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Peroxidative permeabilization of liposomes induced by cytochrome *c*/cardiolipin complex

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ABSTRACT

Interaction of cytochrome *c* with mitochondrial cardiolipin converting this electron transfer protein into peroxidase is accepted to play an essential role in apoptosis. Cytochrome *c*/cardiolipin peroxidase activity was found here to cause leakage of carboxyfluorescein, sulforhodamine B and 3-kDa (but not 10-kDa) fluorescent dextran from liposomes. A marked decrease in the amplitude of the autocorrelation function was detected with a fluorescence correlation spectroscopy setup upon incubation of dye-loaded cardiolipin-containing liposomes with cytochrome *c* and H_2O_2, thereby showing release of fluorescent markers from liposomes. The cytochrome *c*/H_2O_2-induced liposome leakage was suppressed upon increasing the ionic strength, in contrast to the leakage provoked by Fe/ascorbate, suggesting that the binding of cyt *c* to negatively-charged membranes was required for the permeabilization process. The cyt *c*/H_2O_2-induced liposome leakage was abolished by cyanide presumably competing with H_2O_2 for coordination with the central iron atom of the heme in cyt *c*. The cytochrome *c*/H_2O_2 permeabilization activity was substantially diminished by antioxidants (trolox, butylhydroxytoluene and quercetin) and was precluded if fully saturated tetramyristoyl-cardiolipin was substituted for bovine heart cardiolipin. These data favor the involvement of oxidized cardiolipin molecules in membrane permeabilization resulting from cytochrome *c*/cardiolipin peroxidase activity. In agreement with previous observations, high concentrations of cyt *c* induced liposome leakage in the absence of H_2O_2, however this process was not sensitive to antioxidants and cyanide suggesting direct membrane poration by the protein without the involvement of lipid peroxidation.

1. Introduction

Peroxidation of membrane lipids [1–3] has long been considered as one of the harmful consequences of oxidative stress in living cells, manifesting itself, in particular, in perturbing the barrier function of cell membranes. In support of this common idea, experiments with model lipid membranes – planar bilayers and liposomes – have shown that lipid peroxidation results in augmented membrane permeability [4–17]. On the other hand, mechanistic studies of apoptosis have pointed to a key role of cardiolipin peroxidation in the process of cytochrome *c* (cyt *c*) release from mitochondria into cytosol [18–21]. Intriguingly, cardiolipin peroxidation has appeared to be catalyzed by cyt *c* [19,22–24], the peroxidase activity of which [25–32] is dramatically enhanced upon binding to cardiolipin [19,33–35], partial proteolysis [26,36], denaturation [31], dimerization [37], tyrosine nitration [38], methionine (met_{80}) oxidation [39,40] and residues 26 or 41 mutation [41–43]. Induction of lipid peroxidation by cyt *c* has also been reported for other unsaturated lipids [44–49].

In view of the above relationship between lipid peroxidation and membrane permeability, peroxidase activity of cyt *c*/cardiolipin complex may imply its propensity to permeabilize lipid membrane in the presence of hydrogen peroxide. Of note, cyt *c* by itself is also able to induce ion permeability of liposomal [50] and planar bilayer [51] membranes and even leakage of fluorescent dextran and carboxyfluorescein from liposomes, the latter being more pronounced with cardiolipin-containing vesicles [52]. Of relevance to the issue are the data on the cyt *c*-induced formation of non-bilayer structures [53] and morphological transitions [54] in cardiolipin-containing model membranes, as well as permeation of apocytochrome *c* across lipid bilayers [55] along with its ability to induce dye leakage from liposomes [56]. Previously, induction of electrical current across planar bilayer lipid membrane formed from the mixture of soybean phosphatidylcholine and tetraoleoyl-cardiolipin was reported upon the addition of hydrogen peroxide in the presence of cyt *c* [57,58]. To study in detail the membrane-permeabilizing activity of cyt *c* promoted by H_2O_2, we applied here a fluorescence dequenching assay and a fluorescence

Abbreviations: cyt *c*, cytochrome *c*; CF, 5(6)-carboxyfluorescein; SRB, sulforhodamine B; PC, soybean phosphatidylcholine; CL, bovine heart cardiolipin; TMCL, tetramyristoyl-cardiolipin; DPhPC, diphytanoylphosphatidylcholine; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; FCS, fluorescence correlation spectroscopy; G(τ), autocorrelation function

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correlation spectroscopy (FCS) approach using dye-loaded large unilamellar vesicles of different lipid compositions. A combination of cyt *c* with hydrogen peroxide was found to induce dye release from liposomes, provided that both cardiolipin and bulk phosphatidylcholine components of liposomal membranes contained unsaturated acyl tails. Together with sensitivity to antioxidants, these results allowed to relate the cyt *c*/H_2O_2-induced dye leakage to formation of membrane structural defects involving oxidized cardiolipin molecules.

2. Materials and methods

2.1. Materials

Most chemicals including equine heart cytochrome *c*, bovine heart cardiolipin (CL), sulforhodamine B (SRB), 5(6)-carboxyfluorescein (CF), butylated hydroxytoluene (BHT), trolox and quercetin were from Sigma; 1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine (DPhPC) and tetramyristoyl-cardiolipin (TMCL) were from Avanti Polar Lipids (Alabaster, AL).

