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Original Research Communication

Oxidation-Dependent Changes in the Stability and Permeability of Lipid Bilayers

KAZUNORI ANZAI,[1] KUNITAKA OGAWA,[2] YASUKO GOTO,[1] YUMIKO SENZAKI,[1] TOSHIHIKO OZAWA,[1] and HARUHIKO YAMAMOTO[2]

ABSTRACT

Peroxidation-dependent change in the permeability of lipid bilayers was mesaured by using artificial membrane systems, that is, planar lipid bilayers and liposomes. The unsaturated fatty acyl chains of phospholipids in small unilamellar vesicles were peroxidized time-dependently by the hydroxyl radical chemically generated by the reaction of H_2O_2 and $Cu(en)_2$. In contrast, at the same hydroxyl radical concentration and time ranges, no ionic current through the planar lipid bilayers and no release of K^+ from the liposomes were observed. These findings indicate that accumulation of lipid peroxide within lipid bilayers is not responsible for the permeability increase that is often observed in biomembranes exposed to oxidative stresses. Higher concentration of the hydroxyl radical caused break-down of the planar lipid bilayers composed of the mixture (7:3) of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The bilayer containing 100% PE at least at one leaflet of the bilayer (facing the hydroxyl radical-generating solution) was not broken-down by the application of the hydroxyl radical, suggesting that PE stabilizes the planar lipid bilayer against the attack of the hydroxyl radical. Antiox. Redox Signal. 1, 339–347.

INTRODUCTION

RECENTLY, IT HAS BEEN WIDELY ACCEPTED that oxidants regulate cellular responses by affecting signal transduction and gene expression. Reactive oxygen species (ROS) may affect the cellular responses directly by acting as signal transduction messengers (Sen, 1998). ROS may also affect the cellular responses indirectly through changes in intracellular ionic environment, especially in intracellular Ca^{2+} homeostasis (Trump and Berezesky, 1992; Kaneko *et al.*, 1994; Dreher and Junod, 1995). For example, involvement of intracellular Ca^{2+} in oxidant-induced NF-κB activation was reported (Sen *et al.*, 1996). Therefore, it is important to know the effect of ROS on the intracellular ionic concentrations. The ionic concentration is determined by the ion flux through the plasma membrane and/or through the membranes of internal ionic stores. The ion flux occurs either at ion-transporting membrane proteins, such as ion pumps and ion channels, or at lipid bilayer.

There have been few studies that strictly measured oxidation-dependent change in the ion permeability of lipid bilayers (Nakazawa and Nagatsuka, 1980; Kunimoto *et al.*, 1981). Artificial lipid bilayers, *i.e.*, liposomes and pla-

[1]Bioregulation Research Group, National Institute of Radiological Sciences, Japan, and [2]Department of Applied Bioscience, Faculty of Science, Kanagawa University, Japan.

nar lipid bilayers, are good systems to measure the permeability of the lipid bilayer part of membranes independent of the ion-transporting proteins involved in biomembranes. In the present study, by using these artificial membrane systems, we have measured the effects of chemically generated hydroxyl radical (•OH) and peroxyl radical (ROO•) on the peroxidation, stability, and permeability of the lipid bilayers.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipid (Alabaster, AL). The acyl chain composition of the PE was identical to that of the PC, because the PE was the product transesterified from the PC. Cu(II)(ethylenediamine)$_2$ complex (Cu(en)$_2$) was prepared as described previously (Ozawa *et al.*, 1988). 5,5'-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from LABOTEC (Tokyo, Japan). Other chemicals were of analytical grade and used without further purification.

Generation and detection of hydroxyl radical and peroxyl radical

The hydroxyl radical (•OH) was generated chemically by the reaction of the Cu(en)$_2$ complex with hydrogen peroxide (H_2O_2) as described previously (Ozawa and Hanaki, 1991). The generation of •OH was monitored by the ESR (electron spin resonance) spin-trapping technique. The time course of the •OH generation rate was measured as follows. Various concentrations of H_2O_2 and Cu(en)$_2$ were mixed to be 100 and 1 m*M*, 50 and 0.5 m*M*, or 10 and 0.1 m*M*, respectively, with a buffer solution containing 150 m*M* KCl and 10 m*M* Tris-HEPES, pH 7.4. From time to time, a 100-μl aliquot was withdrawn from the reaction solution and mixed with 100 μl of 100 m*M* DMPO solution. The mixture was transferred to an ESR flat cell and ESR spectra were measured with X-band ESR spectrometer (JEOL RE-1X, Tokyo, Japan) within 1 min after the mixing of the sample and DMPO. Typical conditions for the ESR measurement were as follows: magnetic field, 333 ± 5 mT, frequency 9.44 GHz, scan 2 min, response 0.3 sec, and amplitude 2,000. Peroxyl radical (ROO•) was generated from 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) (Niki, 1990).

