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Effect of lipid peroxidation on membrane-bound Ca^{2+}-ATPase activity of the intestinal brush-border membranes

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We have studied lipid peroxidation and Ca^{2+}-ATPase activity of the porcine intestinal brush-border membranes using a oxygen-radical-generating system consisting of dithiothreitol (DTT)/Fe^{2+} and *tert*-butyl hydroperoxide (*t*-BuOOH). The rates of lipid peroxidation were measured by formation of thiobarbituric acid-reactive substances (TBAR) and conjugated diene. Incubation of the membranes with DTT/Fe^{2+} in the absence and presence of *t*-BuOOH resulted in a slight (about 20%) and a marked (about 50%) inhibition of Ca^{2+}-ATPase activity, respectively. The degree of inhibition was dependent on the hydroperoxide concentration. Addition of thiourea effectively protected Ca^{2+}-ATPase activity but catalase and superoxide dismutase showed a slight and no effect on protection of the ATPase activity, respectively. Results of kinetic studies on the ATPase activity with varying ATP and Ca^{2+} concentrations revealed that the decrease in the enzyme activity by treatment with these oxidizing agents is mainly due to decrease of the V_{max} value. Modification of SH groups in the membrane proteins by thiol group reagents such as *N*-ethylmaleimide, monoiodoacetate and monoiodoacetamide did not induce the inhibition of Ca^{2+}-ATPase activity. From these results, it is suggested that inhibition of the ATPase activity of the membranes by treatment with DTT/Fe^{2+} in the presence and absence of *t*-BuOOH is dependent on lipid peroxidation and that oxidative modification of SH groups may be not directly involved to the loss of the ATPase activity. In addition, results of the fluorescence anisotropy measurements of pyrene-labeled membranes suggested that change in the Ca^{2+}-ATPase activity is partly related to a decrease in the membrane lipid fluidity.

Introduction

Lipid peroxidation in biological membrane systems proceeds through a complex process involving rearrangement and destruction of the double bonds in highly unsaturated fatty acids of membrane lipids. Formation of lipid hydroperoxide in membranes would result in damage of the membrane structure and various cellular functions in vitro and in vivo [1–4].

It has been demonstrated by several investigators that lipid peroxidation of biological membranes results in changes in their lipid fluidity [5,6], permeability of ions [7] and activities of membrane-bound enzymes [8–12]. However, an exact mechanism of change in the enzyme activities associated with lipid peroxidation is still unclear.

We have previously reported that the lipid peroxidation of the porcine intestinal brush-border membranes is dependent on the membrane surface charge and/or potential [13] and that the antioxidant action of α-tocopherol is due to stabilization of the lipid organization of the membranes [14].

We have also demonstrated a decrease in the reactivity of SH groups in the membrane proteins for the fluorogenic thiol reagent, *N*-[7-dimethylamino-4-methylcoumarinyl]maleimide [15], and a change in the fluorescence parameters of *N*-(1-pyrene)maleimide-labeled membranes [16] by lipid peroxidation of the membranes. These findings strongly suggest that the conformation of the protein moieties in the membranes is sensitively modified by lipid peroxidation.

Recently we have found that Mg^{2+}-independent Ca^{2+}-ATPase exists in porcine intestinal brush-border

Abbreviations: TBAR, thiobarbituric acid-reactive substances; DTT, dithiothreitol; *t*-BuOOH, *tert*-butyl hydroperoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTNB, 5,5-dithiobis(2-nitrobenzoate); NEM, *N*-ethylmaleimide.

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membranes [17]. In the present study, therefore, we have examined in detail the effect of DTT/Fe^{2+} treatment of the membranes in the presence and absence of a model lipid hydroperoxide, *t*-BuOOH, on the membrane-bound Ca^{2+}-ATPase activity in order to obtain further information about membrane damage by lipid peroxidation.

Materials and Methods

Chemicals. Pyrene, DTT, 2-thiobarbituric acid, 3(2)-*tert*-butyl-4-hydroxyanisole and DTNB were purchased from Wako Pure Chemical Co. ATP (dipotassium salt), *t*-BuOOH, Lubrol PX, NEM, monoiodoacetate, monoiodoacetamide, catalse (3100 U/mg protein) and superoxide dismutase (3000 U/mg protein) were obtained from Sigma. All other reagents used were of the highest purity obtainable and came from commercial sources.