2.2. Preparation of liposomes

Dye-loaded liposomes were prepared by evaporation under a stream of nitrogen of a 2% solution of a mixture of lipids in chloroform followed by hydration with a buffer solution containing appropriate fluorescent marker. Four different lipid mixtures were used: 1) PC, 5 mg soybean phosphatidylcholine (Sigma, Type II-S), 2) PC/CL, 4 mg soybean phosphatidylcholine and 1 mg bovine heart cardiolipin, 3) PC/TMCL, 4 mg soybean phosphatidylcholine and 1 mg tetramyristoyl-cardiolipin, 4) DPhPC/CL, 4 mg diphytanoylphosphatidylcholine and 1 mg bovine heart cardiolipin. Four different marker solutions (0.5 ml each) were used: 1) 100 mM CF adjusted to pH about 8 by Tris, 2) 1 mM SRB in 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4, 3) 3 mg/ml rhodamine-labeled 3-kDa dextran in the same buffer, and 4) 10 mg/ml rhodamine-labeled 10-kDa dextran in the same buffer. The mixture was vortexed, passed through several cycles of freezing and thawing, and extruded through 0.1-μm pore size Nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The unbound marker was then removed by passage through a Sephadex G-50 coarse column (for SRB) or through a Sephadex G-150 coarse column with a buffer solution containing 100 mM KCl, 10 mM Tris, 10 mM MES, and pH 7.4.

2.3. Fluorescence dequenching assay

Fluorescence of liposomes loaded with 100 mM CF was monitored at 520 nm (excitation at 490 nm) with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). The extent of CF efflux was calculated as $(F_t - F_0) / (F_{100} - F_0)$, where F_0 and F_t represent the initial fluorescence intensity and the fluorescence intensity at the time *t*, and F_{100} is the fluorescence intensity after complete disruption of liposomes by addition of the detergent Triton-X100 (final concentration, 0.1% w/w).

2.4. Fluorescence correlation spectroscopy

The home-made setup was described previously [59]. Briefly, fluorescence excitation and detection utilized a Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescent inverted microscope equipped with a 40×, NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence light passed through an appropriate dichroic beam splitter and a long-pass filter and was imaged onto a 50-μm core fiber coupled to an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer Optoelectronics, Vaudreuil, Quebec, Canada). The signal from an output was sent to a PC using a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The data acquisition time was 30 s. The fluorescence was recorded from the confocal volume located at about 50 μm above the coverslip surface with 50 μl of the buffer solution added. Most of the data were collected under the conditions of stirring a suspension by a paddle-shaped 3-mm plastic bar rotated at 600 rpm. To calibrate the setup, we recorded the fluorescence autocorrelation function of Rhodamine 6G solution. Assuming the diffusion coefficient of the dye to be 2.5×10^{-6} cm^2/s, the value of the confocal radius $\omega = 0.42$ μm was obtained. The correlated fluorescence emission signals were fitted to the three-dimensional autocorrelation function [60,61]:

$$G(\tau) = \frac{1}{N}\left(\frac{1}{1+\frac{\tau}{\tau_D}}\right)\left(\frac{1}{\sqrt{1+\frac{\omega^2\tau}{z_0^2\tau_D}}}\right) \quad (1)$$

with τ_D being the characteristic correlation time during which a molecule resides in the observation volume of radius ω and length z_0, given by $\tau_D = \omega^2/4D$, where *D* is the diffusion coefficient, N is the mean number of fluorescent particles in the confocal volume. The amplitude of the autocorrelation function is inversely proportional to the number of fluorescent particles ($N = 1/G(\tau \to 0)$), but is independent of the fluorescence intensity of a single particle (in a system of identical particles) and therefore does not depend on the number of fluorophores per vesicle. Particles can be any fluorescent "point objects" in comparison to the dimension of the observation volume (i.e. about 1 μm). Therefore, particles can be single molecules of dye (i.e. SRB), as well as liposomes carrying different numbers of dye molecules. Initially (before the leakage induction) the system has a limited number of particles per observation volume comprising predominantly several liposomes loaded with the dye. After the leakage, the number of particles increases tremendously, because every liposomal particle produces thousands of particles of free dye leading to a significant decrease in the parameter $G(\tau \to 0)$.

3. Results and discussion

3.1. Cyt c/H_2O_2-induced leakage of carboxyfluorescein from liposomes

Fig. 1A illustrates liposome leakage induced by cyt *c* and H_2O_2, as monitored by release of the fluorophore carboxyfluorescein encapsulated in liposomes at a self-quenching concentration [62], which manifested itself in an increase of CF fluorescence. It is seen that 100 nM cyt *c* in combination with 1.5 mM H_2O_2 caused leakage of CF entrapped in cardiolipin-containing liposomes at low (curve 2), but not at high (100 mM KCl) ionic strength (curve 6). Of note, the H_2O_2-induced change in CF fluorescence depended non-monotonically on cyt *c* concentration (Fig. 1B): the increase in fluorescence became smaller at 1 μM cyt *c* (curve 5), whereas at higher cyt *c* concentrations the addition of H_2O_2 elicited even a decrease in CF fluorescence (curve 6). Earlier the cyt *c*-dependent quenching of fluorescence and the corresponding absorbance loss associated with oxidative damage were observed with the cyanine dye diS-c_3-(5) [63]. Therefore, the cyt *c*/H_2O_2-induced reduction of CF fluorescence was most likely due to destruction of CF caused by cyt *c*-mediated lipid peroxidation. This assumption is supported by the fact that CF itself has been shown to serve as a substrate of a peroxidase [64]. Oxidative instability of CF was also observed previously upon photodynamically induced leakage from liposomes [16].