Liposomes

Small unilamellar vesicles (SUVs) were prepared by sonication with a bath-type sonicator (Iuchi VS-70U, Osaka, Japan) of multilamellar vesicles composed of PE/PC = 7:3 or 100% PC in 150 m*M* KCl and 10 m*M* Tris-HEPES, pH 7.4. Large unilamellar vesicles (LUVs) were prepared by the reverse-phase evaporation method (Kaneda *et al.*, 1987) as follows. Fifty milligrams of PE/PC = 7:3 or 100% PC was dissolved in a 3-ml solution of isopropyl ether and chloroform (64:36). A water-phase solution of 150 m*M* KCl and 10 m*M* Tris-HEPES, pH 7.4, was added to the lipid solution, and the mixture was sonicated briefly with the bath-type sonicator. The organic solvent of the resultant suspension was evaporated with a rotary evaporator and a 10-ml solution of 150 m*M* KCl and 10 m*M* Tris-HEPES, pH 7.4, was added to the residue to form LUVs. It was impossible to form LUVs with 100% PE. For the measurement of K^+ release with an K^+ selective electrode, LUVs were washed twice with 150 m*M* NaCl and 10 m*M* Tris-HEPES, pH 7.4, by centrifugation with an ultracentrifuge (Hitachi SCP70H, Tokyo) at 41,000 rpm for 30 min. The recovery of the LUVs by this treatment was 100% based on the comparison of phosphorus measured with a kit (Wako, Osaka) before and after the centrifugation.

Measurement of phospholipid peroxidation

Formation of peroxidized phospholipids was analyzed using liposomes (SUVs) with the same lipid composition (PE/PC = 7:3) as the planar bilayer membrane. Lipid peroxidation by hydroxyl radical was initiated by the addition of various concentrations of Cu(en)$_2$ and H_2O_2 to the liposomal suspension (2 mg phospholipid/ml). The reaction was stopped at an appropriate time by chelating the Cu with 2 m*M* diethylenetriamine-*N*,*N*,*N'*,*N''*,*N''*-pentaacetic acid (DTPA) and cooling down the so-

lution to 0°C. A 5-ml solution of chloroform/methanol (2:1) was added to the reaction mixture (1 ml), mixed well, and the mixture was centrifuged at 1,000 × *g* for a few min with a centrifuge (05P-21, Hitachi, Tokyo). The upper layer was discarded and 3 ml of distilled water was added to the lower layer. Mixing and centrifugation were repeated as above. From the lower layer, 2.5 ml was withdrawn and the solvent was evaporated. For the diene conjugation assay (Buege and Aust, 1978), the resulting residue was dissolved in cyclohexane and the absorbance at 233 nm was measured with a spectrophotometer (Hitachi U-3210, Tokyo). Lipid peroxidation by ROO• generated by the decomposition of AAPH was performed in a manner similar to the peroxidation by •OH generated by the reaction of $Cu(en)_2$ and H_2O_2 described above. The reaction was stopped just by cooling down the reaction mixture. Iodometric assay of lipid peroxide was occasionally performed according to the literature method (Buege and Aust, 1978; Jessup *et al.*, 1994).

Potassium ion-selective electrode

K^+ release from liposomes was measured with a K^+-selective electrode, which was made according to the literature method (Katsu *et al.*, 1986). At first, a calibration curve was made for the electrode to use with known concentration of KCl. Next, the electrode was immersed in the suspension of LUVs, and the output voltage was continuously measured. The sample was stirred with a magnetic stirrer and kept at 37°C using a cuvette (volume ~2 ml) with a water jacket. At an appropriate time, $Cu(en)_2$ and H_2O_2 or AAPH was added to generate •OH or ROO•. At the end of the measurement, 2 m*M* Triton X-100 was added to the sample to release completely the K^+ in the LUVs. The output voltage was converted to the K^+ concentration with the calibration curve made at the beginning of the measurement.

