AMINOPHOSPHOLIPID GLYCATION CAUSES LIPID BILAYER STRUCTURE ALTERATIONS AND INHIBITION OF MEMBRANE-BOUND Na^+,K^+-ATPase

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In the present study, we have investigated the possibility that aminophospholipid glycation-mediated perturbations of the POPE/POPC lipid bilayer structure affect the activity and structure of the membrane-bound Na^+,K^+-ATPase. It was found that both glucose and glyceraldehyde (GCA*) reacted with aminophospholipid POPE, forming lipid-linked glycofluorophores with the absorbance and fluorescence properties of protein-linked AGEs. The lipid glycation was accompanied by progressive oxidative modification of unsaturated fatty acid residues. Measurements of the steady-state fluorescence anisotropy of TMA-DPH have been a first attempt at detecting distinctive bilayer structural perturbations induced by aminophospholipid glycation. The steady-state fluorescence anisotropy of TMA-DPH increased with the time of glycation, presumably because of the increased lipid order of the bilayer. To improve the definition of structural alterations of the glycated lipid bilayer, we attempted to measure the dynamics of TMA-DPH and DPH fluorescence. The effect of glycation was both to change the membrane dielectric constant (as probed by TMA-DPH and DPH fluorescence lifetimes) and increase the lipid order (as probed by time-resolved fluorescence anisotropy measurements). The aminophospholipid glycation reduced the activity of Na^+,K^+-ATPase, which was incorporated into glycated POPE/POPC vesicles. The enzyme inhibition correlated with the increase in the steady-state fluorescence anisotropy of TMA-DPH but not with the concentration of MDA (*e.g.*, lipid oxidation). Therefore the inhibition of Na^+,K^+-ATPase activity induced by aminophospholipid glycation seems to be related to the modification of the protein molecule conformation through the lipid bilayer structure alterations. The inhibition of Na^+,K^+-ATPase activity was the sum of at least two factors: the increased lipid order and changed membrane dielectric constant. These factors can alter the lipid–lipid and lipid–protein interactions (*e.g.*, electric multipole–multipole interactions) in membrane and thus provoke the inhibition of membrane bound enzymes.

Key words: Fluorescence anisotropy; Membrane fluidity; ATPases; Lipid glycation; Lipid oxidation.

* Abbreviations: POPC, 2-oleoyl-1-palmitoyl-*sn*-glycerol-3-phosphocholine; POPE, 2-oleoyl-1- palmitoyl-*sn*-glycerol-3-phosphoethanolamine; TMA-DPH, trimethyl[4-(6-phenylhexa-1,3,5-trien-1-yl)phenyl]-amonium; DPH, 1,6-diphenylhexa-1,3,5-triene; MDA, malondialdehyde; SUVs, small unilamellar vesicles; GCA, DL-glyceraldehyde; AGE, advanced glycation end product; TBA, thiobarbituric acid; Na^+,K^+-ATPase, ATP phosphohydrolase (EC 3.6.1.3.); τ_i, lifetime of *i*th component; f_i, fractional intensity of *i*th component; $\Sigma f_i\tau_i$, average excited-state lifetime; r_∞, limiting fluorescence anisotropy; ϕ, rotational correlation time.

Glucose (or the other α-hydroxyaldehydes) can slowly condense nonenzymatically with primary amino groups of biomolecules forming, initially, a Schiff base which may rearrange to form the Amadori product. This early stage of the reaction is called "glycation"[1]. The Amadori product subsequently degrades into α-ketoaldehydes such as 1- and 3-deoxyglucosones. These secondary compounds are more reactive than the parent monosaccharide[2] and can react with amino groups of biomolecules to form cross-links, as well as chromo- and fluorophoric adducts called Maillard products (or advanced glycation end products, AGEs)[3] which result in the biomolecule becoming "browned", fluorescent, and cross-linked *in vitro*. This slow reaction is believed to underlie the pathogenesis of aging[4], diabetes[5], and possibly neurodegenerative amyloidal disorders such as Alzheimer's disease[6]. Oxidation reactions involved in glycation can produce the reactive oxygen species ($O_2^{-\bullet}$, H_2O_2, and $^\bullet OH$). These reactive oxygen species cause subsequent damage to proteins, lipids and DNA (ref.[7]).

