Binding of Adriamycin to Liposomes as a Probe for Membrane Lateral Organization

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ABSTRACT A stopped-flow spectrofluorometer equipped with a rapid scanning emission monochromator was utilized to monitor the binding of adriamycin to phospholipid liposomes. The latter process is evident as a decrease in fluorescence emission from a trace amount of a pyrene-labeled phospholipid analog (PPDPG, 1-palmitoyl-2-[(6-pyren-1-yl)]decanoyl-*sn*-glycero-3-phospho-rac-glycerol) used as a donor for resonance energy transfer to adriamycin. For zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes, fluorescence decay was slow, with a half-time $t_{1/2}$ of ~2 s. When the mole fraction of the acidic phospholipid, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-glycerol (POPG), was increased to $X_{PG} \geq 0.04$, the decay of fluorescence became double exponential, and an additional, significantly faster process with $t_{1/2}$ in the range between 2 and 4 ms was observed. Subsequently, as X_{PG} was increased further, the amplitude of the fast process increased, whereas the slower process was attenuated, its $t_{1/2}$ increasing to 20 s. Increasing [NaCl] above 50 mM or [$CaCl_2$] above 150 μM abolished the fast component, thus confirming this interaction to be electrostatic. The critical dependence of the fast component on X_{PG} allows the use of this process to probe the organization of acidic phospholipids in liposomes. This was demonstrated with 1,2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes incorporating PPDPG ($X_{PPDPG} = 0.03$), i.e., conditions where X_{PG} in fluid bilayers is below the required threshold yielding the fast component. In keeping with the presence of clusters of PPDPG, the fast component was observed for gel-state liposomes. At ~34°C (i.e., 6° below T_m), the slower fluorescence decay also appeared, and it was seen throughout the main phase transition region as well as in the liquid-crystalline state. The fluorescence decay behavior at temperatures below, above, and at the main phase transition temperature is interpreted in terms of thermal density fluctuations and an intermediate state between gel and liquid-crystalline states being involved in the phospholipid main phase transition. This is the first observation of a cluster constituted by acidic phospholipids controlling the membrane association of a drug.

INTRODUCTION

Lateral heterogeneity and domain formation in biomembranes are well established (for reviews see Knoll et al., 1991; Rodgers and Glaser, 1991; Kinnunen, 1991; Gascard et al., 1993; Tocanne et al., 1994; Welti and Glaser, 1994) and have been shown to be of importance to the membrane binding and function of proteins such as cytochrome *c* (Vanderkooi et al., 1973; Mustonen et al., 1987) and gelsolin (Janmey and Chaponnier, 1995), in the coupling of G-proteins to their receptors (Neubig, 1994), in the budding of viruses from the plasma membrane (Luan and Glaser, 1994), and in the activation of pancreatic lipase by colipase (Momsen et al., 1995). Considering the coupling between dynamic order and function emphasized in the modern view of biological membranes (see e.g., Kinnunen, 1991; Kinnunen and Mouritsen, 1994; Kinnunen et al., 1994; Mouritsen and Kinnunen, 1996), thorough understanding of the mechanisms causing lateral organization of membranes is clearly of fundamental importance. To this end, several molecular-level mechanisms have by now been established in model membranes, as follows.

Characteristically for liquid crystalline materials, phospholipids can exist in several phases such as gel, liquid crystalline, interdigitated, or inverted nonlamellar phases (Kinnunen and Laggner, 1991). Both the coexistence of gel and fluid (liquid crystalline) phases and fluid-fluid immiscibility have been demonstrated for binary alloys (e.g., Shimshick and McConnell, 1973; Wu and McConnell, 1975; Holopainen et al., 1997). Dehydration-induced phase separation was predicted on theoretical grounds (Bryant and Wolfe, 1989) and was recently verified for binary liquid crystalline bilayers (Lehtonen and Kinnunen, 1995). There is evidence of lateral heterogeneity in lipid membranes being induced by alcohol (Rowe, 1987; Mou et al., 1994). In binary membranes containing charged (anionic or cationic) species, phase separation can be induced by Ca^{2+} (Galla and Sackmann, 1975; Eklund et al., 1988), polycations (Hartmann et al., 1977; Eklund and Kinnunen, 1986), DNA (Kôiv et al., 1994), electric fields (Lee et al., 1994; Lee and McConnell, 1995; Söderlund et al., 1997), and pH (Tilcock and Cullis, 1981). Likewise, several cationic peripheral proteins induce the segregation of acidic phospholipids (Kinnunen et al., 1994). In tertiary alloys the cationic amphiphile sphingosine seems to form microdomains with phosphatidic acid (Mustonen et al., 1993). The mismatch between the lengths of the hydrophobic membrane-spanning segments of integral membrane proteins and those of

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lipids was proposed as a mechanism causing ordering of membranes (Mouritsen and Bloom, 1984). This principle was recently confirmed experimentally for *Escherichia coli* lactose permease (Lehtonen and Kinnunen, 1997) and bacteriorhodopsin (Dumas et al., 1997) reconstituted into liposomes. Hydrophobic matching conditions have also been shown to induce lipid microdomains in fluid, liquid crystalline phospholipid alloys (Lehtonen et al., 1996). Steric elastic strain has been suggested to provide the driving force for the formation of well-defined superlattices in liquid crystalline liposomes (for brief reviews see Kinnunen, 1991; Kinnunen et al., 1993).