Planar bilayer lipid membrane

Planar bilayer membranes were made at a small hole (200 μm in diameter) on a very thin Teflon septum (25 μm thick) in 150 m*M* KCl and 10 m*M* Tris-HEPES, pH 7.4, by the folding method as described previously (Anzai *et al.*, 1991). The lipid composition of the planar bilayer was PE/PC = 7:3 or 100% PE. An asymmetrical bilayer consisting of PE/PC = 7:3 for one leaflet (*cis* side) and 100% PE for the other leaflet (*trans* side) of the bilayer or *vice versa* was also made by the folding method. The Teflon chamber, Teflon septum, and electric setting were the same as described previously (Anzai *et al.*, 1991). $Cu(en)_2$ and H_2O_2 or AAPH was added to one side (*cis* side) of the chamber, and the electric current through the bilayer was measured under a voltage clamp condition at room temperature.

RESULTS

Time course of •OH generation

Hydroxyl radical was generated chemically by the reaction of H_2O_2 and $Cu(en)_2$. The •OH generation rate was detected by the spin trapping technique. Figure 1 shows the time course of the •OH generation rate in several $Cu(en)_2/H_2O_2$ concentrations. The ratio of $Cu(en)_2$ and H_2O_2 was kept constant (1:100). When high concentration of H_2O_2 and $Cu(en)_2$

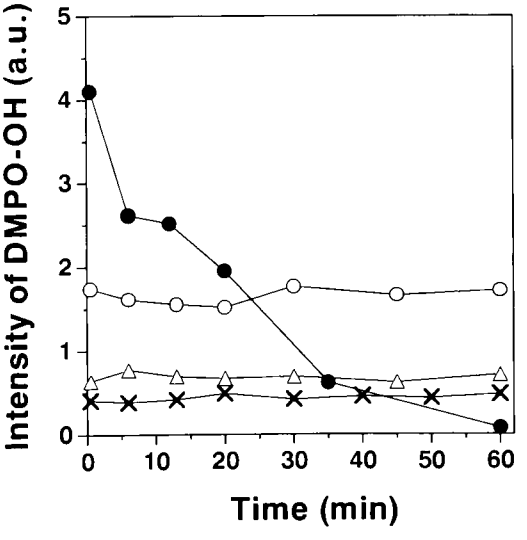

FIG. 1. Time course of hydroxyl radical generation by the reaction of various concentrations of $Cu(en)_2$ and H_2O_2. (●) 100 m*M*/1 m*M*; (○) 50 m*M*/0.5 m*M*; (△) 20 m*M*/0.2 m*M*; and (X) 10 m*M*/0.1 m*M* for the concentrations of $Cu(en)_2/H_2O_2$, respectively. The hydroxyl radical was trapped by 25 m*M* DMPO. The relative intensity of the ESR signal of DMPO-OH adduct to that of MnO, an external standard, is shown.

was used (100 m*M* and 1 m*M*, respectively), the peak height of the DMPO-OH adduct decreased in time and even reached a negligible value in 1 hr. On the other hand, at lower concentrations of the generators, the generation rate was constant and no decrease of the signal was observed at least for 1 hr. At 50 m*M* H_2O_2 and 0.5 m*M* $Cu(en)_2$, the •OH generation gradually decreased after 1 hr, but the rate was still a half of the initial rate even at 4 hr; at 10 m*M* H_2O_2 and 0.1 m*M* $Cu(en)_2$, the •OH generation rate was constant at least for 8 hr (data not shown). The initial generation rates at the initial stage corresponded well to the concentration of the generators.

Lipid peroxidation by •OH and ROO•

Figure 2A shows the increase of conjugated diene in SUVs (PE/PC = 7/3) by the treatment of •OH generated chemically by the reaction of several concentrations of H_2O_2 and $Cu(en)_2$. The peroxidation proceeded rapidly and reached a plateau at 30–60 min. The diene-conjugated assay and the iodometric assay showed similar values for the amount of peroxidized phospholipid (data not shown). The initial peroxidation rate was high for the condition of high •OH generation rate. In contrast, the plateau value was low for such a condition.