Amine-containing phospholipids can react with glucose to initiate the Maillard reaction, forming lipid-linked AGEs and promoting fatty acid oxidation[8,9]. It has been observed that phospholipid glycation and lipid oxidation increase in the course of the chronic hyperglycaemia of diabetes. This supports the concept that lipid glycation can play an important role in initiating lipid oxidation *in vivo*. Thus aminophospholipid glycation could affect membrane functions and all the processes involving aminophospholipids, *e.g.*, the biosynthesis and turnover of the membrane phospholipids[10], physical properties of the membrane (*e.g.*, the lipid order or the lipid hydration)[11,12], and the activity of membrane-bound enzymes that require phospholipids for their functions[13].

The integral membrane proteins require, for their optimal function, a fluid membrane with a minimum bilayer thickness containing unsaturated phospholipid acyl chains[14]. Na^+,K^+-ATPase, also called the sodium pump, is a membrane-bound enzyme responsible for the active transport of Na^+ and K^+ across the plasma membrane of all eukaryotic cells. The enzyme consists of α- and β-subunits, both of which are essential for its function. The lipid bilayer where the Na^+,K^+-ATPase molecules are inserted is widely known to participate in the modulation of the activity of this enzyme[15,16].

In the present study, we have investigated if aminophospholipid glycation-mediated perturbations of the lipid bilayer structure affect the activity and structure of the membrane-bound Na^+,K^+-ATPase. A biologically relevant and well-defined binary lipid mixture system, phosphatidylethanolamine/phosphatidylcholine (POPE/POPC), has been selected. Fluorescent lipid probes, TMA-DPH and DPH, have been used to investigate structural changes of the POPE/POPC vesicles incubated with glucose or glyceraldehyde in physiological conditions. The effect of aminophospholipid glycation was compared with that of lipid oxidation induced by the system L-ascorbic acid/Fe^{2+}.

EXPERIMENTAL

Chemicals

D-Glucose, DL-glyceraldehyde, 2-oleoyl-1-palmitoyl-*sn*-glycerol-3-phosphocholine (POPC), 2-oleoyl-1-palmitoyl-*sn*-glycerol-3-phosphoethanolamine (POPE), malondialdehyde (MDA), and Tris{2-amino-2-(hydroxymethyl)propane-1,3-diol} were obtained from Sigma Chemical Co. 1,6-Diphenylhexa-1,3,5-triene (DPH) and trimethyl[4-(6-phenylhexa-1,3,5-trien-1-yl)phenyl]amonium (TMA-DPH) were purchased from Molecular Probes (Eugene, U.S.A.). All other chemical were of the highest purity commercially available. All solvent used were of spectroscopic grade.

Na^+,K^+-ATPase Preparation

Na^+,K^+-ATPase (EC 3.6.1.3) from outer medulla of pig kidney was prepared by Jørgensen's procedure[17].

Determination of Na^+,K^+-ATPase Activity

Na^+,K^+-ATPase activity was determined according to Schoner *et al.*[18].

Small Unilamellar Vesicles (SUV) Preparation

A suspension of SUVs with the POPE/POPC molar ratio 1 to 2.3 was prepared. Small unilamellar vesicles were prepared by the method of Barenholz *et al.*[19], in which phospholipids were suspended in a buffer containing 20 mM Tris-HCl and 0.02% (w/w) NaN_3 (pH 7.4) and sonicated with a 150 W sonicator (M.S.E., U.K.). After sonication, large multilamellar vesicles were removed by ultracentrifugation at 100 000 g for 1 h. Suspensions of SUV were then diluted to 30 mM concentration.

Preparation of Proteovesicles with Na^+,K^+-ATPase

The glycated or oxidized lipid suspensions were dialyzed to remove unreacted aldehydes. The purified Na^+,K^+-ATPase (100 µl, 12 µg of protein) was added to the suspension of SUVs (5 µl or 10 µl of 15 mM in 20 mM Tris-HCl, 0.02% (w/w) NaN_3, pH 7.4), the total volume was 1 ml. The mixture was kept for 15 min on ice with occasional shaking, then frozen in liquid nitrogen, thawed in a water/ice bath, and sonicated at 50 W for 3×30 s.

Sugar–Lipid Incubations

Suspensions of SUVs were diluted into sterile scintillation vials (final lipid concentration was 15 mM) by buffer containing 20 mM Tris-HCl and 0.02% NaN_3 (w/w), pH 7.4. Afterwards glucose or glyceraldehyde was added (final sugar concentration was 150 mM). The vials were incubated in the dark at 37 °C.

Induction of Lipid Oxidation by System L-Ascorbic Acid/Fe^{2+}

Suspension of SUVs (final lipid concentration was 15 mM) was incubated with 0.4 mM L-ascorbic acid and 0.1 mM $FeSO_4$ in 20 mM Tris-HCl, 0.02% NaN_3 buffer at pH 7.4 for 100 min at 37 °C. The reaction was started by an addition of L-ascorbic acid and stopped by the addition of 5 mM EDTA.