The biological significance of the rich scale of different lipid phases and phase transitions has remained mostly unknown. Phospholipid chain melting at the main transition occurs via the growth of fluid lipid domains in a bulk gel-state membrane. Accordingly, the main transition readily involves dynamic ordering and lateral heterogeneity in the membrane (Mouritsen, 1991, and references therein), which further have profound effects on membrane properties and on lipid-protein interactions, for instance (for a brief review see, e.g., Mouritsen and Kinnunen, 1996). To this end, although dynamic lateral heterogeneity is indeed inherent to any two-dimensional melting process, it is still extremely challenging to describe the relevant processes in terms of molecular interactions (Jutila and Kinnunen, 1997).

Several drugs bearing a positive charge have been shown to interact with acidic phospholipids. In this respect, perhaps the best characterized is adriamycin (e.g., Karczmar and Tritton, 1979; Goormaghtigh et al., 1980a; Henry et al., 1985; de Wolf et al., 1991), a chemotherapeutic drug that is highly soluble in water yet also effectively partitions into membranes. Drug-lipid interactions are believed to be important to the cytotoxicity of adriamycin and may also underlie its clinically most feared, dose-dependent side effect, cardiotoxicity, which involves impairment of mitochondrial function (e.g., Rosenoff et al., 1975; Solem et al., 1996; Doroshow, 1991; Duarte-Karim et al., 1976; Goormaghtigh et al., 1980b, 1982, 1986; Huart et al., 1984; Nicolay and de Kruijff, 1987). The mechanism of adriamycin cytotoxicity appears to be the induction of apoptosis (e.g., Skladanowski and Konopa, 1993; Ling et al., 1993; Zaleskis et al., 1994; Anand et al., 1995; Müller et al., 1997). In this regard, the activation of phospholipase A_2 by adriamycin demonstrated in vitro to result from drug-phospholipid interaction could be relevant (Mustonen and Kinnunen, 1991).

Two types of binding have been suggested to be involved in adriamycin-acidic phospholipid interactions (Karczmar and Tritton, 1979; Henry et al., 1985). In the first mechanism there is an electrostatic association of the amino sugar moiety of the drug with the ionized phosphate of acidic phospholipid, while the dihydroxyanthraquinone ring structures would remain outside the bilayer and may further interact with each other by forming stacks (Goormaghtigh and Ruys-schaert, 1984; Henry et al., 1985; Nicolay et al., 1988). Interaction of the amino sugar with the ionized phosphate is also central in the second mechanism, which additionally involves intercalation of the dihydroxyanthraquinone moiety between the fatty acid chains of phospholipids within the lipid membrane (Dupou-Cézanne et al., 1989; Henry et al., 1985; Fiallo and Garnier-Suillerot, 1986). The above conclusions have been derived from steady-state equilibrium studies (e.g., Karczmar and Tritton, 1979; Goormaghtigh et al., 1980b; Henry et al., 1985; de Wolf et al., 1991).

To our knowledge, the binding of adriamycin to phospholipids has not been assessed by stopped-flow techniques. We report here on the dependence of the rate of the binding of adriamycin to liposomes on their content of the acidic phospholipids. Subsequently, we used the kinetics of drug-membrane interaction to probe membrane lateral order in the course of the main transition of a binary phospholipid alloy.

MATERIALS AND METHODS

Materials

HEPES, EDTA, and adriamycin (doxorubicin) were from Sigma, and NaCl and $CaCl_2$ were from Merck (Merck, Darmstadt, Germany). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were from Avanti Polar Lipids (Alabaster, AL). Pyrene-containing phospholipid analogs, 1-palmitoyl-2-[(6-pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (PPDPC) and 1-palmitoyl-2-[(6-pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphoglycerol (PPDPG), were obtained from K&V Bioware (Espoo, Finland). Dipalmitoylphosphatidylcholine (DPPC) was from Coatsome (Amagasaki, Hyogo, Japan). The purity of the lipids was checked by thin-layer chromatography on silicic acid-coated plates (Merck), using chloroform/methanol/water (65:25:4, v/v) as a solvent. Examination of the plates after iodine staining or, when appropriate, upon fluorescence illumination revealed no impurities. Lipid stock solutions were made in chloroform. Concentrations of the nonfluorescent phospholipids were determined gravimetrically with a high-precision electrobalance (Cahn Instruments, Cerritos, CA), and those of the pyrene-containing phospholipid analogs were determined spectrophotometrically, using the molar extinction coefficient $\epsilon = 42{,}000$ at 344 nm. Adriamycin was dissolved in the buffer, and its concentration was determined spectrophotometrically, using $\epsilon = 11{,}500$ at 480 nm.