Preparation of membrane vesicles. The brush-border membranes were prepared from the porcine small intestine by the calcium-precipitation method as described in our previous paper [18] and suspended in 10 mM Tris-HCl buffer (pH 7.4) at about 20 mg protein/ml. Protein concentration was assayed by the procedure of Lowry et al. [19] using bovine serum albumin.

Lipid peroxidation of the membranes. Lipid peroxidation was generally performed by the incubation of the membranes (1 mg protein/ml) with 20 mM DTT, 10 μM $FeSO_4$ and 5 mM *t*-BuOOH in 30 mM Tris-HCl buffer (pH 7.4) for 30 min at 37°C. The control membranes were incubated in the same manner without oxidizing agents. The reaction was terminated by dilution with a large volume of 10 mM Tris-HCl buffer (pH 7.4) and centrifugation at $25\,000 \times g$ for 20 min. The pellets were washed three times with and resuspended in the same buffer. In measurements of TBAR and conjugated diene formation, the reaction was terminated by addition of 5 mM 3(2)-*tert*-butyl-4-hydroxyanisole (as a final concentration) to the reaction mixture (1 ml). After addition of 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% of 2-thiobarbituric acid (50% CH_3COOH) to each sample, it was heated at 90°C for 60 min and then cooled to room temperature. The amount of TBAR in the supernatant was determined at 530 nm using the molar absorption coefficient of $1.53 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ and expressed as equivalents of malondialdehyde. The amount of conjugated diene formed during the reaction was measured by monitoring the absorbance at 233 nm of the detergent-dispensed membranes (0.02 mg protein/ml in 10 mM phosphate buffer (pH 7.1) containing 1% Lubrol PX) as described in Ref. 20 and calculated using the molar absorption coefficient of $2.52 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [2].

Enzyme assay. Ca^{2+}-ATPase activity was measured by incubating the membranes (9–10 μg protein) in 1 ml of incubation medium containing 30 mM Tris-HCl buffer (pH 7.4), 0.1 mM ouabain, 0.2 mM EGTA, 3 mM ATP and 0.4 mM $CaCl_2$ for 30 min at 37°C as described in our previous paper [17]. Inorganic phosphate liberated was measured by the method of Fiske and SubbaRow [21]. Ca^{2+}-ATPase activity was calculated by subtracting the activity obtained with EGTA alone from that obtained in the presence of Ca^{2+}. The Ca^{2+} concentration-dependence on ATP hydrolysis by the membranes were performed using EGTA-Ca^{2+} buffer. In the calculation of free Ca^{2+} concentration, the dissociation constant of EGTA and Ca^{2+} was estimated to be $2 \cdot 10^{-7}$ M [22].

Labeling of the membranes with pyrene. A suspension of the membranes (1 mg protein/ml) was incubated with 2 μM pyrene (dissolved in ethanol) in 10 mM Tris-maleate buffer (pH 6.85) at 0°C for 30 min. The final concentration of ethanol was 0.33% or less. The reaction was terminated by dilution with a large volume of 10 mM Tris-maleate buffer (pH 6.85) and centrifugation at $25\,000 \times g$ for 20 min. The pellets were washed twice with and resuspended in the same buffer.

Fluorescence measurements. Fluorescence measurements were performed using a Hitachi spectrofluorometer MPF-4 equipped with Rhodamine B quantum counter. The excitation and emission wavelengths used were 340 and 392 nm, respectively. The error due to light scattering of the sample emission could be entirely prevented using a 350 nm cutoff filter. The degree of the steady-state fluorescence anisotropy, r, was calculated according to the equation: $r = (I_V - I_H)/(I_V + 2I_H)$, where I_V and I_H are the fluorescence intensities detected by a polarizer oriented parallel and perpendicular, respectively, to the direction of polarization of the extraction beam.

Results

Lipid peroxidation of the membranes

The effects of increasing concentrations of *t*-BuOOH on TBAR and conjugated diene formation of the intestinal brush-border membranes in the presence and absence of DTT/Fe^{2+} were examined.

Incubation of the membranes with 20 mM DTT and 10 μM Fe^{2+} resulted in slight increases in TBAR and conjugated diene formation. On the other hand, treatment of the membranes with *t*-BuOOH in the presence of DTT/Fe^{2+} caused marked stimulation of TBAR and conjugated diene formation, depending on the hydroperoxide concentration (Fig. 1). In this case, TBAR and conjugated diene formation of the membranes by treatment with either DTT, Fe^{2+} or *t*-BuOOH alone were almost negligible (data not shown).