Analysis of Thiobarbituric Acid-Reactive Substances

At different time intervals (0–35 days) the aliquots (1 ml) were analyzed for the TBA-reactive substances content[20]. Lipids and lipid-soluble products were extracted into 500 μl of chloroform–methanol (2 : 1) to remove unreacted sugar. The tubes were gently shaken for 15 min, the interface was cleared by centrifugation, and the extraction was then repeated twice more. The solvent was evaporated under nitrogen, 1 ml of TBA solution was added, and tubes were incubated at 95 °C for 45 min. After reaction with TBA, samples were extracted into 2 ml of butan-1-ol prior to the fluorescence measurement (emission 553 nm upon excitation at 515 nm). TBA-Reactive substances were quantified by comparison of samples with an MDA standard curve (expressed as nmol of MDA equivalents per mg of lipids).

Absorption and Steady-State Fluorescence Measurements

Absorption measurements were performed on a UVICON 810 spectrophotometer. Steady-state fluorescence data were taken on a Perkin–Elmer LS-5 fluorometer. All measurements were done at 25 °C.

Steady-state fluorescence anisotropy of TMA-DPH measurements. TMA-DPH dissolved in methanol was added to the vesicle suspension (roughly 1 μl of probe stock/ml of vesicle suspension), and the mixture was incubated for 30 min in the dark with continuous stirring. The final probe-to-phospholipid ratio was 1 : 500. Excitation and emission wavelengths were 365 nm and 430 nm, respectively. Two Glan–Thompson polarizers were used for detection of steady-state fluorescence anisotropy values.

Measurements of lipid glycation products fluorescence. To measure the formation of glycation products, an aliquot of SUVs (20 μl) was added to 20 mM Tris-HCl buffer pH 7.4 (2 ml). Fluorescence emission at 425 nm (335 nm excitation) was used as a measure of glycation products formation.

Quenching of tryptophan fluorescence. For the analysis of the tryptophan quenching process in Na^+,K^+-ATPase we assumed independent, equally absorbing fluorophores[22]. We considered all the tryptophan residues buried in the membrane to be totally inaccessible to KI and the others to be accessible having the same value of the Stern–Volmer constant, K_q. At such conditions, the classical Stern–Volmer equation is expressed[22] by Eq. (*1*):

$$F_0/(F_0 - F) = 1/(f_a K_q [Q]) + 1/f_a \quad , \qquad (1)$$

where F_0 and F are fluorescence intensities without and with the quencher (KI), respectively, [Q] is the concentration of the quencher and f_a is the fractional maximum of accessible protein fluorescence. From Eq. (*1*), a plot of $F_0/(F_0 - F)$ *vs* 1/[Q] yields a straight line of slope $1/(f_a K_q)$ and intercept $1/f_a$.

Dynamic Fluorescence Measurements and Data Analysis

The glycated or oxidized lipid suspensions were dialyzed to remove unreacted aldehydes. TMA-DPH and DPH dissolved in methanol and acetone, respectively, were added to the vesicle suspension (roughly 1 μl of probe stock/ml of vesicle suspension), and these mixtures were incubated for 30 min in the dark with continuous stirring. The final probe-to-phospholipid ratio was 1 : 500.

Time-domain fluorescence decay measurements were carried out using the time-correlated single photon counting technique at the Institute of Physics, Charles University. The excitation light pulse source was a cavity dumped dye laser (model 375, Spectra Physics, U.S.A.) synchronously pumped by mode-locked argon ion laser (model 171, Spectra Physics, U.S.A.). Excitation pulses with dura-

tion 10 ps were generated at 352 nm with Pyridine 1 as a laser dye. The emission monochromator was set at 430 nm. Temperature during measurements was controlled by a thermostat and the two components, I_{vv} and I_{vh}, were recorded. The total fluorescence decay corresponds to the weighted sum of the two components:

$$I(t) = I_{vv}(t) + [2\beta I_{vh}(t)] , \qquad (2)$$

where β is a correction factor for the transmission of polarized light by the monochromator at a given wavelength. All measurements were performed at 25 °C and each measurement was repeated three times using separate preparations.