Lipid peroxidation proceeded by the addition of 20 m*M* AAPH at 37°C, but the rate was smaller than that observed in the •OH generating system (Fig. 2B). The peroxidation reaction did not reach a plateau even at 3 hr.

Effect of •OH and ROO• on K^+ permeability through liposomes

Figure 3 shows the effect of •OH generated with 100 m*M* H_2O_2 and 1 m*M* $Cu(en)_2$ (Fig. 3A) and ROO• generated with 20 m*M* AAPH (Fig. 3B) on the K^+ release from the LUVs (PE/PC = 7/3). Although gradual release of K^+ from the LUVs occurred naturally, no significant additional release of K^+ was observed on exposure of the LUVs to either •OH or ROO• at the initial stage (0–60 min) of the time course. However, a significant difference was observed at a later stage (after 60 min) for the sample exposed to •OH.

Effect of •OH on the permeability of planar bilayer membrane

Planar bilayer membranes were used to assess the relationship between lipid peroxida-

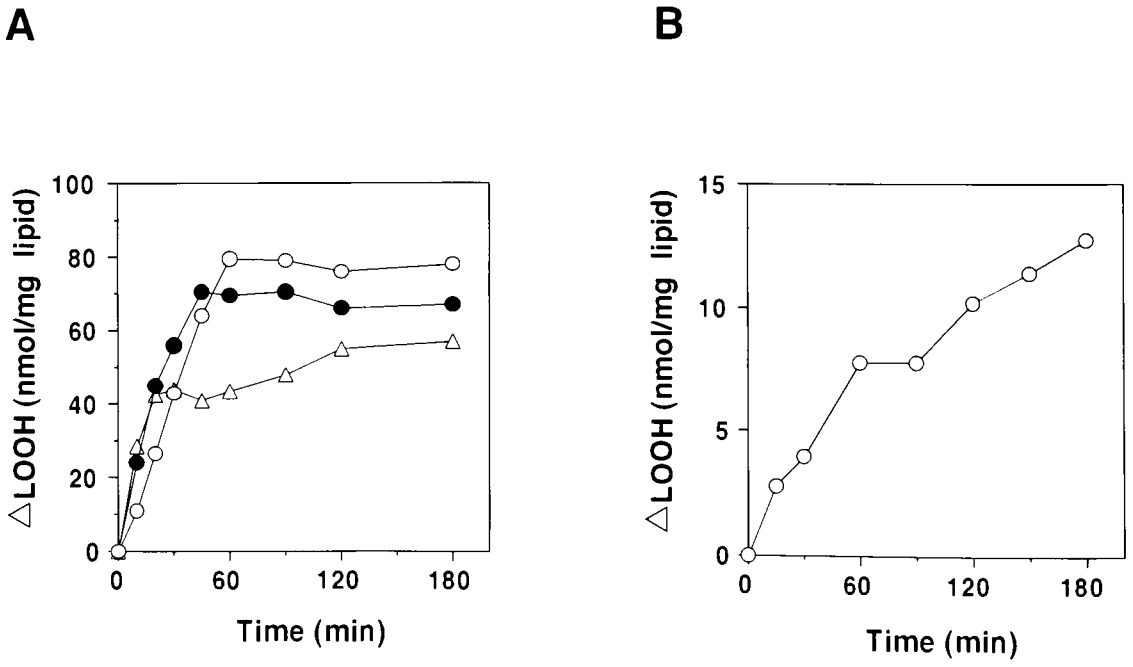

FIG. 2. Formation of phospholipid hydroperoxide in PE/PC (7/3) liposomes. A. Application of hydroxyl radical generated by various concentrations of $Cu(en)_2/H_2O_2$ at 37°C: (△) 100 m*M*/1 m*M*; (●) 50 m*M*/0.5 m*M*; (○) 10 m*M*/0.1 m*M*. **B.** Application of peroxyl radical generated by 20 m*M* AAPH at 37°C.