Change in Ca^{2+}-ATPase activity

As shown in Table I, treatment of the membranes with 20 mM DTT and 10 μM Fe^{2+} for 30 min at 37°C

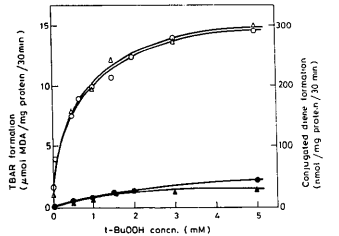

Fig. 1. *t*-BuOOH concentration-dependence of TBAR and conjugated diene formation of the membranes. The concentration of *t*-BuOOH was varied from 0.1 to 5 mM. The conditions and procedures of TBAR and conjugated diene measurements are described in Materials and Methods. ○ and ●, TBAR formation; △ and ▲, conjugated diene formation. Open and closed symbols represent the values in the presence and absence of 20 mM DTT/10 μM Fe^{2+}, respectively. Values are expressed as means of triplicate determinations.

resulted in a slight decrease of Ca^{2+}-ATPase activity (about 20%). Subsequent addition of 5 mM *t*-BuOOH to this system induced a further decrease of Ca^{2+}-ATPase activity (about 50%) and the degree of inhibition of the ATPase activity was dependent on the hydroperoxide concentration (Fig. 2). On the other hand, Ca^{2+}-ATPase activity was not influenced by treatment of the membranes with 10 μM Fe^{2+}, 20 mM or 5 mM *t*-BuOOH alone (0.333 ± 0.005, 0.324 ± 0.003 and 0.310 ± 0.002 μmol P_i/mg protein per min, respectively).

Next we examined the effect of scavengers of the various active oxygen on Ca^{2+}-ATPase activity and TBAR formation.

As shown in Fig. 3, thiourea, the hydroxyl radical scavenger, effectively blocked TBAR formation and substantially reduced the loss of Ca^{2+}-ATPase activity by DTT/Fe^{2+}/*t*-BuOOH treatment of the membranes. On the other hand, catalase, the H_2O_2 scavenger, did not prevent the loss of Ca^{2+}-ATPase activity but slightly inhibited TBAR formation. Superoxide dismutase, the superoxide anion scavenger, had no effect on the loss of Ca^{2+}-ATPase activity and TBAR formation.

TABLE I

Effect of lipid peroxidation on Ca^{2+}-ATPase activity

The conditions and procedure of lipid peroxidation were the same as described in Materials and Methods. Each value is expressed as mean ($n = 5$) ± S.E.

Membranes	Peroxidizing conditions	Ca^{2+}-ATPase activity (μmol P_i/mg protein per min)
Control	–	0.310 ± 0.002 (100%)
Peroxidized	DTT/Fe^{2+}	0.255 ± 0.005 (82%)
	DTT/Fe^{2+}/*t*-BuOOH	0.160 ± 0.008 (52%)

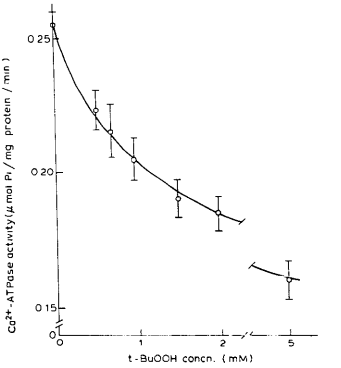

Fig. 2. *t*-BuOOH concentration-dependence of Ca^{2+}-ATPase activity. In this experiment, the membranes (1 mg protein/ml) were incubated with various concentrations of *t*-BuOOH (0–5 mM) in the presence of 20 mM DTT and 10 μM Fe^{2+} at 37°C for 30 min. Ca^{2+}-ATPase activity was measured after incubation of the membranes with corresponding concentrations of *t*-BuOOH and DTT/Fe^{2+}. The membrane protein concentration employed in the ATPase measurement was 10 μg. Values are expressed as mean ($n = 12$–13) ± S.E.

