Control of a Redox Reaction on Lipid Bilayer Surfaces by Membrane Dipole Potential

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ABSTRACT Nitro-2,1,3-benzoxadiazol-4-yl (NBD) group is a widely used, environment-sensitive fluorescent probe. The negatively charged dithionite rapidly reduces the accessible NBD-labeled lipids in liposomes to their corresponding nonfluorescent derivatives. In this study both the phospholipid headgroup and acyl chain NBD-labeled L-α-1,2-dipalmitoyl-*sn*-glycero-3-phospho-[*N*-(4-nitrobenz-2-oxa-1,3-diazole)-ethanolamine] (DPPN) and 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC), respectively, were employed. The correlation of both the rate coefficient k_1 of the redox reaction and the fluorescence properties of the two probes with the membrane dipole potential Ψ in fluid dipalmitoylglycerophosphocholine (DPPC) liposomes is demonstrated. When Ψ of the bilayer was varied (decreased by phloretin or increased by 6-ketocholestanol), the value for k_1 decreased for both DPPN and NBD-PC with increasing Ψ. For both fluorophores a positive correlation to Ψ was evident for the relative fluorescence emission intensity (RFI, normalized to the emission of the fluorophore in a DPPC matrix). The relative changes in emission intensity as a function of Ψ were approximately equal for both NBD derivatives. Changes similar to those caused by phloretin were seen when dihexadecylglycerophosphocholine (DHPC) was added to DPPC liposomes, in keeping with the lower dipole potential for the former lipid compound compared with DPPC. These effects of Ψ on NBD fluorescence should be taken into account when interpreting data acquired using NBD-labeled lipids as fluorescent probes.

INTRODUCTION

7-Nitro-2,1,3-benzoxadiazol-4-yl (NBD) is a widely used fluorescent moiety in investigations on biological systems (for review see Chattopadhyay, 1990). In studies on biomembranes NBD-labeled lipids have been employed in applications ranging from the visualization of domain morphology by epifluorescence microscopy of lipid monolayers (Weis, 1991) to the measurement of temperature in living cells (Chapman et al., 1995), detection of bilayer-to-hexagonal phase transition (Hong et al., 1988. Stubbs et al., 1989. Han and Gross, 1992), studies of organizational changes in membranes (Mukherjee and Chattopadhyay, 1996), and detection of interdigitation (Li and Kam, 1997). Simple NBD derivatives have three major absorbance bands in the visible and near UV region, at ~420, 306–360, and 225 nm (Lancet and Pecht, 1977; Fery-Forgues et al., 1993). The first absorption peak corresponds to the band of NBD-labeled lipids near 470 nm (Fig. 1) and is due to an intramolecular charge-transfer (ICT) type transition (Paprica et al., 1993; Fery-Forgues et al., 1993), which is associated with a large (~4 Debye) change in the dipole moment (Mukherjee at al., 1994). The 306–360-nm absorbance band (for NBD-lipids, ~335 nm) corresponds to an ordinary $\pi^* \leftarrow \pi$ transition (Fery-Forgues et al., 1993). Regardless of the absorbance band used for excitation, the maximum emission wavelength λ_{max} of NBD-labeled lipids lies at 520–535 nm (Fig. 1). Absorbance, fluorescence intensity, and both absorbance maximum wavelength of the ICT transition and the corresponding emission λ_{max} are sensitive to the polarity and hydrogen bonding capability of the environment and to the presence of charge transfer donors (Lancet and Pecht, 1977; Fery-Forgues et al., 1993; Lin and Struve, 1991; Saha and Samanta, 1998).

NBD-labeled lipids have been used also to study phospholipid asymmetry (McIntyre and Sleight, 1991). More specifically, when added to liposomes containing NBD-labeled lipids, either dithionite or its negatively charged radical form can rapidly reduce NBD to the the nonfluorescent product 7-amino-2,1,3-benzoxadiazol-4-yl (ABD; Fig. 2), thus allowing for the assessment of the content of the probe in the outer leaflet of the bilayer. Simultaneously with this rapid reaction dithionite ion diffuses slowly across the bilayer, so as to reduce also the NBD groups in the inner leaflet. The reduction of NBD-labeled lipids by dithionite has been further used, e.g., to make asymmetrically labeled liposomes (McIntyre and Sleight, 1991), to monitor lipid flip-flop (Williamson et al., 1995), and to study the properties of membranes undergoing phase transitions (Langner and Hui, 1993).

The electric properties of lipid bilayers are currently gaining increasing attention. Of particular interest is the dipole potential Ψ, which is caused by the oriented arrays of electric dipoles in the membrane and in the membrane-water interface. However, a more complex picture arises when the contributions of the interfacial water molecules, phospholipid headgroups, glycerol ester groups, and the

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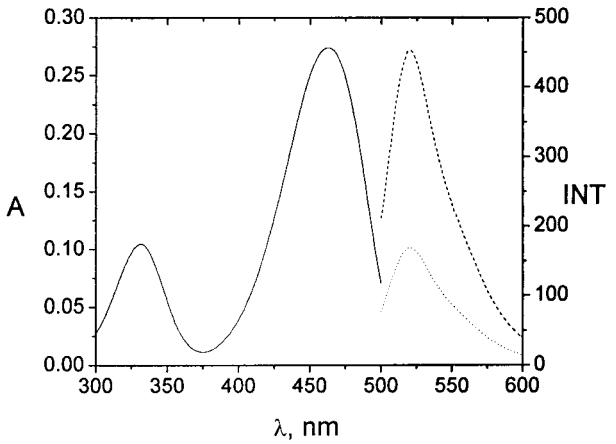

FIGURE 1 Absorption (——) spectrum and emission spectra of DPPN in methanol. The upper emission spectrum (– –) was recorded with excitation at 470 nm, and the lower spectrum (- - -) with excitation at 335 nm. Spectra were measured at ambient temperature.