Thus, we found significant stimulation of cyt *c*-induced carboxyfluorescein leakage from liposomes by hydrogen peroxide. However, a quantitative study of cyt *c*/cardiolipin peroxidase effect on the fluorescence of liposomes loaded with CF is hampered by oxidative instability of this dye.

3.2. FCS study of cyt c/H_2O_2-induced liposome leakage

In recent publications [16,65–67], permeability of vesicle membranes to fluorescent dyes has been studied by FCS, the approach which does not require loading of liposomes with dyes at very high,

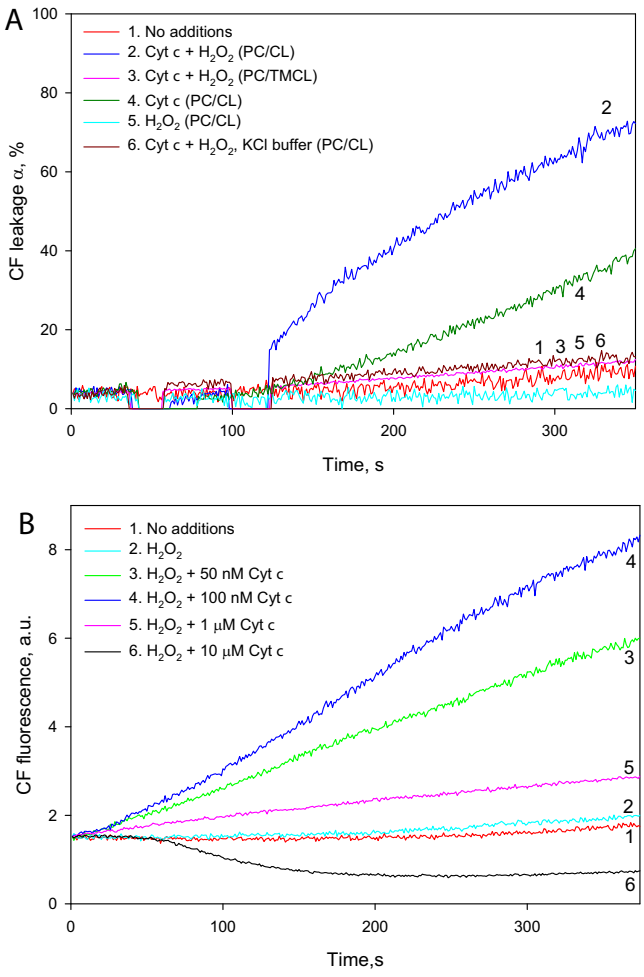

Fig. 1. A. Effect of cytochrome *c* and H_2O_2 on carboxyfluorescein leakage (α denotes the extent of the CF leakage) from liposomes composed of PC/CL (curves 2, 4, 5, 6) or PC/TMCL (curve 3). The concentration of cyt *c* was 100 nM, the concentration of H_2O_2 — 1.5 mM. B. Changes in fluorescence of CF-loaded PC/CL liposomes induced by the addition of H_2O_2 at different concentrations of cytochrome *c*. Curve 1, no additions; curve 2, 1.5 mM H_2O_2; curve 3, cyt *c* 50 nM and 1.5 mM H_2O_2; curve 4, cyt *c* 100 nM and 1.5 mM H_2O_2; curve 5, cyt *c* 1 μM and 1.5 mM H_2O_2; curve 6, cyt *c* 10 μM and 1.5 mM H_2O_2. The sucrose buffer contained 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4. The KCl buffer contained 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4. Lipid concentration, 10 μg/ml.