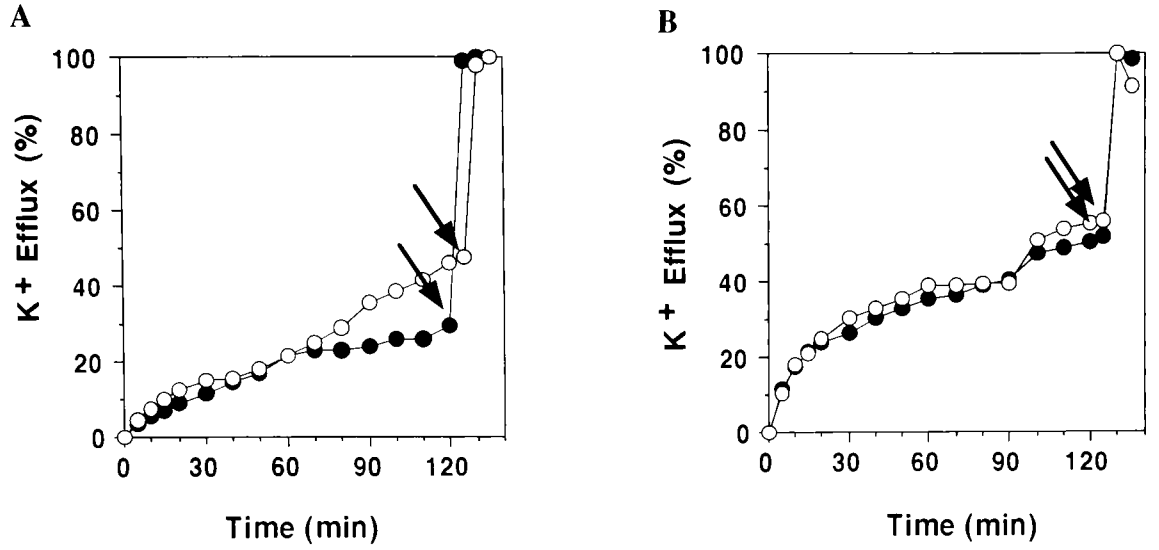

FIG. 3. Effects of hydroxyl radical and peroxyl radical on the K^+ permeability of PC liposomes. A. The time-course of K^+ release from the liposomes was measured with a K^+-selective electrode in the presence of hydroxyl radical generated by 100 m*M* H_2O_2/1 m*M* $Cu(en)_2$ (○) and in its absence (●). **B.** The time-course of K^+ release from the liposomes was measured with a K^+-selective electrode in the presence of peroxyl radical generated by 20 m*M* AAPH (○) and in its absence (●).

tion and the integrity and permeability of the lipid part of bilayer membranes. Figure 4 shows no change in the membrane current for more than 40 min after application of 50 m*M* H_2O_2 and 0.5 m*M* $Cu(en)_2$ (trace A) or 10 m*M* H_2O_2 and 0.1 m*M* $Cu(en)_2$ (trace B). No current change was observed up to 90 min in these conditions (data not shown).

When higher concentration of •OH was generated by 100 m*M* H_2O_2 and 1 m*M* $Cu(en)_2$, the membrane current gradually increased at about 10 min (Fig. 5). The current increased exponentially with increased noise, and the membrane collapsed suddenly at around 16 min.

The stability of the planar lipid bilayer was dependent on the composition of the polar head group of the bilayer lipid. When a symmetrical bilayer was formed by PE/PC = 7:3, application of a high concentration of •OH by 100 m*M* H_2O_2 and 1 m*M* $Cu(en)_2$ increased the membrane current and produced bilayer breakdown (Fig. 5). The bilayer made of 100% PE was, however, relatively stable and membrane breakdown did not occur until 70 min after the application of •OH (Fig. 5B). When an asymmetrical bilayer with PE/PC = 7:3 for the leaflet of *cis* side (the side of H_2O_2 and $Cu(en)_2$ addition) and 100% PE for the leaflet of *trans* side was used, high concentration of •OH collapsed the bilayer within 30 min (Fig. 5C). On the other hand, when an asymmetrical bilayer with 100% PE for the *cis* side and PE/PC = 7:3 for the *trans* side was used, the asymmetrical bilayer was more stable and no breakdown of the bilayer occurred within 60 min.