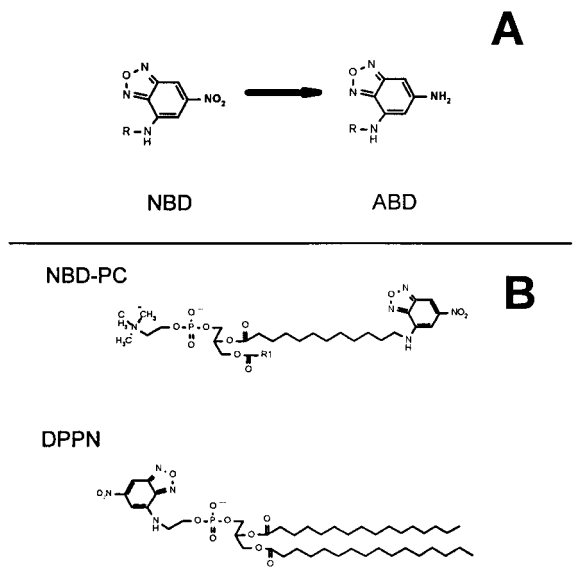

FIGURE 2 (*A*) Chemical structures of NBD and its reduction product ABD. (*B*) Also shown are the chemical structures of the NBD-labeled lipids used in this study. R1 represents an alkyl chain.

terminal methyl groups of the acyl chains are considered (Brockman, 1994). Recent molecular dynamics simulations (Chiu et al., 1995; Marrink et al., 1996) suggest that in didimyristoylglycerophosphocholine (DMPC) and dipalmitoylglycerophosphocholine (DPPC) bilayers Ψ originates from the oriented water molecules overbalancing the contribution of the lipid dipole moments. In both simulations the absolute individual contributions of lipid and water were many times greater than that of the net dipole potential, and the slight overbalancing by water is caused by the steric constraints imposed by the optimization of hydrogen bonding between the water molecules intercalated into the interfacial region. The thickness of the region with the most polarized water molecules (Marrink et al., 1996) agrees quite well with the experimental value of 0.5 nm or less for the effective separation of the layers of partial charges that generate dipole potential (Gross et al., 1994). Considering that the strength of the immense electric fields generated by dipole potential can be as high as 1 GV/m (Gross et al., 1994), it is not surprising that Ψ has been suggested to play a functional role in biomembranes (e.g., Andersen et al., 1976; Cafiso, 1998; Cladera and O'Shea, 1998; Reyes and Benos, 1984; Rokitskaya et al., 1997; Silvestroni et al., 1997; Starkov et al., 1997).

The electronic transitions of the fluorescent probes confined into the interfacial region can be expected to be sensitive to dipole potential. In this study we used phloretin and 6-ketocholestanol (6-KC) to vary Ψ of fluid DPPC liposomes while monitoring the changes in the fluorescence behavior of L-α-1,2-dipalmitoyl-*sn*-glycero-3-phospho-[*N*-(4-nitrobenz-2-oxa-1,3-diazole)-ethanolamine] (DPPN) and 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC). In DPPN the NBD moiety is linked to the headgroup, thus restricting its movements and maintaining it in the interface. In NBD-PC the NBD moiety is linked to the acyl chain. We also measured the dependency of the rate of reduction of NBD by dithionite as a function of Ψ.

MATERIALS AND METHODS

Materials

DPPC, 6-KC, and phloretin were purchased from Sigma Chemical Co. (St. Louis, MO), Hepes was from Boehringer Mannheim (Mannheim, Germany), and sodium dithionite, sodium carbonate, and sodium hydrogen carbonate were from Merck (Darmstadt, Germany). NBD-PC was from Avanti Polar Lipids (Alabaster, AL), DPPN was from Molecular Probes (Eugene, OR), and DHPC was from Bachem (Budendorf, Switzerland). Stock solutions were prepared in chloroform, except for phloretin and NBD-PC, which were dissolved in ethanol and in toluene:ethanol (1:1, v/v), respectively. The concentrations of fluorescent lipids were determined spectrophotometrically using the molar absorptivities ϵ_{463} = 21,000 M^{-1} cm^{-1} (in CH_3OH) and ϵ_{465} = 19,000 M^{-1} cm^{-1} (in C_2H_5OH) for DPPN and NBD-PC, respectively. The concentrations of the nonlabeled lipids were determined gravimetrically with a high precision electrobalance (Cahn Instruments, Cerritos, CA).

Preparation of liposomes

Appropriate amounts of the lipid stock solutions were mixed in chloroform, evaporated to dryness under a gentle nitrogen stream, and then maintained under reduced pressure for a minimum of 1.5 h to remove any residual solvent. The dry lipid residues were hydrated with 5.0 mM Hepes, pH 7.4, after which the suspensions were extensively vortexed, incubated for 30 min at 50°C with continuous shaking, sonicated for 2 min with a bath sonicator, and incubated for another 30 min at 50°C. To produce unilamellar vesicles hydrated lipid solutions were extruded with a LiposoFast pneumatic low-pressure homogenizer (Avestin, Ottawa, Canada) 19 times through Millipore (Bedford, MA) polycarbonate filters (pore size 100 nm)

at a temperature well above the main-phase transition temperature of the lipid. After extrusion, the liposome solutions were kept on ice until used.

Steady-state fluorescence measurements

Fluorescence emission spectra were recorded using a Perkin-Elmer LS50B spectrofluorometer with a magnetically stirred cuvette compartment thermostatted with a circulating waterbath (Haake C25, Haake, Germany). The temperature in the cuvette was measured with an immersed probe (HH42 Digital Thermometer, Omega Engineering, Stamford, CT). Excitation and emission bandpasses were set at 5 nm. The fluorometer is connected to a Pentium 133-MHz PC and the data were analyzed by the dedicated software from Perkin-Elmer. Individual spectra were collected as averages of five scans. The total lipid concentration in all the steady-state fluorescence measurements was 100 μM.