We used the "wobble-in-a-cone" model to describe the decay of fluorescence anisotropy[23]. Time-resolved fluorescence anisotropy studies of rodlike probes such as 1,6-diphenylhexa-1,3,5-triene (DPH) in lipid bilayers have demonstrated that the rotational motion of these probes is hindered and distinctly different from the probe motion in isotropic oils[24,25]. Immediately after the flash, the anisotropy equals r_0, the maximal fluorescence anisotropy, and decays towards a finite level, r_∞, the limiting fluorescence anisotropy. A useful approximation of this decay behaviour is

$$r(t) = r_\infty + (r_0 - r_\infty) \exp(-t/\phi) , \qquad (3)$$

where $r(t)$ is the fluorescence anisotropy at time t after the flash and φ is the rotational correlation time. The values of r_∞ and φ were obtained by nonlinear, least-squares fitting.

RESULTS

Generation of Lipid-Derived Glycofluorophores and Lipid Oxidation

Suspensions of POPE/POPC vesicles were incubated with glucose or glyceraldehyde (GCA) under physiological conditions for increasing time intervals. All samples were observed to react with glucose or GCA to form products with absorbance and fluorescence properties of protein AGEs. The glucose-lipid glycation products showed an excitation maximum at 345 nm, an emission maximum at 424 nm, while the GCA-lipid glycation products showed an excitation maximum at 335 nm and an emission maximum at 439 nm. Progress curves for the development of lipid-derived glycofluorophores are shown in Fig. 1 along with curves for generation of malondialdehyde (MDA). Monounsaturated fatty acid residues are 10 to 30 times less susceptible to oxidation than polyunsaturated fatty acids[26] but, once oxidized, can undergo β-scission to form reactive aldehydes (*e.g.*, MDA) that are characteristic for initiation reactions. POPE and POPC used in our experiments contained esterified oleic acid (monounsaturated fatty acid) and palmitic acid (saturated fatty acid). As shown in Fig. 1, lipid oxidation products formed at a rate that was higher but parallel to the rate of glycofluorophores formation for both glucose and GCA.

Effect of Aminophospholipid Glycation on Steady-State Fluorescence Anisotropy of TMA-DPH

TMA-DPH is thought to be anchored with the positively charged trimethylammonium group in the lipid water interface and the phenylhexatriene portion in the acyl chain interior. Thus this probe can reflect molecular motion in head-group region of the bilayer. To observe the effect of aminophospholipid glycation on the lipid bilayer structure, we measured the steady-state fluorescence anisotropy of TMA-DPH as a function of the time of incubation, both for glucose and GCA (Fig. 2). The fluorescence anisotropy of TMA-DPH increased significantly with the time of incubation for both sys-

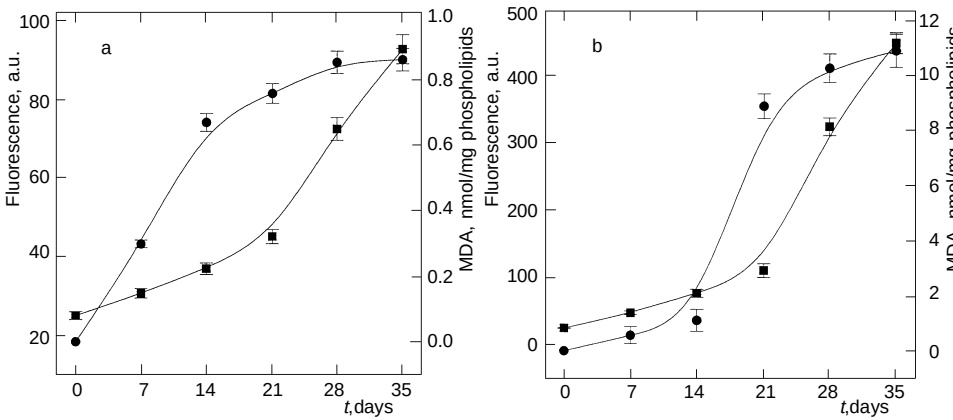

FIG. 1

Progress curves for the development of lipid-derived glycofluorophores along with curves for generation of malondialdehyde (MDA, ●). Fluorescence (■) emission at 425 nm (335 nm excitation) was used as a reflection of glycofluorophores formation. Graph a shows SUVs (30% POPE) glycated by glucose, graph b SUVs (30% POPE) glycated by glyceraldehyde. Experimental points are averages of three independent measurements