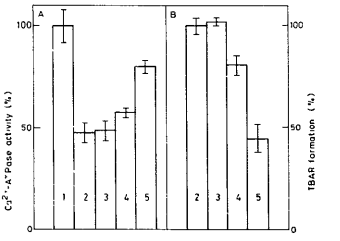

Fig. 3. Effects of active oxygen scavengers on Ca^{2+}-ATPase activity and TBAR formation. The concentrations of superoxide dismutase, catalase and thiourea were 30 μg/ml, 30 μg/ml and 30 mM, respectively. (A) Ca^{2+}-ATPase activity; (B) TBAR formation. Ca^{2+}-ATPase activity and TBAR formation were expressed as relative values to those of the control membranes and of the complete system, DTT/Fe^{2+}/*t*-BuOOH, respectively. 1, Control membranes; 2, complete system; 3, complete system plus superoxide dismutase; 4, complete system plus catalase; 5, complete system plus thiourea. Values are expressed as means ($n = 3$) ± S.D.

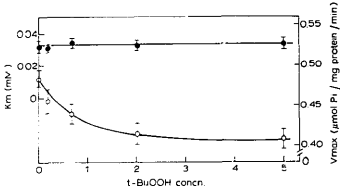

Fig. 4. *t*-BuOOH concentration-dependence of the kinetic parameters of Ca^{2+}-ATPase activity. The ATP concentration was varied from 0.01 to 0.25 mM. The conditions of lipid peroxidation were the same as those described in the legend to Fig. 2, except for the use of 5 mM *t*-BuOOH. Ca^{2+}-ATPase activity was measured for 10 min after addition of the membranes to the ATPase assay medium. ○, V_{max} value; ●, K_m value. Values are expressed as means ($n = 3$) ± S.E.

TABLE II

Effect of thiol group reagents on Ca^{2+}-ATPase activity

The membranes (0.5 mg protein/ml) were incubated with thiol group reagents (1 mM of each) in 30 mM Tris-HCl buffer (pH 7.4) for 30 min at 37 °C. The reaction was terminated by the addition of 2-mercaptoethanol (18–20 mM as a final concentration) to the reaction mixture. Then 20 μl of the reaction mixture was added to 0.98 ml of the ATPase assay medium and incubated for 30 min at 37 °C. In this experiment, 2-mercaptoethanol (20 mM) did not affect the Ca^{2+}-ATPase activity. Values are expressed as means ($n = 3$–6) ± S.E.

Reagent	Ca^{2+}-ATPase activity (μmol P_i/mg protein per min)
No addition	0.330 ± 0.009
NEM	0.319 ± 0.018
Monoiodoacetate	0.410 ± 0.024
Monoiodoacetamide	0.406 ± 0.017

Kinetic study of Ca^{2+}-ATPase activity

To investigate what kinds of factor are related to inhibition of Ca^{2+}-ATPase activity by treatment of the membranes with DTT/Fe^{2+} in the presence and absence of *t*-BuOOH, the effect of these treatments on the kinetic parameters of the ATPase activity was examined.

The ATP concentration-dependence profiles (0.01–0.25 mM) of ATP hydrolysis by the control and peroxidized membranes revealed simple saturation kinetics. From the plots of [S]/*v* versus [S] of the ATPase activity (*v*) of the control membranes, the K_m and V_{max} values were determined to be 0.036 ± 0.003 mM and 0.658 ± 0.010 μmol P_i/mg protein per min, respectively. On the other hand, the K_m and V_{max} values of the membranes treated with 20 mM DTT/10 μM Fe^{2+} in the absence and presence of 5 mM *t*-BuOOH were 0.034 ± 0.005 mM and 0.484 ± 0.009 μmol P_i/mg protein/min, and 0.032 ± 0.003 mM and 0.408 ± 0.011 μmol P_i/mg protein per min, respectively.

The effect of increasing concentrations of *t*-BuOOH on the kinetic parameters of the ATPase activity of the membranes with DTT/Fe^{2+} was presented in Fig. 4.

From these results, it is clear that lipid peroxidation of the membranes causes a decrease of the V_{max} value but does not affect the K_m value.