Stopped-flow fluorescence spectroscopy

The kinetics of the reduction of NBD-labeled lipids by dithionite were measured using a stopped-flow spectrofluorometer (Olis RSM 1000F, On-Line Instruments, Bogart, GA) equipped with a rapid scanning monochromator and a water-cooled 450-W Xe lamp as described previously (Söderlund et al., 1999). Two pneumatically driven (gas pressure 7 bar) parallel-mounted syringes inject the reactants into the rapid mixing quartz glass fluorescence observation chamber (20-mm path length, 1.5 mm diameter). The temperature in the fluorescence observation chamber and in the syringes was controlled by a circulating water bath. One of the syringes was loaded with a liposome solution with a total lipid concentration of 100 μM in 5.0 mM Hepes buffer, pH 7.4. The other syringe contained 10 mM dithionite. To slow down its initial reaction with protons upon dissolving, the latter was made by first dissolving sodium dithionite to a 0.2 M solution in 2.5 mM sodium carbonate buffer (pH 10.5). Subsequently, this was diluted to 10 mM with 5.0 mM Hepes, pH 7.4. The 0.2 M and 10 mM solutions were used within 45 min. Within this time period, no errors in reaction rates caused by the oxidation of dithionite were evident. The concentrations in the observation chamber were 5.0 mM for dithionite and 50 μM for lipid. The mole fraction X of the NBD-labeled lipids was 0.03 yielding 1.5 μM concentration of the indicated fluorescent markers. A thermocouple was used to measure temperature in the observation chamber. An excitation wavelength of 470 nm was selected with a monochromator and the beam was passed through two subsequent 6.32-mm slits. Between 21 and 62 spectra per second were measured by passing the beam through a 3.16-mm slit and a rotating spoke wheel. Fluorescence emission between 473.5 and 626.5 nm was recorded by a photomultiplier tube. Photomultiplier output was digitized by a Pentium PC equipped with an A/D converter. A representative trace for the reduction of NBD by dithionite is illustrated in Fig. 3. The fast bimolecular reaction of the NBD groups located on the outer surface with dithionite was found to obey pseudo-first-order kinetics, in keeping with the NBD and dithionite concentrations of 1.5 μM and 5.0 mM, respectively, in the mixing chamber. The slower component consisting of the diffusion of dithionite through the membrane and the subsequent reduction of the NBD groups located on the inner surface could be fitted as a first-order reaction, as shown previously by Langner and Hui (1993). Accordingly, two independent first-order reactions provided the simplest model yielding good agreement with the measured data. Software provided by instrument manufacturer was used to fit data by the equation

$$U = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (1)$$

where k_1 and k_2 represent rate coefficients (Steinfeld et al., 1998) for the fast and slow components, respectively, A_1 and A_2 represent the corresponding amplitudes, and U represents the photomultiplier tube output voltage. The rate coefficients were derived from the collected data using nonlinear least-squares fitting procedures by both Levenberg-Marquardt algorithm and Successive Integration method.

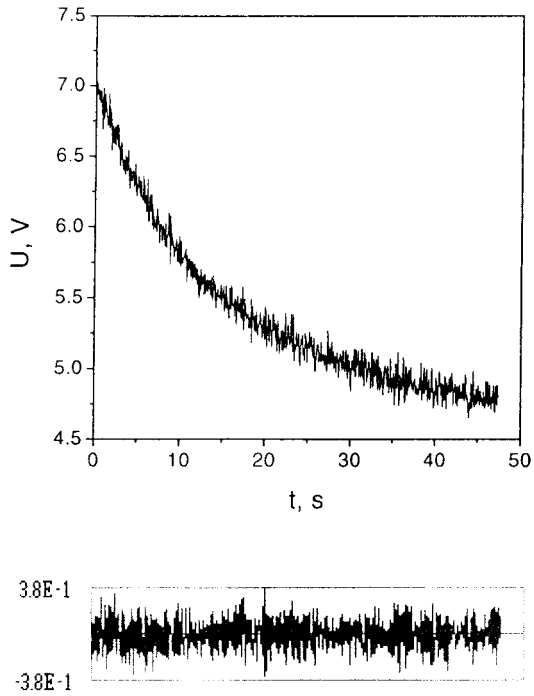

FIGURE 3 (*A*) A representative fluorescence intensity trace of the reduction by 5 mM dithionite of the 1.5 μM NBD moiety of DPPN (X = 0.03) residing in DPPC liposomes. Total lipid concentration is 50 μM in 5 mM Hepes, pH 7.4. Temperature was maintained at 45°C. (*B*) The residuals of the fit as two concurrent first-order reactions.

RESULTS

Effects of 6-KC and phloretin

The sensitivity of fluorescence characteristics of NBD to its environment is well established (Fery-Forgues et al., 1993; Lancet and Pecht, 1977; Lin and Struve, 1991; Paprica et al., 1993; Saha and Samanta, 1998). However, to our knowledge, their dependence on membrane dipole potential of vesicles has not been investigated previously. Accordingly, Ψ for DPPC large unilamellar vesicles (LUVs) was either decreased or increased by including increasing contents of either phloretin (PHL) or 6-KC (up to X = 0.30), respectively (Franklin and Cafiso, 1993), while studying the changes in the fluorescence emission of DPPN (X = 0.03). The values for the changes in Ψ were derived from EPR measurements of hydrophobic anion and cation binding and translocation rates, the effective dipole moments calculated from these values, and a model that assumes the additives to be dipoles within the membrane (Franklin and Cafiso, 1993). PHL and 6-KC have been previously used in a similar way to characterize the response of fluorescent probe di-8-ANEPPS to the membrane dipole potential (Gross et al., 1994; Clarke and Kane, 1997). In DPPN the

NBD moiety is linked to the nitrogen of the phosphoethanolamine headgroup (Fig. 1 *B*) and should thus be sensitive to the electric fields imposed over the interface. All measurements were carried out at 45°C, causing the liposomal membranes to be in the liquid crystalline state (Fig. 4 *A*). Under these conditions the values of RFI, i.e., the maximum emission intensities (at 521–523 nm) relative to the maximum emission intensity of DPPN in neat DPPC matrix, increase and decrease when 6-KC or PHL, respectively, are included in the vesicles so as to vary Ψ. The correlation with $\Delta\Psi$ is nearly linear, the values for RFI increasing with the dipole potential (Fig. 4 *A*).