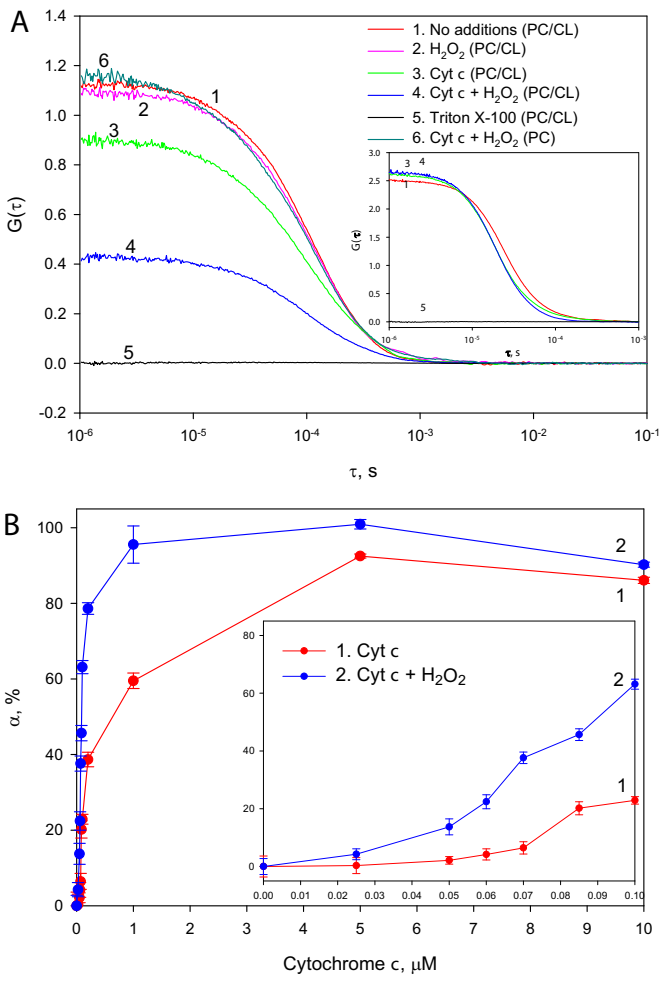

Fig. 2. Effect of cytochrome *c* and H_2O_2 on sulforhodamine B leakage from liposomes. A. Autocorrelation functions of SRB-loaded liposomes formed from PC/CL, 80/20% w/w (curves 1–5), or SRB-loaded liposomes formed from PC only (curve 6) measured after 30-min incubation with cyt *c* (100 nM) and/or H_2O_2 (1.5 mM) in sucrose buffer. Curve 1, no additions; curve 2, H_2O_2; curve 3, cyt *c*; curve 4, cyt *c* and H_2O_2; curve 5, 0.1% Triton X-100; curve 6, cyt *c* and H_2O_2. Inset: similar experiments performed in KCl buffer. B. The dependence of α (the extent of the SRB leakage) on the concentration of cytochrome *c* after 30-min incubation with (blue curve) and without (red curve) 1.5 mM H_2O_2. The sucrose buffer contained 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4. The KCl buffer contained 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4. Lipid concentration, 2 μg/ml.

self-quenching concentrations. Here we studied the effect of a combination of H_2O_2 and cyt *c* on the fluorescence intensity autocorrelation function (G(τ)) of the water-soluble dye sulforhodamine B encapsulated in cardiolipin-containing liposomes (Fig. 2A). Importantly, this fluorescent dye has been shown to be resistant to oxidative damage [16]. According to [60,61], the amplitude of G(τ) in the limit $\tau \to 0$ is determined by the reciprocal of a mean number (N) of fluorescent particles in the observation volume. In dye-leakage experiments, N comprises dye-loaded liposomes and free dye molecules released from liposomes. To measure $G(\tau \to 0)$ more precisely, we performed FCS experiments under stirring conditions [59]. As seen from Fig. 2A, the $G(\tau \to 0)$ amplitude of SRB-loaded cardiolipin-containing liposomes measured after incubation with H_2O_2 alone (curve 2) did not differ from the control level (curve 1). Incubation of SRB-loaded liposomes in the presence of cyt *c* led to a decrease in the $G(\tau \to 0)$ amplitude (curve 3), which was much more pronounced if cyt *c* was combined with H_2O_2 (curve 4). Disruption of liposomes by the addition of Triton X-100 resulted in a drop of $G(\tau \to 0)$ to nearly zero (curve 5). The reduction of the $G(\tau \to 0)$ amplitude apparently reflected an increase in the number of fluorescent particles due to SRB release from liposomes. Of note, the contribution of different fluorescent species (here dye-loaded liposomes and free dye molecules released from liposomes) to $G(\tau \to 0)$ is proportional to the square of their brightness [65,66]. With cardiolipin-lacking liposomes, no sizeable changes in the $G(\tau \to 0)$ amplitude was detected upon incubation with cyt *c* and H_2O_2 (Fig. 2A, curve 6).

Fig. 2B displays cyt *c* concentration dependences of the extent of liposome leakage α after 30-min incubation of liposomes with cyt *c* calculated from the Eq. (2) (see Appendix A):

$$\alpha(t) = 1 - \sqrt{\frac{G^t(\tau \to 0)}{G^0(\tau \to 0)}} \qquad (2)$$

where $G^0(\tau \to 0)$ and $G^t(\tau \to 0)$ represent $G(\tau)$ in the limit $\tau \to 0$ at the moment of cyt *c* addition (zero time) and t min after the addition, respectively. It is seen that H_2O_2 markedly enhanced the dye-releasing effect of cyt *c*.

The non-linear profiles of the concentration dependence in Fig. 2B suggest the involvement of the interaction of cyt *c* molecules with each other in the permeabilization process. It is relevant that cyt *c* oligomerization has recently been shown to promote its binding to

negatively charged lipid membranes [68] and facilitate the induction of its peroxidase activity [37].

3.3. Comparison of cyt c/H_2O_2-induced and Fe/ascorbate-induced liposome leakage

To test specificity of the cyt c/H_2O_2 permeabilizing activity, we compared its sensitivity to cyanide (known to bind the cytochrome heme group), a ferrous chelator and ionic strength (Fig. 3A) with that of the Fe/ascorbate-induced membrane permeabilization (Fig. 3B). Similar to the effect of KCN on cyt *c*-dependent lipid peroxidation described earlier [28,46,47,69], the cyt c/H_2O_2-induced SRB release from liposomes was prevented by cyanide (Fig. 3A), whereas the Fe/ascorbate-provoked SRB release was completely insensitive to it (Fig. 3B). The free iron chelator EDTA had no effect on the cyt c/H_2O_2-induced liposome leakage (Fig. 3A), but inhibited the Fe/ascorbate-induced leakage (Fig. 3B). In line with the data on the ionic strength effect on cyt *c* binding to cardiolipin-containing lipid bilayers and monolayers [33,34,51,70–76], supplementing the medium with 100 mM KCl suppressed the effect of cyt *c* and H_2O_2 on $G(\tau \rightarrow 0)$ (Fig. 3A), which indicated that the membrane binding of cyt *c* is a key step in the cyt c/H_2O_2 permeabilizing activity. By contrast, a drop in the $G(\tau \rightarrow 0)$ amplitude caused by the Fe/ascorbate addition did not change upon increasing the ionic strength (Fig. 3B). These data excluded the possibility that the cyt c/H_2O_2-induced SRB release from liposomes was a result of heme decomposition by H_2O_2.