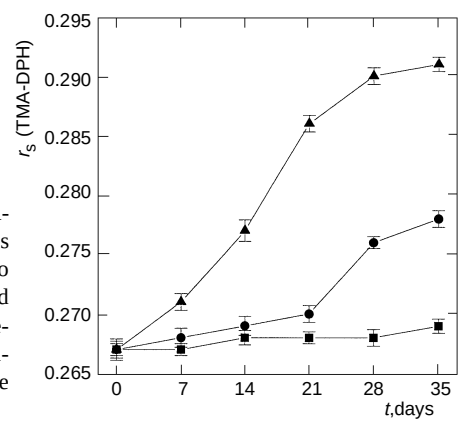

FIG. 2

The steady-state fluorescence anisotropy of TMA-DPH as a function of the incubation time of SUVs (30% POPE) for glucose and GCA. Probe to phospholipid ratio was 1 : 500. Excitation and emission wavelengths were 365 and 430 nm, respectively. Experimental points (■ control, ● glucose, ▲ glyceraldehyde) are averages of three independent measurements

tems, presumably because of the increased lipid order of the bilayer. Figure 3 shows the steady-state fluorescence anisotropy of TMA-DPH as a function of the lipid oxidation for SUVs treated with glucose (line *1*), GCA (line *2*) and the system L-ascorbic acid/Fe^{2+} (line *3*).

Analysis of Fluorescence Lifetimes and Time-Resolved Fluorescence Anisotropy for TMA-DPH

To determine the motion properties of TMA-DPH and DPH in SUVs treated by glycation (with glucose or GCA) or oxidation (induced by the system L-ascorbic acid/Fe^{2+}), the time-resolved fluorescence anisotropy decays were measured using the time-correlated single photon counting technique. The results of dynamic fluorescence data analysis for TMA-DPH are listed in Table I. It was found that fluorescence decay of TMA-DPH could be described adequately by assuming two lifetime components. The contribution of the short lifetime component increased for both glycated and oxidized SUVs. The lifetimes associated with both components decreased, therefore the average lifetime decreased. However, in comparison with glycation, the lipid oxidation (induced by the system L-ascorbic acid/Fe^{2+}) had only slight effect on the TMA-DPH lifetime. Glycation but not oxidation significantly increased the limiting fluorescence anisotropy r_∞, while the rotational correlation time ϕ remained practically unchanged. The influence of the lipid oxidation (induced by the system L-ascorbic acid/Fe^{2+}) was again weaker in comparison with the effect of lipid glycation.

Analysis of Fluorescence Lifetimes and Time-Resolved Fluorescence Anisotropy for DPH

Table II shows the dynamic fluorescence data analysis for DPH. The fluorescence decay of DPH could be also described adequately by assuming two lifetime compo-

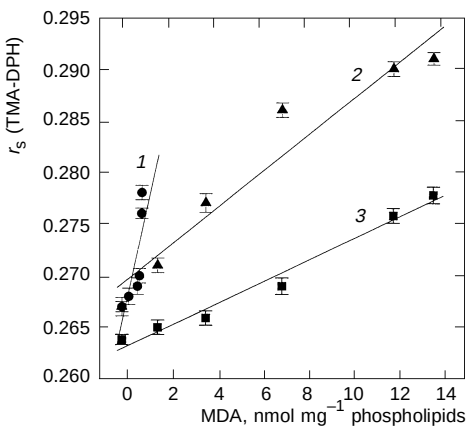

FIG. 3
The steady-state fluorescence anisotropy of TMA-DPH as a function of the lipid oxidation (MDA concentration) for SUVs treated with glucose (line *1*), GCA (line *2*), and the system L-ascorbic acid/Fe^{2+} oxidation system (line *3*). Experimental points are averages of three independent measurements

TABLE I
Summary of TMA-DPH lifetimes and dynamics in glycated and oxidized vesicles

Parameter	SUVs Control	SUVs + Glucose	SUVs + GCA	SUVs + L-Ascorbic acid/Fe^{2+}
f_1	0.385 ± 0.021	0.498 ± 0.015	0.458 ± 0.031	0.499 ± 0.018
τ_1, ns	1.61 ± 0.08	1.48 ± 0.04	1.22 ± 0.08	1.59 ± 0.05
f_2	0.615 ± 0.020	0.502 ± 0.016	0.542 ± 0.030	0.501 ± 0.016
τ_2, ns	5.08 ± 0.06	4.95 ± 0.08	4.17 ± 0.07	5.05 ± 0.07
$\Sigma f_i\tau_i$, ns	3.74 ± 0.23	3.22 ± 0.20	2.81 ± 0.28	3.32 ± 0.26
χ^2	1.09	1.14	1.18	1.12
ϕ, ns	2.72 ± 0.35	2.86 ± 0.41	2.98 ± 0.43	2.79 ± 0.35
r_∞	0.124 ± 0.003	0.134 ± 0.003	0.143 ± 0.004	0.129 ± 0.003