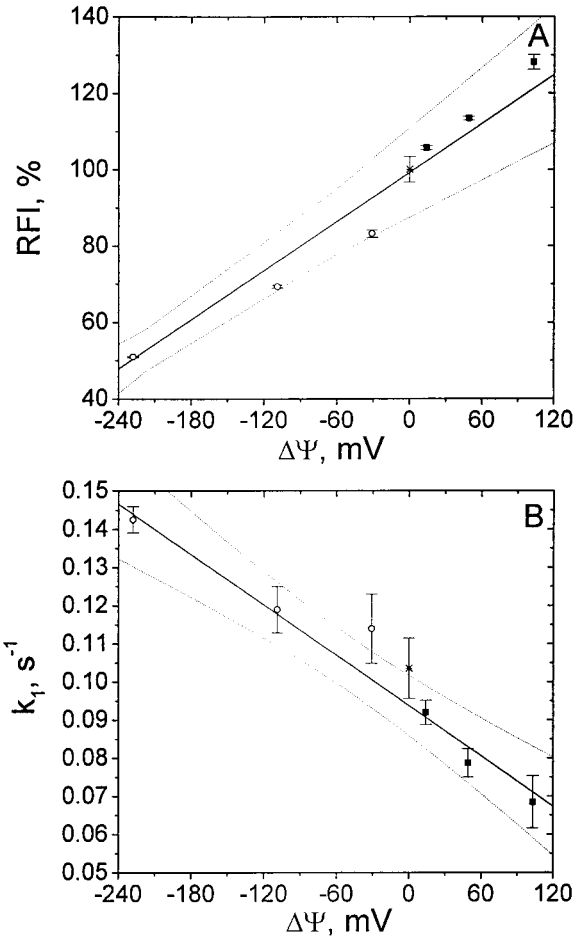

FIGURE 4 The dependency of DPPN fluorescence on dipole potential Ψ, varied by adding either 6-KC (■) or PHL (○) into DPPC liposomes. The mole fractions of the above modulators of Ψ were 0.05, 0.15, and 0.30. The value measured for neat liposomes is marked by an asterisk. Buffer is 5 mM Hepes, pH 7.4, and temperature is 45.0°C ± 0.2°C. Other rate coefficients correspond to averages of 9 measurements, but for X_{PHL} = 0.30 of 7 measurements and for DPPC LUVs to an average of 16 measurements. Error bars represent standard deviation. The solid line represents linear fit weighed with error bars, and dotted lines represent 99% confidence limits. (*A*) RFI measured as a function of $\Delta\Psi$ at λ_{exc} = 470 nm. Correlation coefficient for linear fit is 0.9Ψ. (*B*) The rate coefficient plotted against $\Delta\Psi$. Correlation coefficient for linear fit is −0.987.

In the next series of experiments we investigated the effect of dipole potential on the reduction of DPPN by dithionite. The latter carries a net charge of −2 and its approach to the NBD moiety of the DPPN can therefore be expected to be sensitive to the prevailing electric fields in the vicinity of the surface. The rates for the fast reaction were measured for membranes containing different mole fractions of either PHL or 6-KC. The measured rate coefficients k_1 versus $\Delta\Psi$ are illustrated in Fig. 4 *B*. The nearly linear correlation suggests that in fluid DPPC membranes the rate of this redox reaction is modulated by the dipole potential. Accordingly, an increase in Ψ by 6-KC decreases k_1, whereas a decrease in Ψ by PHL has the opposite effect.

The pK_a of PHL in solution is 7.35 (Reyes et al., 1983), close to the pH of the aqueous buffer used. The active form of PHL in membranes is known to be the neutral state (Jennings et al., 1976; De Levie et al., 1979; Cseh and Benz, 1999), so the main question concerns its partitioning. Yet the local, interfacial pH on the surface of the liposomes is much less due to the presence of the acidic DPPN (X = 0.03). This and the good correlation with the calculated dipole potential changes suggest that most of the phloretin is partitioned into the bilayer.

To test whether solution screening affects the system, similar experiments as described above were carried out in a buffer containing 1.0 M NaCl. However, no significant effects on RFI were revealed. Likewise, the effects on k_1 of 6-KC and PHL persisted, although for all four membrane compositions used the values for k_1 were increased. This is probably due to the screening of the respective negative charges of the dithionite and DPPN.

Effect of ester carbonyls

Dipole potential is strongly affected also by the phospholipid structure. To this end, the deletion of the ester bond carbonyls of DPPC to yield the ether lipid DHPC decreases Ψ by approximately 118 ± 15 mV, from 227 to 109 mV (Gawrisch et al., 1992). As an independent test to the effects of Ψ on DPPN fluorescence DPPC was progressively replaced by DHPC in fluid LUVs while recording RFI (Fig. 5 *A*). In keeping with the above measurements on the effects of 6-KC and PHL this change in membrane composition decreased RFI. Subsequently, we assessed the effect of ether bonds on the rate of reduction of DPPN by dithionite. These stopped-flow fluorescence measurements revealed k_1 to increase from 0.094 s^{-1} to 0.130 s^{-1}, upon the gradual substitution of DPPC for DHPC (Fig. 5 *B*). On the basis of the linear fit of the 6-KC and PHL data this change in k_1 corresponds to a decrease in Ψ by 166 mV. Combining the error limits of the linear fit parameters in Fig. 4 and the error limits for our measurements, the estimated decrease in dipole potential is 76–124 mV on the basis of RFI and 121–219 mV on the basis of the values for k_1, in good