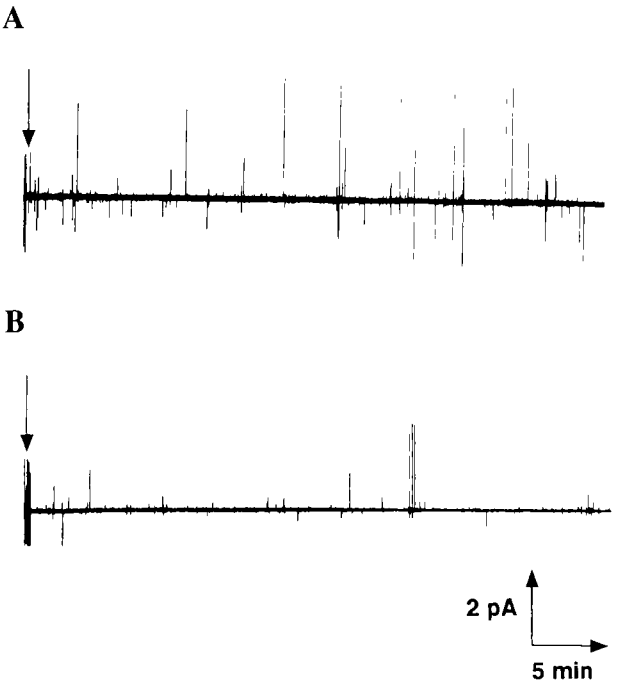

FIG. 4. Effect of hydroxyl radical generated by (A) 0.5 mM $Cu(en)_2$ and 50 mM H_2O_2 and (B) 0.1 mM $Cu(en)_2$ and 10 mM H_2O_2 on the current through the symmetrical planar lipid bilayer of PE/PC (7/3). The arrows indicate the time of the addition of $Cu(en)_2$ and H_2O_2 to the *cis* compartment of the chamber.

DISCUSSION

A conjugated diene was formed time-dependently by the exposure of liposomes to •OH or ROO•. A similar time course of the formation of peroxidized lipid was observed by the iodometric assay (data not shown). Therefore, the acyl chains of PC and PE in SUVs were peroxidized by the exposure to •OH or ROO• The peroxidation reached a plateau at about 60 min by the application of •OH (Fig. 2), whereas it still proceeded even at 180 min when ROO• was applied from 20 m*M* AAPH at 37°C. During the first 60 min, •OH was constantly generated, except at high concentrations of H_2O_2 and $Cu(en)_2$ (100 m*M* and 1 m*M*, respectively), by which the generation rate decreased gradually with time and reached almost zero at 60 min. The formation of ROO• by the decomposition of AAPH and additional reaction of O_2 was reported by Niki (1990). Radical formation from AAPH was detected by spin-trapping method (Sato *et al.*, 1995; Krainev and Bigelow, 1996).

The apparent saturation of the peroxidation seen in Fig. 2 at 100 m*M* H_2O_2 and 1 m*M* $Cu(en)_2$ is not the real saturation, because a slow increase of peroxidized lipid is seen after 1 hr. The peroxidation level at 1 hr was higher when lower concentrations of the •OH generators were used. Because the •OH generation proceeded for more than 4 hr when 50–10 m*M* H_2O_2 and 0.5–0.1 m*M* $Cu(en)_2$ was used, the saturation of lipid peroxidation observed for these conditions must be due to the consumption of the substrate (unsaturated double bonds), whereas the apparent saturation at 30 min observed for 100 m*M* H_2O_2 and 1 m*M* $Cu(en)_2$ should be ascribed to the stop of the •OH generation.

The acyl chain composition of PC was reported as 32% $C_{16:0}$, 1.0% $C_{16:1}$, 16.0% $C_{18:0}$,