Fig. 4 shows the dependence of the cyt c/H_2O_2-induced SRB release from liposomes on the concentration of NaCN. The measurements were carried out after 30-min incubation in the presence of cyt c/H_2O_2. The inhibiting effect of cyanide could be related to CN binding to the heme group of cyt *c*. Following [69], we believe that cyt *c* with the CN coordinated to the central Fe atom of the heme is unable to perform peroxidative activity, presumably because of the involvement of the Fe atom of the heme in the binding of H_2O_2 to cyt *c*. The pattern of the dependence of the cyt c/H_2O_2-induced SRB release on the cyanide concentration could be associated with the protein heterogeneity regarding the depth of heme insertion into the lipid bilayer, which results in variation of heme accessibility to cyanide.

3.4. Dependence of cyt c/H_2O_2-induced liposome leakage on lipid composition

Time courses of SRB leakage induced by cyt *c* and H_2O_2 were measured for liposomes of different lipid compositions (Fig. 5). It appeared that either substitution of fully saturated tetramyristoyl cardiolipin

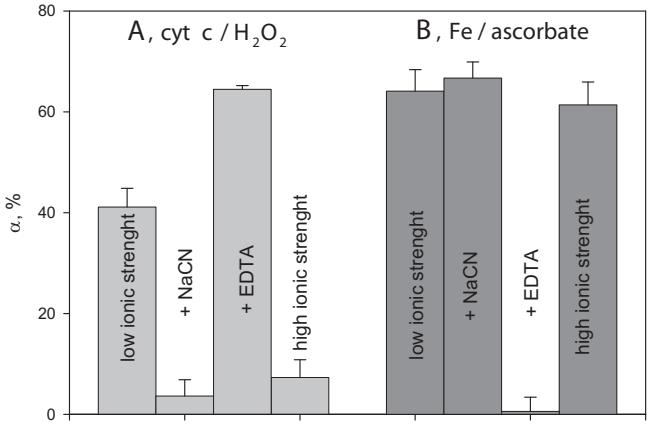

Fig. 3. Effect of sodium cyanide, EDTA and ionic strength on the SRB leakage from PC/CL liposomes induced by cytochrome c/H_2O_2 (A) or Fe/ascorbate (B). Lipid, 2 μg/ml; cyt *c*, 100 nM; H_2O_2, 1.5 mM; $FeSO_4$, 5 μM; ascorbate, 100 μM; NaCN, 1 mM; EDTA, 1 mM. The buffer solution of 10 mM Tris, 10 mM MES, pH 7.4 contained 200 mM sucrose for low ionic strength conditions or 100 mM KCl for high ionic strength conditions.

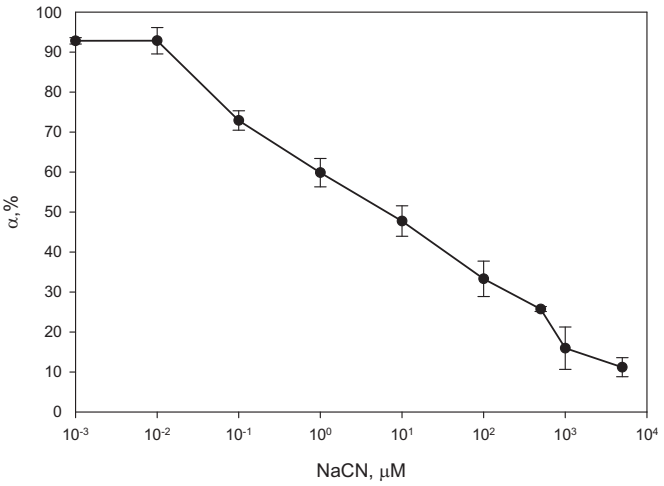

Fig. 4. Dependence of the cyt c/H_2O_2-induced SRB leakage on the concentration of sodium cyanide measured after 30-min incubation with cyt c/H_2O_2. Lipid, 2 μg/ml; cyt *c*, 100 nM; H_2O_2, 1.5 mM. The solution was 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4.

(curve 2) for bovine heart cardiolipin (predominantly containing tetralinoleyl-cardiolipin) (curve 1) or substitution of fully saturated diphytanoyl phosphatidylcholine (curve 3) for soybean phosphatidylcholine (curve 1) led to dramatic suppression of the cyt *c*-induced leakage, becoming close to that in the absence of cyt *c* and H_2O_2 (curve 4). Similar dependence on lipid composition was also found with CF-loaded liposomes (Fig. 1A). These effects of changing lipid composition on membrane permeabilization could not be associated with phase transitions, because diphytanoyl phosphatidylcholine, although fully saturated, is known to be in the fluid state at room temperature [77] and the addition of 20% of fully saturated TMCL to highly unsaturated egg PC could also hardly alter its fluid state. Thus, the requirement of the presence of unsaturated fatty-acid tails in both cardiolipin and diacyl phosphatidylcholine for cyt *c*/cardiolipin membrane-permeabilizing activity was most likely due to the involvement of lipid peroxidation in this activity.