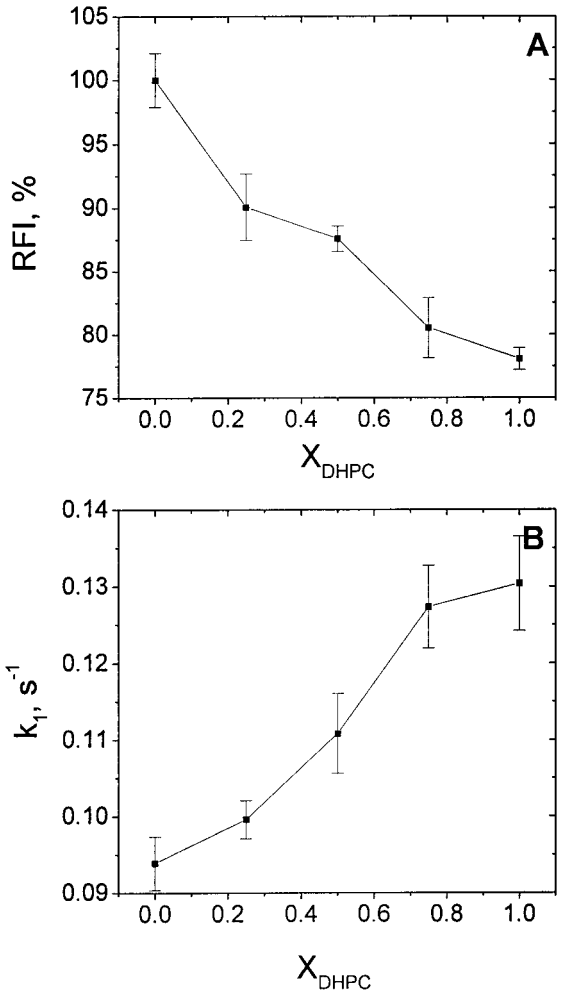

FIGURE 5 Effects of substituting DPPC for DHPC, with DPPN (X = 0.03) as a fluorescent marker. The mole fraction of DHPC was progressively increased. Temperature was maintained at 45°C±0.2°C. (*A*) RFI for DPPN as a function of X_{DHPC}. Fluorescence intensities are averages of three separate measurements for three liposome samples. Error bars represent standard deviation. (*B*) The rate coefficient k_1 is shown as a function of X_{DHPC}. Rate coefficients represent averages (n = 9–12).

agreement with the measurements by Gawrisch et al. (1992).

Comparison with the chain-labeled NBD-lipid

In the light of the above data on the headgroup-labeled DPPN it was of considerable interest to study also the properties of the chain-labeled NBD-PC. Studies using the parallax method have suggested that the NBD moiety attached to the acyl chain causes the acyl chain to loop back toward the water phase, so that the NBD moiety of NBD-PC resides at approximately the same depth within the membrane as does that of DPPN (Chattopadhyay and London, 1987; Abrams and London, 1993). For the NBD-PC probe residing in LUVs, RFI responds in a nearly linear fashion to $\Delta\Psi$ upon inclusion of 6-KC and PHL (Fig. 6 *A*).[7] Interestingly, the linear fits for DPPN and NBD-PC have approximately equal slopes of 0.239 ± 0.021 and 0.205 ± 0.015, respectively. As the actual intensities at 45°C are quite different, ~680 and 320 in arbitrary units for DPPN and NBD-PC, respectively, the relative but not the absolute responses to $\Delta\Psi$ are the same for both probes. The values for the rate coefficient k_1 for the reduction of NBD-PC by dithionite exceed those for DPPN. The error bars in Fig. 6 *B* are large enough to make comments about qualitative similarity ambiguous. However, we have calculated the χ^2 values and the probability that all the values measured for all the different membrane compositions could be explained using a single k_1 value. The p value is ~5%. It thus seems unlikely that the average values of k_1 for the different membrane compositions could be interpreted as deviations from a single rate coefficient. This is further supported by the calculation of the probability that the data can be presented with best linear fit, which yields $p > 50\%$. We thus argue that although the linearity of the change may be questionable, it is likely that k_1 is indeed changing when the membrane composition is altered. Judged by the k_1 versus $\Delta\Psi$ data the effect of Ψ appears to be quite small (Fig. 6 *B*). Yet the absolute values for the changes of k_1 for NBD-PC and DPPN are approximately equal (Fig. 6 *C*). Although it is possible that the correspondence between absolute changes is just coincidental, it is also possible that it reflects the actual behavior of the system. We may thus conclude also that NBD-PC responds to Ψ in a qualitatively similar manner as DPPN.

DISCUSSION

The molecular level origins of dipole potential have been extensively discussed (Brockman, 1994; Langner and Kubica, 1999). The present data demonstrate that the fluorescence emission intensity of the NBD moiety covalently linked to either the headgroup (DPPN) or the acyl chain (NBD-PC) of a phospholipid is positively correlated to the membrane dipole potential Ψ varied by the inclusion of either 6-KC or PHL into the bilayer. In contrast, the rate coefficient k_1 for the reduction by dithionite of the NBD in these probes shows a negative correlation with Ψ. Dipole potential is sensitive also to the lipid structure. The magnitude of $\Delta\Psi \approx 100$ mV estimated from the RFI of DPPN for the substitution of the ester linkages of DPPC by ether bonds to yield DHPC is close to the value of 118 ± 15 mV reported previously (Gawrisch et al., 1992). In contrast to the large changes in their quantum yields (Fery-Forgues et al., 1993), the maximum emission wavelength λ_{max} of NBD is known to be relatively insensitive to the solvent polarity (Chattopadhyay and London, 1988; Fery-Forgues et al., 1993). In keeping with the above, despite the pronounced differences in RFI, the differences in λ_{max} were negligible, and no significant spectral broadening was seen upon varying Ψ.

