPC dissolved in ethanol was added to the buffer solution containing liposomes (final lipid concentration 50 μg/ml). Incorporation of pyPC into the outer membrane leaflet was followed by measuring the ratio of fluorescence intensities of monomers and excimers at 395 and 480 nm, respectively. The fluorescence was excited at 344 nm.

The permeation of weak acids and bases through a planar bilayer lipid membrane (BLM) was measured by a previously developed technique based on monitoring steady-state pH shifts in the unstirred layers (USLs) near the BLM [45,47–50]. Planar BLMs were formed on a 0.55-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 100 mM KCl, 1 mM MES, 1 mM Tris at pH 6.3. The membrane-forming solutions contained 10 mg eggPC and 3 mg cholesterol in 1 ml n-decane. Control experiments were done with membranes made of 10 mg diphytanoylphosphatidylcholine (DPhPC) in 1 ml n-decane. Gradients of pH on the BLM (ΔpHs) were measured according to [45,47–50] by the method of recording an open-circuit potential in the presence of a protonophore (10 μM TTFB) which was added at both sides of the BLM. The open-circuit BLM potentials were recorded with the help of Keithley 301 amplifier, digitized by a LabPC 1200 (National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). The electrical current (I) was measured with a Keithley 428 amplifier. Ag–AgCl electrodes were connected to membrane-bathing solutions via agar–agar bridges filled with 100 mM KCl.

3. Results and discussion

It was found in our previous work [20] that photodynamic treatment (i.e. illumination in the presence of a photosensitizer) of liposome membranes containing unsaturated lipids caused selective perturbation of their barrier properties, so that the resulting leakage from liposomes varied for different markers: for example, there was pronounced leakage of CF, whereas calcein leakage was negligibly small, which agreed with the absence of photosensitized leakage of calcein from eggPC liposomes found in [51]. To probe the dependence of this effect on membrane composition, we performed similar experiments with liposomes formed of eggPC/Chol mixture (77/23 w/w%), bearing in mind that cholesterol substantially impedes lipid bilayer permeability for nonelectrolyte compounds [52,53]. EggPC/Chol liposomes subjected to illumination in the presence of a photosensitizer displayed substantial leakage with CF as a marker (Fig. 1A, B), but not with calcein (Fig. 1C, D). Similar to the leakage pattern observed previously without cholesterol [20], the magnitude of the CF leakage depended on the photosensitizer: chlorin e6 proved to be much more effective than $AlPcS_3$ (Fig. 1B). Taking into account the two-fold difference between calcein and CF in the molecular mass and the number of carboxylic groups (calcein having 4, while CF — only 2), we supposed previously [20] that the selectivity of the photosensitized leakage of liposomes is associated with formation of membrane pores of certain size, which are permeable for CF, but impermeable for calcein.

Here, we decided to test this hypothesis by measuring ionic leakage from liposomes, as it was reasonable to expect that CF-permeable pores would be highly permeable to small inorganic ions like potassium cation and chloride anion. To measure KCl leakage by conductometry, we placed eggPC/Chol liposomes loaded with 0.5 M KCl into KCl-depleted buffer solution. Fig. 2 displays typical time courses of electric conductance of such samples after illumination in the presence of chlorin e6. It is seen that the photodynamic treatment produced practically no effect on the KCl conductivity, except for a slight increase during the very period of light exposure. Similar results were obtained when using $AlPcS_3$ as a photosensitizer (the data not shown). Therefore, the hypothesis of certain-sized pores is not compatible with the conductometry data. An alternative reason of selective photosensitized leakage of CF as compared to calcein and KCl may lie in their diverse lipophilicity, with potassium and chloride ions being the most hydrophilic species and calcein having less lipophilicity than carboxyfluorescein due to a larger number of negative charges.

Assuming the variation in the observed degree of oxidatively induced membrane permeabilization to be associated with lipophilicity of markers, it was reasonably to expect the largest effect of the photodynamic treatment on the translocation of compounds across lipid bilayers to be found in the case of transmembrane movement of labeled lipids, i.e. flip-flop. Here we monitored lipid flip-flop by tracking the evolution of the ratio of excimer/monomer fluorescence intensities of pyrene-labeled phosphatidylcholine (pyPC) [46]. If added to water solution, pyPC inserts solely into an outer monolayer of a liposome membrane, and induction of flip-flop leads to redistribution of the labeled lipid between the two monolayers, manifesting itself in a reduction of the I_E/I_M ratio due to a decrease in a local pyPC concentration [46]. Fig. 3 shows time courses of pyPC flip-flop initiated by photodynamic treatment of liposomes. A rather fast (on a minute scale) drop (Fig. 3A, black curves) of the I_E/I_M ratio was recorded after exposure of eggPC/Chol liposomes to red light even in the presence of a low concentration of $AlPcS_3$, whereas practically no change of the I_E/I_M ratio was observed after similar treatment of liposomes formed from the saturated lipid DPhPC (Fig. 3A,