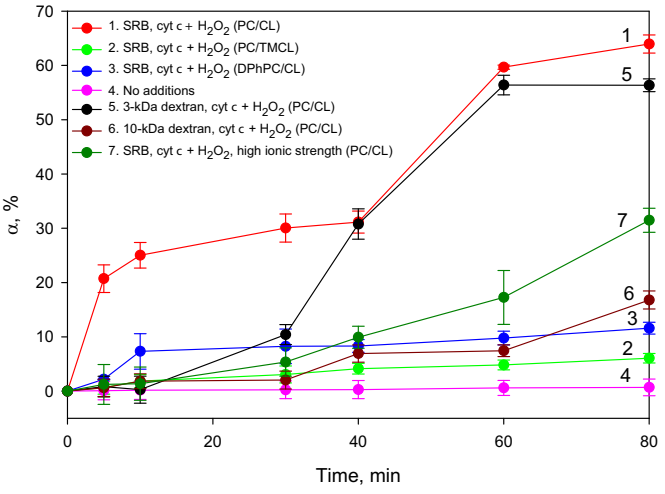

Fig. 5. Time courses of leakage of SRB (curves 1–4, 7), 3-kDa dextran (curve 5) and 10-kDa dextran (curve 6) from liposomes of different lipid compositions (PC/CL, DPhPC/CL, PC/TMCL) induced by cytochrome *c* and H_2O_2. Experiments were carried out in the low ionic strength buffer containing 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4, except for curve 2 where 100 mM KCl was used instead of 200 mM sucrose. Lipid, 2 μg/ml; cyt *c*, 100 nM; H_2O_2, 1.5 mM.

3.5. Cyt c/H_2O_2-induced leakage of fluorescent dextrans from liposomes

To estimate the size of conductive defects in membranes formed in the presence of cyt *c* and H_2O_2, we measured leakage of fluorescently labeled dextrans from cardiolipin-containing soybean phosphatidylcholine liposomes. Initially the leakage of 3-kDa dextran induced by the combination of cyt *c* and H_2O_2 was noticeably slower than that of SRB, although at longer time the extent of leakage for 3-kDa dextran became close to that for SRB (Fig. 5, curve 5). This observation could be related to lateral expansion of pores described for amphipathic antimicrobial peptides [78]. The leakage of 10-kDa dextran (2.4-nm Stokes radius [79]) was markedly suppressed (curve 6), suggesting the size of cyt *c*-induced membrane defects being approx. 5 nm.

3.6. Effect of antioxidants on cyt c/H_2O_2-induced liposome leakage

To test the relation of the cyt *c*/cardiolipin permeabilizing activity to lipid peroxidation, we studied the effect of various antioxidants on cyt *c*/H_2O_2-induced SRB leakage from liposomes. As follows from Fig. 6A, the addition of trolox, butylated hydroxytoluene (BHT) or quercetin significantly suppressed the SRB leakage. These free radical-scavengers were earlier found to inhibit cyt *c*/cardiolipin peroxidase activity [35]. Panel B of Fig. 6 shows the effect of trolox and sodium cyanide on the SRB leakage induced by a high concentration of cyt *c* (1 μM) without H_2O_2. Both compounds did not affect the extent of the leakage under these conditions, thereby indicating that mechanisms of the leakage differed in the two cases.

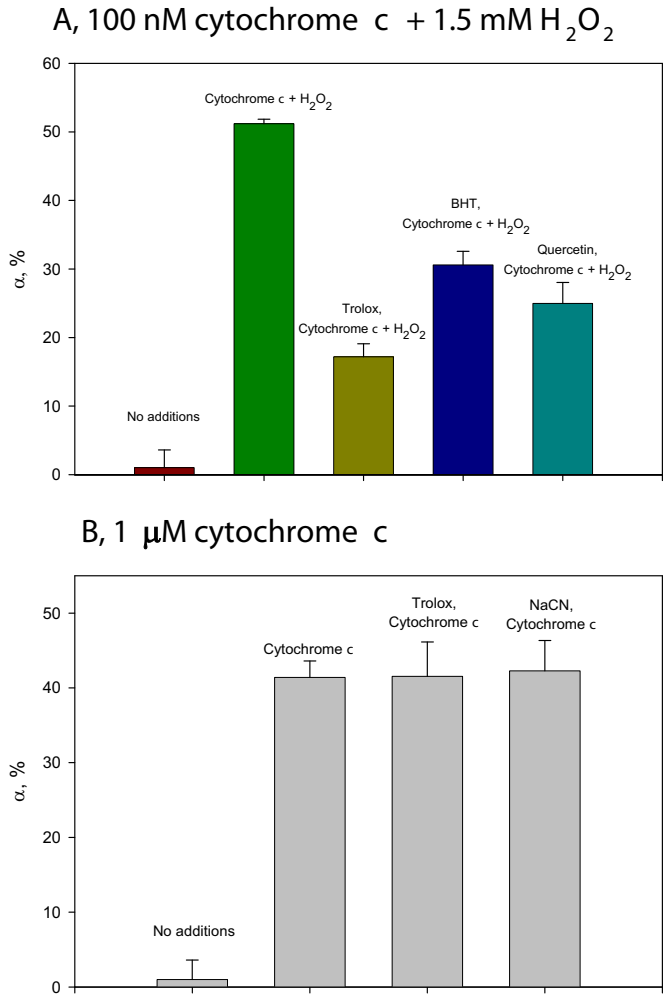

Fig. 6. A. Effect of antioxidants on the cytochrome *c*-induced SRB leakage from PC/CL liposomes in the presence of H_2O_2. Lipid, 2 μg/ml; cyt *c*, 100 nM; H_2O_2, 1.5 mM, trolox, 100 μM; BHT, 1 mM; quercetin, 20 μM. The solution was 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4. B. Effect of Trolox (100 μM) and KCN (1 mM) on the cytochrome *c*-induced SRB leakage from PC/CL liposomes without H_2O_2. Cyt c was 1 μM. Other conditions as in panel A.