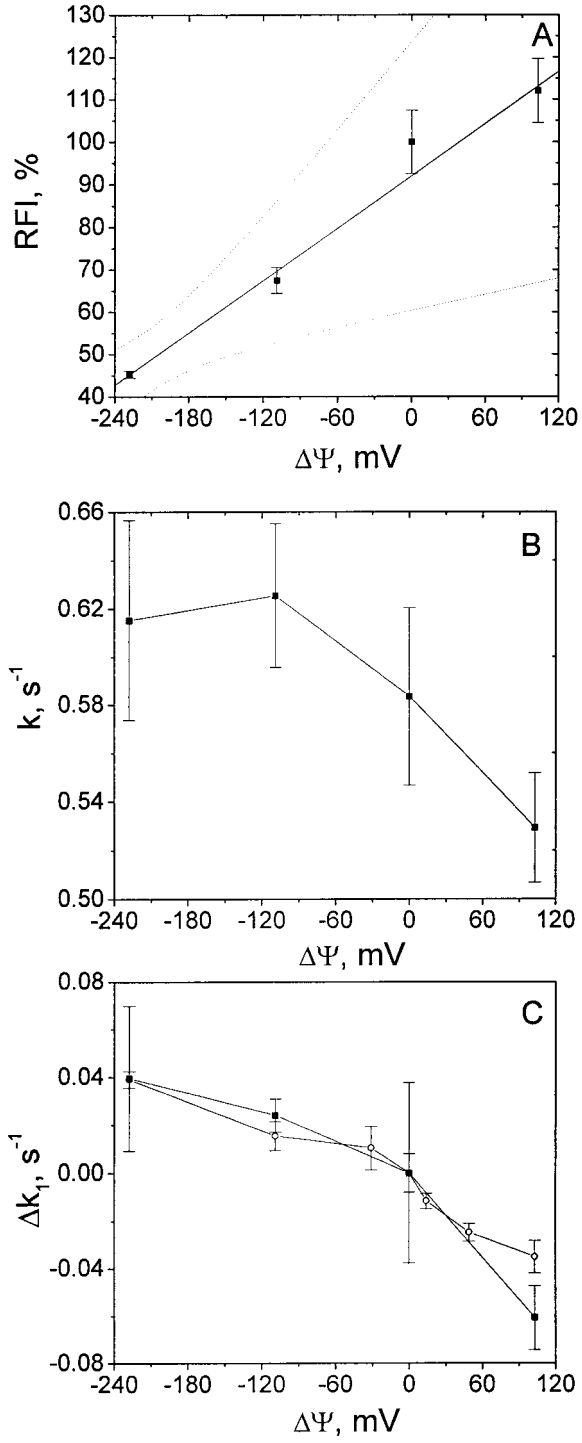

FIGURE 6 The dependence of NBD-PC fluorescence and the rate coefficient k_1 for the reduction of NBD on dipole potential Ψ, varied by adding either 6-KC or PHL into DPPC liposomes. The mole fractions of the above modulators of Ψ were 0.30 for 6-KC, and 0.15 and 0.30 for PHL. Buffer is 5 mM Hepes, pH 7.4, and temperature is 45.0°C ± 0.2°C. RFI values correspond to averages of three samples. The values for the rate coefficient correspond to averages of 18–19 measurements, with error bars showing standard deviation. The solid line represents linear fit weighed with error bars, and dotted lines represent 99% confidence limits. (*A*) RFI measured as a function of $\Delta\Psi$ at λ_{exc} = 470 nm. Correlation coefficient for linear fit is 0.922. (*B*) The rate coefficient plotted against $\Delta\Psi$. (*C*) The absolute changes in rate coefficient k_1 plotted against $\Delta\Psi$. ■, absolute changes for NBD-PC; ○, those for DPPN. The error bars are larger for NBD-PC due to much smaller relative changes.

Mechanism(s) of the dependence of the RFI of NBD on dipole potential

In an isotropic solution the orientation of solvent molecules around the solute fluorophore is determined by the interactions between their dipoles. The damping of electric fields by field-induced dipoles or by polar solvent molecules orienting themselves against the field is described by the relative permittivity of the solvent. The above is contrasted by the highly anisotropic environment in the surface of lipid bilayers. As supported by anisotropic fluctuations of the interfacial molecules seen in molecular dynamics simulations (Shinoda et al., 1998), and by theoretical work based on continuum assumptions (Raudino and Mauzerall, 1984), the permittivities parallel and perpendicular to the plane of the interface should be different. Consequently, the effective solvent environment can be expected to depend on the orientation of the transition dipoles of the dye. For NBD the angle between the absorption and emission dipoles is ~25° (Thompson et al., 1984), and thus the effective environment may differ for the absorption and emission. There is also a steep polarity gradient across the interface (White and Wimley, 1998). Accordingly, changes in the vertical position and orientation of the fluorophore would lead to changes in the environment experienced by the fluorophore residing within the interfacial region. Because the effects of $\Delta\Psi$ on the fluorescence of DPPN and NBD-PC in fluid DPPC matrices are approximately equal, this would suggest that the permanent dipole moment of NBD can maintain a significant fraction of the NBD moieties in both DPPN and NBD-PC in the same average orientation in the membrane. Considering possible changes in NBD orientation or its vertical position with respect to the plane of the bilayer, the similar responses of RFI to $\Delta\Psi$ for both DPPN and NBD-PC would further require the dislocations of NBD to be similar irrespective of the site of the linkage of the dye moiety to the phospholipid structure in these analogs.

The changes in Ψ could mirror alterations in the freedom of movement and anisotropy of the interfacial water molecules. Keeping in mind that NBD is nearly nonfluorescent in water (Fery-Forgues at al., 1993) changes in the hydration shell might be important. However, this seems unlikely when the relation between dipole potential modifiers, such as PHL, DHPC, 6-KC, and cholesterol (Szabo, 1974), and hydration is taken into account. More specifically, several studies have demonstrated a correlation between dipole potentials measured for monolayers and the hydration pressure between bilayers (Simon and McIntosh, 1989; McIntosh et al., 1996). This is the case also for such modifiers of dipole potential as PHL (Jendrasiak et al.,

1997) and 6-KC (Simon et al., 1992). However, if DPPC is replaced with the ether lipid analog DHPC the hydration pressure remains nearly unaltered, despite the ~118-mV decrement in Ψ (Gawrisch et al., 1992). Assuming that hydration pressure is related to water structure on the membrane surface and not caused entirely by, e.g., protrusion and undulation forces (Israelachvili and Wennerström, 1992), for instance, the possible effects of water structure on the membrane surface as a mechanism for the changes observed for NBD can be excluded. Furthermore, a recent study has shown that neither PHL nor 6-KC affect water anisotropy deeper within the membrane, at the level of the carbonyl groups (Diaz et al., 1999).

The phosphocholine headgroup orientation is known to be modulated by, e.g., surface potential due to external electric fields (Stulen, 1981), charged species in the membrane (Scherer and Seelig, 1989), PHL (Bechinger and Seelig, 1991a), and DHPC (Siminovitch et al., 1983). However, as similar changes in RFI were seen for both DPPN and NBD-PC, only indirect effects of the headgroup orientation on the measured parameters would be possible.