The SUVs were glycated by incubation with glucose or GCA for 35 days in the dark at 37 °C. To oxidize the vesicles on the same level as glycation by GCA, the time of incubation of SUVs with the oxidation system L-ascorbic acid/Fe^{2+} was 100 min. Component lifetimes (τ_i) were obtained by non-linear, least-squares analysis (f_i is the fractional intensity of *i*th component, $\Sigma f_i\tau_i$ is the average excited-state lifetime, r_∞ is the limiting fluorescence anisotropy, and ϕ is rotational correlation time). Quality of fit is indicated by the reduced mean squared deviations of calculated from experimental values (χ^2); values close to 1 indicate an adequate description of the data. Parameter uncertainties are standard deviations based on three independent measurements.

TABLE II
Summary of DPH lifetimes and dynamics in glycated and oxidized vesicles

Parameter	SUVs Control	SUVs + Glucose	SUVs + GCA	SUVs + L-Ascorbic acid/Fe^{2+}
f_1	0.071 ± 0.012	0.056 ± 0.014	0.206 ± 0.024	0.088 ± 0.012
τ_1, ns	2.95 ± 0.09	2.79 ± 0.06	2.35 ± 0.07	2.73 ± 0.05
f_2	0.929 ± 0.013	0.944 ± 0.014	0.794 ± 0.022	0.912 ± 0.012
τ_2, ns	9.19 ± 0.08	8.98 ± 0.07	7.29 ± 0.09	8.99 ± 0.06
$\Sigma f_i\tau_i$, ns	8.75 ± 0.25	8.63 ± 0.24	6.27 ± 0.32	8.44 ± 0.27
χ^2	1.07	1.11	1.19	1.10
ϕ, ns	2.37 ± 0.36	2.49 ± 0.41	2.58 ± 0.45	2.52 ± 0.38
r_∞	0.073 ± 0.003	0.075 ± 0.004	0.088 ± 0.005	0.089 ± 0.003

For details, see Table I (footnote).

nents. The contribution of the long lifetime component and the average lifetime decreased significantly only for SUVs incubated with GCA. Incubation of SUVs with glucose had no influence on motion parameters of DPH. However, both glycation by GCA and oxidation by the system L-ascorbic acid/Fe^{2+} increased significantly the limiting fluorescence anisotropy r_∞, while the rotational correlation time ϕ remained unchanged.

Inhibition of Na^+,K^+-ATPase

To observe the effect of lipid bilayer glycation or oxidation on activity of Na^+,K^+-ATPase, two types of proteovesicles of Na^+,K^+-ATPase, with glycated and oxidized SUVs were prepared. The first group of proteovesicles contained the Na^+,K^+-ATPase mixed with treated lipids in the ratio 1 : 1 (*e.g.*, 1 mg of protein plus 1 mg of lipids), the second group contained the Na^+,K^+-ATPase mixed with lipids in the ratio 1 : 2. The results of enzyme activity, steady-state fluorescence anisotropy of TMA-DPH and lipid oxidation measurements are summarized in Table III. As can be seen from the table, the activity of Na^+,K^+-ATPase was significantly affected by the presence of glycated lipids in the membrane. The enzyme inhibition correlated with the increase in steady-state fluores-

TABLE III
Results of Na^+,K^+-ATPase activity, steady-state fluorescence anisotropy of TMA-DPH and lipid oxidation measurements

System	Ratio protein/lipids	Activity, u/mg	r_S	MDA nmol/mg of phospholipids
Control	1 : 1	56.7 ± 2.1	0.267 ± 0.002	2.62 ± 0.19
	1 : 2	56.5 ± 2.2	0.267 ± 0.002	2.67 ± 0.21
Glucose	1 : 1	50.1 ± 1.6	0.275 ± 0.002	2.47 ± 0.23
	1 : 2	48.5 ± 2.0	0.276 ± 0.003	3.14± 0.27
GCA	1 : 1	41.4 ± 2.2	0.285 ± 0.003	8.31 ± 0.42
	1 : 1	38.3 ± 3.2	0.288 ± 0.004	9.15 ± 0.49
L-Ascorbic	1 :1	55.1 ± 1.8	0.272 ± 0.003	8.04 ± 0.45
acid/Fe^{2+}	1 : 2	54.8 ± 1.9	0.272 ± 0.003	9.26 ± 0.44

SUVs were glycated by glucose (or glyceraldehyde) or oxidized by the L-ascorbic acid/Fe^{2+} oxidation system. Then the vesicles with purified Na^+,K^+-ATPase were mixed with SUVs (weight ratio was 1 : 1 and 1 : 2) and enzyme activity, steady-state fluorescence anisotropy of TMA-DPH (r_S) and lipid oxidation measurements were done. Parameter uncertainties are standard deviations based on three independent measurements.

cence anisotropy of TMA-DPH but not with the concentration of MDA (the oxidized lipids slightly influenced Na^+,K^+-ATPase activity).