3.7. Mechanism of cyt c/H_2O_2-induced liposome leakage: toroidal protein–lipid pore requiring oxidized cardiolipin versus pure lipidic pores

There has been an enormous body of evidence revealing changes in cyt *c* tertiary structure associated with extensive unfolding of the protein upon binding to lipid vesicles [80,81], especially those containing cardiolipin (see [82] and refs. therein). Furthermore, insertion or partial penetration of cyt *c* into membranes have been proposed [52,83–90]. It is the membrane-penetrating capacity of cyt *c* that presumably results in its ability to permeabilize lipid membranes in the absence of hydrogen peroxide [51,52]. Electrophysiological measurements revealed the cyt *c*-induced fast current flickering at low protein concentrations and stable giant pores at micromolar cyt *c* concentrations suggesting different mechanisms of the membrane permeabilization [51]. According to our data, this cyt *c*-induced leakage proved to be insensitive to antioxidants and cyanide (Fig. 6B). Therefore, it was not associated with peroxidase activity of cyt *c* and induction of lipid peroxidation.

Based on the obligatory presence of unsaturated alkyl chains in cardiolipin as a prerequisite to observe cyt *c*/H_2O_2-induced permeabilization (Fig. 5) and its sensitivity to antioxidants and cyanide (Figs. 3, 4, 6), it seems reasonable to suggest the involvement of oxidized cardiolipin molecules in the formation of membrane defects associated with cyt *c*/H_2O_2-induced perturbation of bilayer structure. According to electrophysiological data reported in [58], the combination of cyt *c* and H_2O_2 induced low-amplitude ion channels with a lifetime in a timescale of seconds, which differed substantially from those found with cyt *c* only [51]. Our experiments revealed the significant permeability of the liposomal membranes to 3-kDa dextran caused by cyt *c*/H_2O_2, which could be tentatively ascribed to evolution of structural defects into hydrophylic pores, presumably of the toroidal protein–lipid nature, with pore walls formed by protein helices intercalated by lipid head-groups [91–97,52] (see Fig. 7 depicting insertion of cyt *c* α-helices into a membrane driven by cyt *c*/cardiolipin peroxidase activity which results in toroidal pore formation). Favoring this model, lipid oxidation was shown to dramatically stimulate membrane pore formation induced by the water-soluble protein colicin E1 [98]. Alternatively, cyt *c*/H_2O_2-caused permeabilization may be ascribed to formation of pure lipidic pores involving oxidized lipids [16,99–101] or trivial membrane rupture resulting from lipid peroxidation. However, our FCS experiments with rhodamine-labeled liposomes showed that the cyt *c*-promoted dye leakage could proceed during a long time without membrane disruption (data not shown), and no stable pores were observed upon lipid peroxidation [102], which makes the pure oxidized lipid pore model of cyt *c*/H_2O_2-induced permeabilization less probable than the toroidal one involving protein helices. The striking differences in the sensitivity of cyt *c*/H_2O_2-induced and Fe/ascorbate-induced liposome leakage to cyanide (forming the cyanide–ferricytochrome *c* complex) and a ferrous chelator (Fig. 3) support direct participation of the protein in cyt *c*/H_2O_2-caused permeabilization. The preventing effect of cyanide on the cyt *c*/H_2O_2-induced permeabilization of liposomes could be attributed to the competitive binding of hydrogen peroxide and cyanide to the sixth ligand position of the heme iron in cyt *c*, as described in [69].

The importance of studying here the liposome leakage induced by Fe/ascorbate was associated with the possibility that H_2O_2 could provoke considerable damage to cyt *c* which might include iron release from the heme. Actually, this process was described in [103]. Of note, it was known from early studies that Fe/ascorbate could bring about an increase in membrane permeability resulting in dextran leakage from liposomes [7]. Our experiments with Fe/ascorbate and the applied

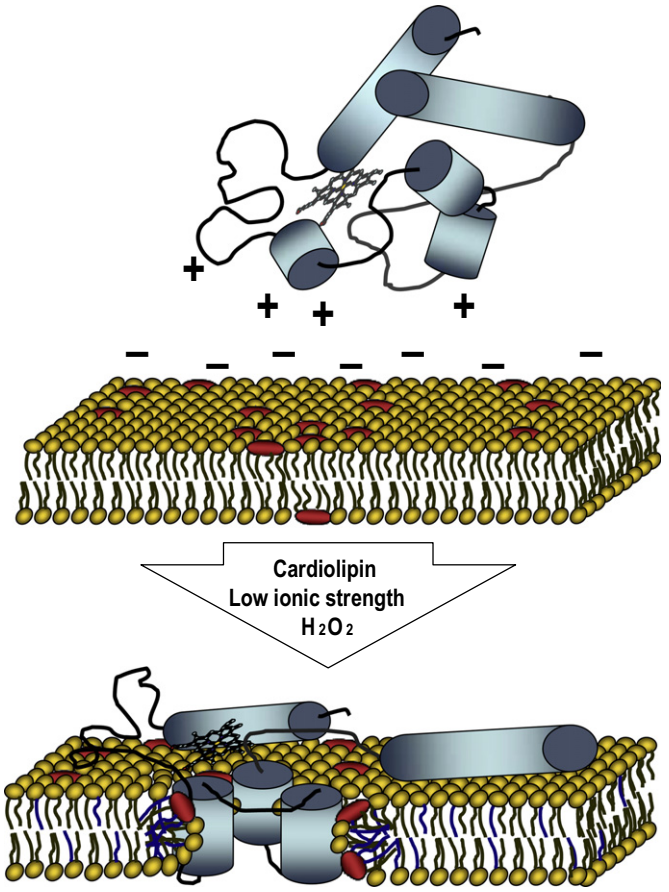

Fig. 7. Scheme of cardiolipin-dependent lipid membrane permeabilization by cytochrome *c* and H_2O_2. Electrostatic binding of cyt *c* to cardiolipin-containing membranes followed by conformational changes of the protein leading to the induction of peroxidase activity, lipid oxidation and formation of a protein–lipid pore permeable for SRB.

inhibitory analysis proved that the possible release of iron was not involved in the liposome permeabilization process induced by cyt *c*/H_2O_2.