Lastly, the observed changes in the emission intensity of NBD upon variation in Ψ could arise from solvatochromic effects involving the formation of hydrogen bonds together with other interactions (Suppan and Ghoneim, 1997) between the solvent and the fluorophore, and electric fields generated by polarized solvent molecules surrounding the dye (Liptay, 1969). The latter are also known as reaction fields, the magnitudes of which can be as high as 1–10 GV/m (Lombardi, 1998). Direct effects of external electric fields, i.e., electrochromic effects, are usually small (Suppan and Ghoneim, 1997), mainly due to the weakness of external electric fields compared with the strengths of reaction fields (Liptay, 1969). Electrochromism has been suggested to play a role in both the absorbance and fluorescence response of probes subject to the immense electric fields present in lipid membranes (Reich and Schmidt, 1972; Loew et al., 1978, 1979; Gross et al., 1994). The electric fields caused by dipole potential can be as high as 1 GV/m (Gross et al., 1994). For LUVs at $X_{6\text{-KC}} = 0.30$ and at $X_{PHL} = 0.30$ the value for $\Delta\Psi$ is ~330 mV, corresponding to a difference of almost 0.7 GV/m. Fluorescence intensities for DPPN and NBD-PC are directly proportional to Ψ (Fig. 4 *C*). Linear response of the emission intensity to the strength of the external field has been reported for other fluorophores (Ohta et al., 1997). The electrochromic effect could thus represent a feasible mechanism causing the observed behavior of NBD in response to varying Ψ. However, the other possibilities discussed above cannot be unambiguously excluded at this stage.

Although it has been reported that the effects, other than those related to dipole potential, of 6-KC on membranes are quite small (Simon et al., 1992) and although PHL is known to decrease the main-phase transition temperature without significant effects on the transition cooperativity (Cseh et al., 2000), we observed the fluorescence anisotropy of DPPN to increase with the addition of either PHL or 6-KC. Thus, fluidity-related effects seem unlikely. Effects related to lateral organization seem also unlikely, as for DPPN the increase in self-quenching would be accompanied by increased local charge density due to the DPPN molecules themselves. Thus, if the effects were due to changes in the lateral organization, we would expect a decrease in the rate coefficient with a decrease in intensity, whereas the opposite was observed.

Obviously, it is not possible to completely rule out possible other effects that PHL and 6-KC might have. However, due to the relatively good correlation with the dipole potential values for 6-KC, PHL, and DHPC, it seems reasonable to assume that the effects seen are related to the general effects of dipole potential, either directly or indirectly.

Correlation between k_1 and Ψ

The observed decrease in the rate coefficient with increasing Ψ is opposite to the effects of Ψ on the rate of transport of hydrophobic anions across black lipid membranes (Andersen et al., 1976; Malkov and Sokolov, 1996). It is important to note the principal differences between the two systems. More specifically, the reaction of dithionite with NBD on the outer surface of the liposome is different from the above transport studies in two respects. First, the reaction does not necessitate the penetration of dithionite across the membrane but instead only penetration sufficiently deep into the polar zone. Second, the dithionite ion itself is hydrophilic. Different effects of dipole potential on the penetration of a hydrophobic anion across the bilayer and penetration of a hydrophilic anion into the polar zone of the outer leaflet of the bilayer are to be expected. This is supported by recent theoretical treatments of ion diffusion across membranes with soft polar interfaces under short-circuit conditions (Aguilella et al., 1996; Levadny et al., 1998). Although the latter analysis as such is not directly applicable to our data, these authors suggest the surface dipoles to have variable effects on the rate of diffusion depending on the circumstances and characteristics of the system, e.g., depending on whether it is the hydrophobic or polar zone that is constituing the rate-limiting barrier. Taking into account the free energy profile for tetraphenylborate (Franklin and Cafiso, 1993), it is evident that the free energy maximum at the hydrophobic core acts as the main barrier for the diffusion of the hydrophobic ions across the bilayer. As further discussed by Franklin and Cafiso, due to the positive partial charge of dipole potential residing inside the bilayer, the free energy barrier of the hydrophobic zone for the negatively charged tetraphenylborate is considerably lower than for the positively charged tetraphenylphosphonium. For an increasing dipole potential, the positive potential inside the membrane is increased, further favoring the partitioning of tetraphenylborate into the membrane inte-

rior, thus lowering the free energy barrier, and increasing rate constant. In our system, however, the above free energy barrier is not involved, for it is not necessary for the hydrophilic dithionite to enter the hydrocarbon region to react with the NBD, which resides within the polar zone of the bilayer on the outer surface of the liposomes. Thus, all the effects on k_1 in our system should primarily depend on changes within the polar zone.

Compared with the above changes in RFI, the mechanistic basis for the reciprocal dependency on Ψ of the rate coefficient k_1 for the reduction of DPPN by dithionite is more easily rationalized. As outlined above for the variation in RFI, changes in the structure of water in the interface, or reorientation of headgroups may perhaps be excluded. The pseudo-first-order rate coefficient k_1 describes the combined rate for a series of time-dependent processes and changes such as the diffusion of the reducing agent to the membrane interface, its intercalation between lipid headgroups, and the actual reaction steps. Yet membrane interface is a truly complex system, and detailed analysis would require estimation of several unknown parameters and their use in solving the complicated material balance equation accounting for the above processes. In our case only the initial boundary conditions for the concentration gradient of dithionite are known. It thus becomes warranted to use rough approximations in estimating the feasibility of direct electrostatic effects, as follows. Two extreme possibilities as to the rate-limiting steps are the diffusion of dithionite into the interface and the formation of pre-equilibrium for the interfacial dithionite concentration at the level of NBD. In the former case k_1 would be proportional to the flux J of dithionite per unit area per unit time into the interface, which is described by