Protein Structural Alterations

To explain the inhibition of Na^+,K^+-ATPase after the insertion into the glycated bilayer, we have attempted to measure the accessibility of tryptophan residues to the polar quencher KI (Fig. 4). Modified Stern–Volmer plots of fluorescence quenching for Na^+,K^+-ATPase mixed with non-treated vesicles (line *1*), vesicles glycated by glucose (line *2*), vesicles glycated by GCA (line *3*), and oxidized vesicles (line *4*) are shown in Fig. 4. Table IV contains quenching parameters K_q and f_a, which were calculated using the modified Stern–Volmer plots.

DISCUSSION

In this work, we have investigated the effect of aminophospholipid glycation on the lipid bilayer structure and the activity of membrane-bound Na^+,K^+-ATPase using the fluorescence approach. Suspension of POPE/POPC SUVs (molar ratio 1 : 2.33) has been either incubated with glucose or glyceraldehyde (GCA), or with the oxidation system L-ascorbic acid/Fe^{2+}. It was found that both glucose and GCA reacted with POPE, forming lipid-linked glycofluorophores with absorbance and fluorescence properties of protein-linked AGEs. Glyceraldehyde was considerably more reactive than glucose, presumably due to the presence of the three-carbon chain and impossibility of forming a hemiacetal ring. The lipid glycation was accompanied by progressive oxidative modification of unsaturated fatty acid residues. MDA (the main product of lipid oxidation) was formed at a rate that was parallel but shifted forward to the rate of glycofluorophores formation for both glucose and glyceraldehyde. This means that the lipid oxidation was initiated mainly during the "early" phase of the

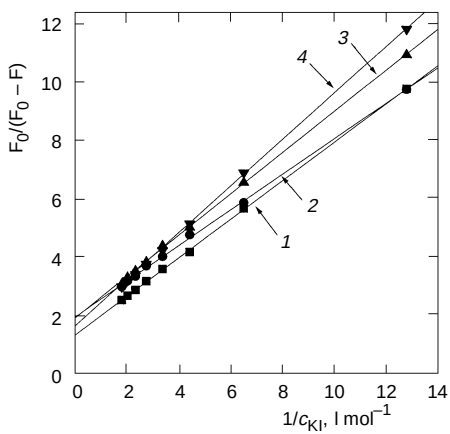

FIG. 4
Modified Stern–Volmer plots of tryptophan fluorescence quenching for Na^+,K^+-ATPase mixed with non-treated vesicles (line *1*), vesicles glycated by glucose (line *2*), vesicles glycated by GCA (line *3*), and oxidized vesicles (line *4*). Experimental points are averages of three independent measurements

glycation reaction and the initiation of oxidation depended on the hydroxyaldehyde reactivity.

Steady-state fluorescence anisotropy of TMA-DPH measurements were a first attempt to detect distinctive bilayer structural perturbations induced by aminophospholipid glycation. The steady-state fluorescence anisotropy of TMA-DPH increased with the time of incubation (Fig. 2), presumably because of the increased lipid order of the bilayer[27]. Nevertheless, the steady-state fluorescence anisotropy can be affected by a change of the fluorescence lifetime. So, to clarify the effect of phospholipid glycation on the lipid order, it is necessary to measure the dynamic fluorescence.

The glycation (Maillard reaction) affects in particular the aminophospholipid head groups[8,9], while the lipid oxidation perturbs the membrane core[28]. Both these processes can change the volume occupied by the phospholipid molecule in the bilayer as well as lipid–lipid interactions and thus the lipid order[28,29]. The difference in slopes for the dependence of the steady-state fluorescence anisotropy of TMA-DPH on the degree of lipid oxidation (Fig. 3) (for glycation and oxidation) has certain implications. Thus, upon aminophospholipid modification by glucose or GCA, the resulting lipid oxidation cannot be used to explain the total increase in the steady-state fluorescence anisotropy of TMA-DPH (*e.g.*, the increase of lipid order). We suppose that the total increase in the steady-state fluorescence anisotropy of TMA-DPH in the case of the lipid glycation seems to be the sum of more effects.