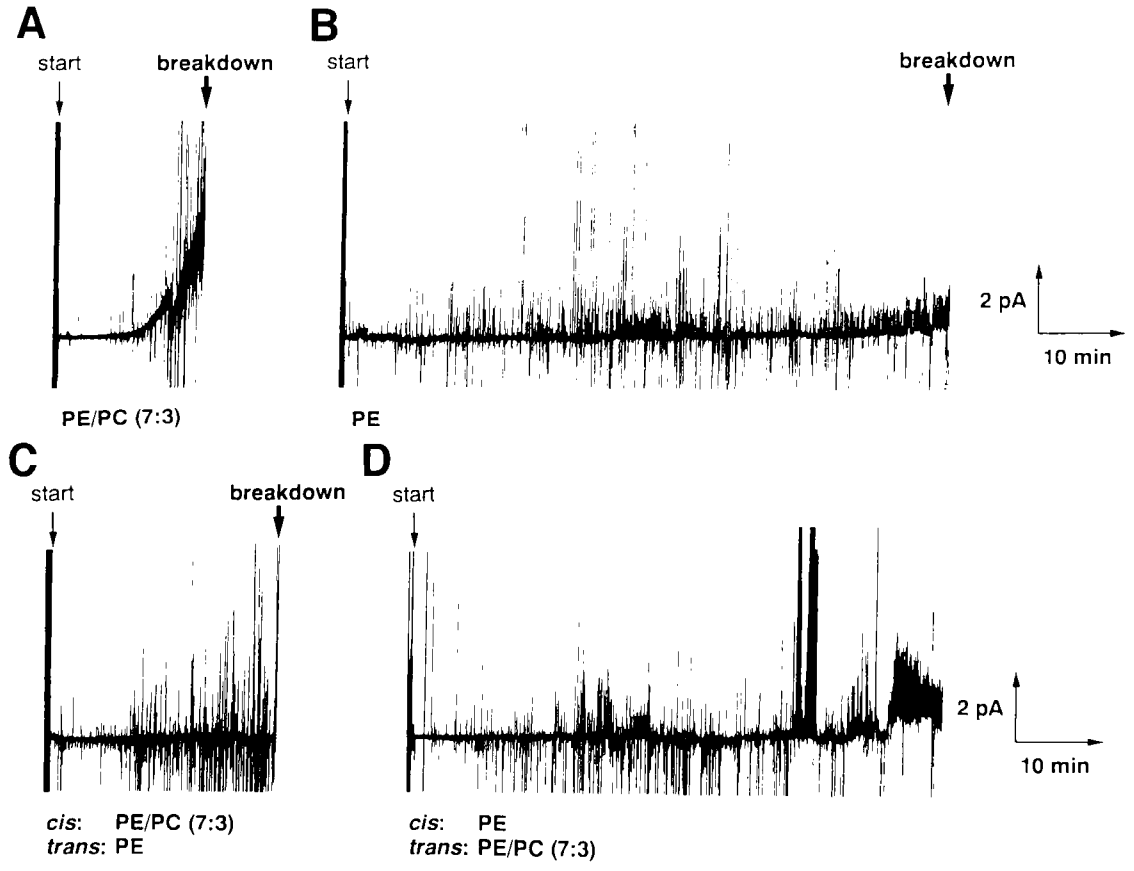

FIG. 5. Effect of hydroxyl radical generated by 1 m*M* $Cu(en)_2$ and 100 m*M* H_2O_2 on the current through: the (**A**) symmetrical planar lipid bilayer of PE/PC (7/3); (**B**) symmetrical planar lipid bilayer of PE only; (**C**) asymmetrical planar lipid bilayer (*cis*, PE/PC (7/3); *trans*, PE only); and (**D**) asymmetrical planar lipid bilayer (*cis*, PE only; *trans*, PE/PC (7/3)).

30.0% $C_{18:1}$, 17.0% $C_{18:2}$, and 3.0% $C_{20:4}$ (White, 1973). Because the PE we used was the product transesterified from PC, the acyl chain composition should be identical to that of PC. We assume that $C_{18:2}$ and $C_{20:4}$ contribute mainly to the conjugated diene formation; that is, about 20% of the lipid undergoes peroxidation. Assuming that the average molecular weight of the phospholipids is 800 and all the $C_{18:2}$ and $C_{20:4}$ are peroxidized, the saturation level of the conjugated diene is calculated to be 250 nmol/mg lipid. The saturation level observed (about 75 nmol/mg lipid) was about 30% of the calculated value. Because the •OH-generating agents are water soluble, •OH should be generated outside the liposomes, causing peroxidation of only the outer leaflet of the liposomes. In addition, the liposomes we used were not completely unilamellar. These factors may explain the observed peroxidation level of 30% of the total peroxidizable part of the acyl chains. The lower saturation level at 100 m*M* H_2O_2 and 1 m*M* $Cu(en)_2$ may represent a progress of the reaction to decompose the formed lipid peroxide. This kind of decomposition also explains the lower level of peroxidation than expected. The increase of the absorbance at 270 nm supports the occurrence of further decomposition (data not shown).