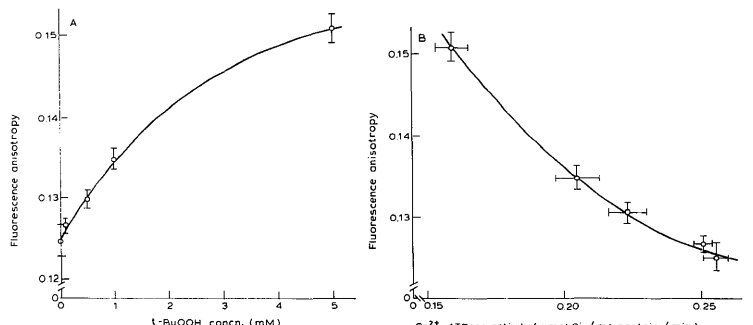

Fig. 5. (A) Effect of *t*-BuOOH on fluorescence anisotropy of DTT/Fe^{2+}-treated pyrene-labeled membranes. The membrane protein concentration was 0.05 mg/ml. *t*-BuOOH concentration was varied from 0.1 to 5 mM. The fluorescence anisotropy of pyrene-labeled control membranes at 25 °C was 0.115 ± 0.002. Values are expressed as means ($n = 3$) ± S.D. (B) Relationship between Ca^{2+}-ATPase activity and the fluorescence anisotropy. The values of the fluorescence anisotropy and Ca^{2+}-ATPase activity were obtained from Figs. 5(A) and Fig. 2, respectively.

Ca^{2+} concentration-dependence

We have previously reported [17] that there are two kinetic forms with a high affinity and a low affinity for Ca^{2+} stimulation of the ATPase activity. Therefore, the effect of lipid peroxidation on the Ca^{2+} concentration-dependence of ATP hydrolysis by the membranes was examined in the range of free Ca^{2+} concentration from 0.0665 to 250 μM.

From the Eadie plots of the ATPase activity (v) of the control and peroxidized membranes against $v/[Ca^{2+}]$, the K_m values for Ca^{2+} of the high and low affinity components of the membranes were determined to be 0.36 and 102 μM for the control membranes, 0.44 and 110 μM for the DTT/Fe^{2+}-treated, and 0.34 and 116 μM for the DTT/Fe^{2+}/*t*-BuOOH-treated membranes, respectively. This result suggests that the affinity for Ca^{2+} is not or scarcely influenced by lipid peroxidation.

Effect of SH modification on Ca^{2+}-ATPase activity

In order to clarify whether SH groups have an important role on the ATPase activity, we examined the effect of several thiol group reagents on Ca^{2+}-ATPase activity of the membranes.

As shown in Table II, exposure of the membranes to NEM did not affect on Ca^{2+}-ATPase activity. On the other hand, treatment of the membranes with monoiodoacetate or monoiodoacetamide resulted in an increase of the ATPase activity by 20–25%.

Relationship between Ca^{2+}-ATPase activity and fluorescence anisotropy of pyrene-labeled membranes

As shown in Figs. 5A and B, the degree of the fluorescence anisotropy of pyrene-labeled membranes increased depending on the concentration of *t*-BuOOH and it was shown that there is a good correlation between the degrees of increase of the fluorescence anisotropy and inhibition of Ca^{2+}-ATPase activity. On the other hand, treatment of the labeled membranes with DTT, Fe^{2+} or *t*-BuOOH alone did not show appreciable change in the fluorescence anisotropy (data not shown).

Discussion

The results of this study suggest that the inhibition of Ca^{2+}-ATPase activity of the porcine intestinal brush-border membranes by treatment with DTT/Fe^{2+} in the presence and absence of *t*-BuOOH may be due to lipid peroxidation of the membranes.

As shown in Fig. 1, incubation of the membranes with *t*-BuOOH in the presence of DTT/Fe^{2+} resulted in a marked enhancement of TBAR and conjugated diene formation. As is well-known [23–25], reaction of *t*-BuOOH in the presence of Fe^{2+} causes formation of *t*-butoxyl and *t*-butyl peroxy radicals, which then react with membrane lipids to initiate peroxidation. Therefore it could be considered that the effect of *t*-BuOOH observed in the present study may be due to stimulation of lipid peroxidation by these radicals produced during the reaction. In fact, TBAR formation of the membranes by treatment with DTT/Fe^{2+}/*t*-BuOOH was effectively prevented by the addition of thiourea, which can scavenge hydroxyl and *t*-butoxyl radicals [26,27] (Fig. 3).

Inhibition of Ca^{2+}-ATPase activity of the membranes induced by DTT/Fe^{2+} treatment was further strengthened in the presence of *t*-BuOOH, depending on the hydroperoxide concentration (Fig. 2). This inhibition was also effectively prevented by the addition of thiourea (Fig. 3). On the other hand, treatment of the membranes with 10 μM Fe^{2+}, 20 mM DTT or 5 mM *t*-BuOOH alone had no appreciable influence on the ATPase activity. From these results, it can be considered that inhibition of the enzyme activity by DTT/Fe^{2+} or DTT/Fe^{2+}/*t*-BuOOH treatment is related to lipid peroxidation. Treatment of the membranes with DTT/Fe^{2+} in the absence of *t*-BuOOH induced slight decreases in Ca^{2+}-ATPase activity (Table I) and the V_{max} value, in cases where TBAR formation is very little (Fig. 1). This result suggests that Ca^{2+}-ATPase activity of the membranes is sensitively modified even at low levels of lipid peroxidation, because DTT or Fe^{2+} alone did not affect the ATPase activity at the same concentration.