The detailed nature of these structural alterations is not clear from these steady-state anisotropy data. To improve definition of these structural changes, we attempted to measure the dynamics of TMA-DPH and DPH fluorescence. It was found that lipid glycation by GCA significantly decreased the fluorescence lifetime of both probes

TABLE IV
Accessibility of tryptophan residues of Na^+,K^+-ATPase inserted into non-treated, glycated and oxidized lipid bilayer to iodide quencher

System	Ratio protein/lipid	K_q, $dm^3\ mol^{-1}$	f_a
Control	1 : 1	1.685 ± 0.017	0.77 ± 0.02
	1 : 2	1.679 ± 0.020	0.77 ± 0.02
Glucose	1 : 1	1.463 ± 0.012	0.57 ± 0.03
	1 : 2	1.395 ± 0.021	0.52 ± 0.03
GCA	1 : 1	1.417 ± 0.033	0.57 ± 0.02
	1 : 2	1.334 ± 0.023	0.53 ± 0.02
L-Ascorbic acid/Fe^{2+}	1 : 1	1.622 ± 0.015	0.63 ± 0.02
	1 : 2	1.598 ± 0.020	0.62 ± 0.03

Parameter uncertainties are standard deviations based on three independent measurements.

(Tables I and II). The fluorescence lifetime of the fluorophore in the lipid bilayer depends on its dielectric environment provided that no excited state reactions such as excimer formation occur. Small alterations in the membrane dielectric constant have been shown to significantly affect the fluorescence lifetime due to fluorophore excited state–water interactions[13,17–19]. We assume that the effect of aminophospholipid glycation was to change the dielectric constant both in the head-group and acyl chain region of the membrane (in particular glycation by GCA) as probed by the decrease in average TMA-DPH and DPH fluorescence lifetimes ($\Sigma f_i \tau_i$). This contrasted with the effect of the system L-ascorbic acid/Fe^{2+}, which had a weak effect on the average fluorescence lifetimes (*e.g.*, the membrane dielectric constant). The change in the membrane dielectric constant could alter all lipid–lipid and lipid–protein interactions (*e.g.*, electric multipole–multipole interaction) and thus properties of the lipid bilayer[29].

Both aminophospholipid glycation and oxidation were found to increase the limiting fluorescence anisotropy of DPH (r_∞). However, the effect of glycation (in particular GCA) was more potent in comparison with the oxidation promoted by the system L-ascorbic acid/Fe^{2+} (Tables I and II). These results also showed that aminophospholipid glycation affected mainly the head-group region of the membrane, while the oxidation system L-ascorbic acid/Fe^{2+} increased order only in the acyl chain region. This difference could be explained by the glycation-mediated modification of head-groups of POPE molecules which is accompanied by the increase in the head-group volume, steric head-group interactions and also electrostatic interactions between charged groups[29]. On the other hand, the lipid oxidation, which degrades the unsaturated acyl chain of phospholipids, can influence mainly the order in the acyl chain region of the membrane[28,30].

It has been reported that the lipid order and lipid hydration are not simply related quantities[12,31]. For example, cholesterol increases both the lipid order and the hydrophobicity in the acyl chain region of the bilayer. However, in the head-group region cholesterol increases lipid hydration. Modification of the acyl chain region and resultant alterations of the lipid order cannot be used as a reliable indicator of effects at the head-group region, at least in terms of hydration. The different effects of glycation and oxidation on the lipid hydration and lipid order (Tables I and II) support the recent hypothesis that the lipid order cannot exactly predict the functional effects on membrane proteins, which may also be affected by altered hydration at the head-group region of the bilayer.

It is well known that the change of the membrane lipid order (*e.g.*, membrane fluidity) can influence membrane-bound proteins, resulting in their changed structural and functional properties[27]. It may be supposed that the increase in lipid order can affect the protein conformation[28–30]. We found that aminophospholipid glycation increased the limiting anisotropy of both probes and changed the membrane dielectric constant. Therefore the inhibition of Na^+,K^+-ATPase activity induced by aminophos-

pholipid glycation seems to be related to the modification of the protein conformation through the lipid bilayer alterations. The collisional quenching of protein tryptophan residues by KI showed that after lipid glycation, more tryptophan residues are inaccessible to KI (Table IV). This conformational change is probably a consequence of the increase in the lipid order, which caused the "compression" of the protein molecule in the membrane. But the inhibition of Na^+,K^+-ATPase activity could be the sum of at least two factors: an increased lipid order and a changed membrane dielectric constant. These factors can alter the lipid–lipid and lipid–protein interactions (*e.g.*, electric multipole–multipole interaction) in membranes and thus provoke the inhibition of membrane bound enzymes.

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