$$J = -(D\nabla c_d + z/|z| \times \mu c_d E) \qquad (2)$$

where D is the diffusion coefficient, c_d is [dithionite], z is the number of charges, μ is electrochemical mobility of dithionite, and E is electric field (Steinfeld et al., 1998). Both D and E are functions of position. The concentration gradient and thus also the flux change with time. The above equation describes the combined effect of electric fields and concentration gradient on J for any given concentration gradient. In this case, the driving force of the reaction derives from the concentration gradient, whereas changes in the electric field modify the opposing force. Neglecting small time-dependent changes in Ψ due to dithionite binding to the membrane, we may assume that the magnitude of the field does not vary with time. We then attribute the changes in k_1 caused by the dipole potential modifiers to changes in the viscosity gradient of interface and changes in the magnitude of the electric field. If large changes in the latter dominate over the former, we would expect a nearly linear relationship between the field strength and the flux of dithionite per unit area per unit time. The magnitude of E in turn is directly proportional to separated partial charges, as exemplified, e.g., for the square lattice of dipoles (Israelachvili, 1991). Accordingly, the field magnitude is directly proportional to Ψ. It is noteworthy that this holds even for more complex treatments as the same arguments apply also for the derivatives and integrals of E with respect to the position. To conclude, the approximately equal absolute changes for DPPN and NBD-PC (Fig. 6 *C*) as well as the linearity of k_1 versus $\Delta\Psi$ data would be compatible with direct electrostatic effects being rate limiting.

As an additional test, k_1 and RFI values were recorded also in 1.0 M NaCl. Perhaps somewhat unexpectedly, the above effects were independent of the presence of salt. With respect to the screening of the partial charges it is noteworthy that whereas cations screen the negative partial charges on the outer surface, and in fact have been also shown to bind to membranes and decrease Ψ (Clarke and Lüpfert, 1999), the resulting screening is not complete. Accordingly, there still remains an excess of partial charges, as evidenced by the fact that dipole potential decreases only slightly upon the increase of the cation concentration in the bulk phase. In screening of the electric potentials of the surface the accumulation of the cations in the interface certainly plays an important role especially with respect to the surface potential caused by the charged lipid species. Due to the short decay length of the electric field of the dipolar systems, the screening by cations and thus the formation of an electrical double layer should screen the partial charges efficiently, abrogating the effects of the dipole-potential-induced fields in the water phase. However, the membrane interface is not sharp but rather a dynamic zone of a diffuse change from bulk water to hydrocarbon core, further dividable into subzones. The location of the negative zone of the dipole potential should thus be taken into account when considering the screening by ions present in the interface. The minor effects of hydrophilic ions on Ψ suggest their limited penetration into the membrane, thus preventing efficient screening of the dipole potential deeper within the interface. The screening in the bulk water phase on the other hand is certainly sufficient to prevent any electrostatic effects of dipole potential extending further from the interface. The direct effects of electric field could, however, perhaps manifest deeper within the interface within a diffuse, viscous zone, where a limited number of screening ions is present. Accordingly, the possibility of direct effects of the electric fields cannot be excluded.

An increase in Ψ corresponds to an interface that is more negatively charged on the surface and more positively charged within the hydrocarbon region. The rate coefficient k_1 should be attenuated by an increase in the density of the negative partial charges on the membrane surface, expelling dithionite from the interface and reducing its effective concentration in the interface, at the level of NBD. It was reported recently that anions decrease Ψ, presumably due to binding to sites in the inner positive region of the dipole-

potential-generating layer (Clarke and Lüpfert, 1999). Increasing Ψ should thus promote anion binding, in keeping with the coincidence of maxima in Ψ and minima in zeta potential in binary phospholipid membranes (Luzardo et al., 1998). Consequently, the equilibrium concentration of the anions in the layer of positive partial charges would increase with Ψ, but so would also the potential barrier for the diffusion of dithionite into that layer. Although the magnitude of dipolar fields decays rapidly with increasing distance, direct electrostatic interactions could be large enough to explain the observed effects on k_1. It is also possible that the changes in Ψ lead to a vertical redistribution of the NBD moieties so as to influence the access of dithionite to NBD. Location of the NBD moiety of DPPN deeper in the interface would necessitate the penetration of dithionite through a layer of negative partial charges. Studies using parallax method have suggested that in dioleoylphosphatidylcholine vesicles the NBD moieties of both DPPN and NBD-PC reside at the border between the hydrocarbon region and the polar interfacial layer (Chattopadhyay and London, 1987; Abrams and London, 1993). Moreover, the red-edge excitation shift for the dye moiety of DPPN suggests that it is accommodated in a viscose, polar environment (Chattopadhyay and Mukherjee, 1993). Our observation that changes in Ψ affect both RFI and k_1 for DPPN and NBD-PC in a similar fashion suggests the fluorescing fraction of NBD moieties in both probes to be exposed to the electric field associated with Ψ. Although the approximately equal absolute changes in the rate coefficient k_1 for the DPPN- and NBD-PC-containing membranes (Fig. 6 *C*) could be a mere coincidence, it would also be compatible with direct electrostatic effects on the access of dithionite into the NBD-containing zone of the interface.

In the light of the values measured in the presence of NaCl, the redistribution of NBD moieties would provide another plausible explanation. Regardless of the mechanism, the independence of the effects caused by NaCl concentration and Ψ is somewhat unprecedented. If the screening by NaCl plays a significant role in the electrostatic effects due to the Ψ, this should affect both possible electrostatic effects on NBD distribution as well as possible direct electrochromic effects and direct electrostatic effects on the reaction rate. The mechanistic basis for the effects of $\Delta\Psi$ on k_1 thus remain elusive.

CONCLUSIONS

The present data demonstrate that the emission intensity of the NBD moiety in the headgroup-labeled DPPN and the acyl-chain-labeled NBD-PC is sensitive to the bilayer dipole potential Ψ. Moreover, the rate of the reduction of NBD in these probes by dithionite can be controlled by dipole potential. Although electrochromic and direct electrostatic effects provide appealing explanations for the above effects, these observations may well involve other mechanisms, such as redistribution of the NBD moieties due to changes in Ψ. In near ideal conditions, where perturbation by other factors affecting NBD fluorescence intensity is insignificant, these responses of DPPN could be used to estimate the magnitude of changes in Ψ. However, NBD emission is sensitive to a number of environmental factors, thus limiting the use of DPPN for this purpose. DPPN also responds to surface charges (Shrive et al., 1996). Because NBD probes are widely used, this response of NBD fluorescence to Ψ also represents a potential caveat and should be taken into account.

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