Recently, several investigators have reported that oxidation of SH groups is an important factor in oxygen-radical-mediated inhibition of Ca^{2+}-ATPase activities of sarcoplasmic reticulum [10] and hepatocyte plasma membranes [12]. In the present study, Ca^{2+}-ATPase activity of the intestinal brush-border membranes was not inhibited by treatment with NEM (Table II), suggesting that the SH groups are not required for the ATPase activity as reported in Ca^{2+}-ATPase of rat liver plasma membranes [28]. On the other hand, incubation of the membranes with monoiodoacetate or monoiodoacetamide resulted in an increase in the ATPase activity. It seems that this discrepancy may be due to a difference in their reactivity. In any event, these results indicate that modification of SH groups in the membrane proteins does not induce the inhibition of Ca^{2+}-ATPase activity. Therefore it seems that the loss of Ca^{2+}-ATPase activity after treatment of the membranes with DTT/Fe^{2+} or DTT/Fe^{2+}/*t*-BuOOH is not directly related to a thiol-dependent mechanism.

Results of kinetic studies of Ca^{2+}-ATPase activity revealed that decrease of the ATPase activity is mainly due to decrease of the V_{max} value (Fig. 4) and that the affinity of Ca^{2+} for the membranes is not influenced by lipid peroxidation.

We have previously reported [6] that lipid fluidity of the membranes decreases depending on the degree of

lipid peroxidation induced by ascorbic acid/Fe^{2+}. In the present study, we have also observed an increased fluorescence anisotropy of pyrene-labeled membranes after treatment with DTT/Fe^{2+} in the presence and absence of *t*-BuOOH (Fig. 5A). In general, the degree of the fluorescence anisotropy of fluorophore attached to macromolecules depends on the degree of restriction of the dye molecules [29]. Therefore it seems that an increased fluorescence anisotropy of pyrene-labeled membranes after lipid peroxidation may reflect a restricted motion of the dye molecules in the membrane lipids, i.e., decrease in lipid fluidity. This interpretation was further supported by an increased fluorescence anisotropy of pyrene molecules embedded in the liposomes prepared from the extracted lipids of the peroxidized membranes ($r = 0.106 \pm 0.001$, 0.112 ± 0.001 and 0.124 ± 0.003 for the control, DTT/Fe^{2+}-treated and DTT/Fe^{2+}/*t*-BuOOH-treated membranes, respectively).

As is well-known [30], malondialdehyde produced as a peroxidation byproduct is a cross-linking agent of membrane components. Therefore we have examined the effect of malondialdehyde treatment on the fluorescence anisotropy of pyrene-labeled membranes. Our results revealed that the degree of the fluorescence anisotropy of the complex was unchanged by reaction of the membranes with 500 μM malondialdehyde ($r = 0.115 \pm 0.003$ and 0.111 ± 0.002 for the control and treated membranes, respectively). This result suggests that an increased anisotropy of pyrene-labeled membranes is not attributed to cross-linking formation of both lipids and proteins in the membranes.

It is of interest that there is a correlation between the degrees of Ca^{2+}-ATPase activity and the fluorescence anisotropy of pyrene-labeled membranes (Fig. 5B). It has been proposed [31,32] that mobility of membrane-bound proteins in biological membranes is directly modulated by lipid fluidity, presumably through changes in fatty acid unsaturation. In addition, recently, it has been reported by several investigators [33–35] that membrane lipid composition is more important a factor than previously thought in the regulation of certain protein-mediated activities in biological membranes. In the previous paper [15], we have reported that there is a good correlation between changes in the protein conformation and lipid fluidity of the membranes associated with lipid peroxidation. Therefore it seems that alteration in Ca^{2+}-ATPase activity induced by lipid peroxidation is partly related to modification of the ATPase molecules through changes in lipid–protein interactions due to alteration of the lipid environment around the enzyme, although the exact molecular mechanism for how a decreased lipid fluidity influences Ca^{2+}-ATPase activity is unclear at present. Further detailed experiments such as reconstitution study could shed additional light on this problem.

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