In summary, cyt *c*/cardiolipin peroxidase activity is shown to result in leakage of fluorescent markers from liposomes, inhibitable by cyanide and antioxidants. Requirement of unsaturated cardiolipin for the leakage points to the involvement of oxidized cardiolipin in the formation of cyt *c*/H_2O_2-induced membrane defects.

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Appendix A

According to [66], G(τ) of a system of a dye in and out of vesicles (V) can be described by the following equation (Eq. (4) of the reference):

$$G(\tau)=\frac{\left\langle N_{dye}\right\rangle}{\left(\left\langle N_{dye}\right\rangle+\widetilde{B}\langle N_v\rangle\right)^2}\left[1+\frac{\tau}{\tau_{dye}}\right]^{-1}\left[1+\left(\frac{r_0}{z_0}\right)^2\frac{\tau}{\tau_{dye}}\right]^{-\frac{1}{2}}+\frac{\widetilde{B}^2\langle N_v\rangle\left(1+\widetilde{B}^{-1}\right)}{\left(\left\langle N_{dye}\right\rangle+\widetilde{B}\langle N_v\rangle\right)^2}\left[1+\frac{\tau}{\tau_v}\right]^{-1}\left[1+\left(\frac{r_0}{z_0}\right)^2\frac{\tau}{\tau_v}\right]^{-\frac{1}{2}}$$

Where N_{dye} and N_V are the number of dye and vesicle particles in the observation volume with dimensions r_0 and z_0, τ_{dye} and τ_V are diffusion times of the dye and the vesicles, $\widetilde{B}$ is the mean number of dyes per vesicle at a given time. In the limit $\{\tau \to 0\}$ we have

$$G(\tau\to 0)=\frac{\left\langle N_{dye}\right\rangle}{\left(\left\langle N_{dye}\right\rangle+\widetilde{B}\langle N_V\rangle\right)^2}+\frac{\widetilde{B}^2\langle N_V\rangle\left(1+\widetilde{B}^{-1}\right)}{\left(\left\langle N_{dye}\right\rangle+\widetilde{B}\langle N_V\rangle\right)^2}=\frac{\left\langle N_{dye}\right\rangle+\widetilde{B}^2\langle N_V\rangle\left(1+\widetilde{B}^{-1}\right)}{\left(\left\langle N_{dye}\right\rangle+\widetilde{B}\langle N_V\rangle\right)^2}.$$

Parameters N_{dye} and N_V are not independent. At the beginning of leakage experiments $N_{dye}=0$ and $\widetilde{B}=B_0$, afterwards

$$\left\langle N_{dye}\right\rangle=\left(B_0-\widetilde{B}\right)\langle N_V\rangle$$

because the appearance of the dye molecules in the solution is a consequence of their efflux from the vesicles. Therefore:

$$G(\tau\to 0)=\frac{\left(B_0-\widetilde{B}\right)\langle N_V\rangle+\widetilde{B}^2\langle N_V\rangle\left(1+\widetilde{B}^{-1}\right)}{\left(\left(B_0-\widetilde{B}\right)\langle N_V\rangle+\widetilde{B}\langle N_V\rangle\right)^2}=\frac{1}{\langle N_V\rangle}\frac{B_0+\widetilde{B}^2}{\widetilde{B}_0{}^2}=\frac{1}{\langle N_V\rangle}\left(\frac{1}{B_0}+\left(\frac{\widetilde{B}}{B_0}\right)^2\right).$$

Since $G^{t=0}(\tau\to 0)=\frac{1}{\langle N_V\rangle}$,

$$G^t(\tau\to 0)=G^{t=0}(\tau\to 0)\left(\frac{1}{B_0}+\left(\frac{\widetilde{B}}{B_0}\right)^2\right)\quad\text{and}\quad\frac{\widetilde{B}}{B_0}=\sqrt{\frac{G^t(\tau\to 0)}{G^{t=0}(\tau\to 0)}-\frac{1}{B_0}}.$$

The estimated value of B_0 (number of dye molecules per vesicle) in our experiments was about 10^3. This estimation corresponds to the ratio of the initial value of $G(\tau \to 0)$ and the value after the addition of Triton X-100. This means that at low and intermediate leakage extents one can use the approximation

$$\frac{\widetilde{B}}{B_0}\cong\sqrt{\frac{G^t(\tau\to 0)}{G^{t=0}(\tau\to 0)}}.$$

The conventional definition of the leakage extent α is a percentage of dye outside of vesicles, i.e.

$$\alpha=1-\frac{\widetilde{B}}{B_0}=1-\sqrt{\frac{G^t(\tau\to 0)}{G^{t=0}(\tau\to 0)}}.$$

This equation gives a simple relationship between the decrease in $G(\tau \to 0)$ and the dye leakage. The assumption $1/B_0 \ll \widetilde{B}/B_0$ makes the deviation from the equation less than 1% in the region of $\alpha < 50\%$ keeping in mind that $B_0 > 100$.

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