The K^+-selective electrode detected a slow release of K^+ ion entrapped inside the liposomes. Application of •OH or ROO• did not enhance the leak out of K^+ ion significantly in the first 60 min. This result indicates that the formation of peroxidized lipid in the lipid bilayer does not necessarily increase the ion permeability of the bilayer. When high concentrations of the •OH generating system were used (100 m*M* H_2O_2 and 1 m*M* $Cu(en)_2$), significant increase of K^+ efflux was observed (Fig. 3A). Because the •OH generation stopped after 60 min in this condition, this increase should be due to some after effect such as deformation or disintegration of liposomes. Also, 100 m*M* H_2O_2 alone or 1 m*M* $Cu(en)_2$ alone did not enhance the K^+ efflux (data not shown), indicating that the enhancement was caused not by H_2O_2 or $Cu(en)_2$ but by •OH.

When •OH generated from $H_2O_2/Cu(en)_2$ (50/0.5 m*M* or 10/0.1 m*M*) was applied to one side of the planar bilayer composed of PE/PC = 7/3, no increase of the membrane current was observed (Fig. 4). Because •OH generated from these concentrations of $H_2O_2/Cu(en)_2$ peroxidized the acyl chain of the phospholipid (Fig. 2), this observation supports the idea based on the result of K^+ efflux from liposomes: that is, the formation of lipid peroxide itself does not cause the increase of the ion permeability of the lipid bilayer membrane.

When high concentrations of $H_2O_2/Cu(en)_2$ (100/1 m*M*) were used, the membrane current began to increase at about 10 min after the application of •OH to the PE/PC (7/3) bilayer, and the bilayer was suddenly broken down at about 16 min (Fig. 5A). When the planar bilayer made of 100% PE was used, no increase of the membrane current was observed and the bilayer was broken down only at about 70 min (Fig. 5B). Therefore, the stability of the planar lipid bilayer against •OH was dependent on the composition of the head group of the phospholipids. Because •OH was produced at the *cis* side of the planar bilayer, •OH should first attack the phospholipid molecules of the leaflet facing the •OH generating system (*cis* side). The asymmetrical bilayer made of 100% PE and PE/PC (7/3) for the *cis* and *trans* leaflet of the bilayer, respectively, was more stable than the asymmetrical bilayer of opposite composition. The head group of PE may protect the unsaturated fatty acyl chains from the attack by •OH. Formation of a Schiff base between PE head groups and aldehyde formed by the decomposition of lipid peroxide and/or the cross-linking between the ethanolamine head groups may also contribute to the more stable bilayer containing more PE (van Duijn *et al.*, 1984). The composition of the head group at the *cis* leaflet is an important factor determining the stability of the planar bilayer.

Many reports showed that the ion permeability of biological membranes was increased under the condition of oxidative stress. According to the results of this study, the increase of the ion permeability is not due to the increase at the lipid bilayer by the formation of lipid peroxide. Rather, the degradation products of peroxidized lipid, such as various aldehydes, are important to the change of membrane permeability through modification of membrane ion-transporting proteins (Halliwell and Gut-

teridge, 1989). In addition, direct modification of membrane proteins would be also important for the increase of the ion permeability of the membrane induced by the oxidative stress. Indeed, several reports suggested that modification of sulfhydryl groups of ion channel proteins increased the ion permeation through the channels (Anzai *et al.*, 1998; Xu *et al.*, 1998; Bouzat *et al.*, 1991; Abramson *et al.*, 1995; Serre *et al.*, 1995).

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ABBREVIATIONS

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; $Cu(en)_2$, Cu(II)(ethylenediamine)$_2$ complex; DMPO, 5,5'-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriamine- *N,N,N',N'',N''*-pentaacetic acid; ESR, electron spin resonance; H_2O_2, hydrogen peroxide; LUVs, large unilamellar vesicles; $^{\bullet}OH$, hydroxyl radical; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; $ROO^{\bullet}$, peroxyl radical; ROS, reactive oxygen species; SUVs, small unilamellar vesicles.

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Address reprint requests to:
Dr. Kazunori Anzai
Bioregulation Research Group
National Institute of Radiological Sciences
4-9-1 Anagawa, Inage-ku,
Chiba 263-8555, Japan

E-mail: anzai@NIRS.go.jp

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