Preparation of liposomes

Lipids were mixed in the indicated molar ratios. Unless otherwise indicated, the content of the fluorescent probe (PPDPC or PPDPG) was $X = 0.03$, which was chosen as a probe concentration to enhance signal-to-noise ratio. In experiments on large unilamellar vesicles (LUVs) with $X_{PG} \geq 0.03$, PPDPG ($X = 0.03$) was preferred to assess more directly the correlation between half-time of the fluorescence decay ($t_{1/2}$) for the binding and the distribution of the probe (representing phosphatidylglycerol, PG) in the membrane. This does not affect the appearance of the fast component, which for fluid membranes was evident only at $X_{PG} \geq 0.04$. The lipid mixtures were evaporated to dryness under a stream of nitrogen and then kept under reduced pressure for at least 2 h to remove traces of solvent. Dry residues were hydrated in buffer for 30 min at ~15°C above the gel → liquid crystalline transition temperature (T_m) of lipid used. Large unilamellar liposomes (diameter 80 ± 25 nm; MacDonald et al., 1991) were made by extruding the lipid dispersions 17 times through two Millipore polycarbonate filters (average pore size 100 nm; Millipore, Bedford, MA), using a LiposoFast (Avestin, Ottawa, Canada) small-volume homogenizer.

Liposomes were stored in a refrigerator (+4°C) and were used within 24 h after hydration.

Stopped-flow fluorescence spectroscopy

Electronic energy is efficiently transferred from a fluorescent energy donor to a suitable acceptor up to a distance of 60 Å, with the rate of the transfer depending on the reciprocal of the sixth power of the average distance between the donor and the acceptor. Furthermore, orientation of the donor and acceptor relaxation and excitation dipoles and the magnitude of their spectral overlap determine the efficiency of the energy transfer process (Stryer, 1978). Adriamycin has a broad absorption peak centered at 480 nm that overlaps the emission spectrum of pyrene (Mustonen and Kinnunen, 1991). With excitation at 344 nm and measurement of pyrene (donor) emission in the 375–525 nm range, it is possible to measure the binding of adriamycin (acceptor) to lipid membranes embedding pyrene containing a phospholipid analog, e.g., PPDPC or PPDPG (Mustonen and Kinnunen, 1991, 1993; Kôiv and Kinnunen, 1994); the membrane association of the drug is readily evident as a decrease in pyrene emission intensity.

The binding of adriamycin to liposomes was 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 xenon lamp. Two pneumatically driven (gas pressure 7 bar) syringes were mounted in parallel to inject the reactants into the rapid-mixing quartz glass fluorescence observation chamber (20-mm path length, 1.5-mm diameter). The temperature in the reaction chamber and in the syringes was regulated by a computer-controlled circulating waterbath (Neslab Instruments, Portsmouth, NH). The initial concentration of phospholipid was 50 μM and that of adriamycin 8 μM, thus yielding the corresponding final concentrations of 25 and 4 μM in the mixing chamber. An excitation wavelength of 344 nm was selected with a monochromator, and the beam was passed through subsequent 6.12-mm and 3.16-mm slits. A maximum of 1000 spectra/s was measured by passing the beam through a 1.24-mm slit and a rotating spoke wheel. Fluorescence emission between 375 and 525 nm was recorded with a photomultiplier tube. Photomultiplier output was digitized by a Pentium PC equipped with an A/D converter. The fluorescence decays for each sample were monitored by using scanning rates of 1000, 62, and 10 scans/s, with the respective collection times of 0.25, 5, and 96 s.

Values for the given half-times of the reactions represent averages from at least three separate measurements. Data with two fluorescence decay processes were fitted by the equation

$$Y = A_1 * e^{-k_1 \cdot t} + A_2 * e^{-k_2 \cdot t}$$

which was solved with nonlinear least-squares fitting procedures by both the Levenberg-Marquardt algorithm and the successive integration method, using the routines of the software provided by the instrument manufacturer. The best-fit parameters A_1 and A_2 represent the amplitudes of the processes, and k_1 and k_2 the respective kinetic rate constants. Representative traces for the binding of adriamycin to phospholipid vesicles with their respective residuals are illustrated in Fig. 1. In longer measurements (96 s) photobleaching of pyrene due to the 450-W xenon lamp irradiation became evident as an additional slow process. As this bleaching was very slow ($t_{1/2} \approx 115$ s), it did not interfere with the recording of the fluorescence intensity decays due to the membrane association of adriamycin. However, because of the large difference in the half-times of the different components, the routines of the software did not allow us to obtain accurate values for relative amplitudes for the two exponential processes measured. Yet, the relative amplitude of the fast fluorescence decay (RFI) seen within the 250-ms time domain could be obtained from the data as RFI = $\Delta I/I_0$, where ΔI is the amplitude of the rapid fluorescence decay, and I_0 is the initial level of fluorescence of the LUVs before the rapid quenching by adriamycin. The apparent rate constants (k_1 and k_2) obtained from the measurements ranged from 0.055 to 550 s^{-1}, with the corresponding errors of ± 0.00045 and ± 100 s^{-1}. These values convert to the respective half-times of 18.18 s and 1.82 ms, with the corresponding average errors of ± 100 and ± 0.8 ms ($t_{1/2} = k^{-1}$).

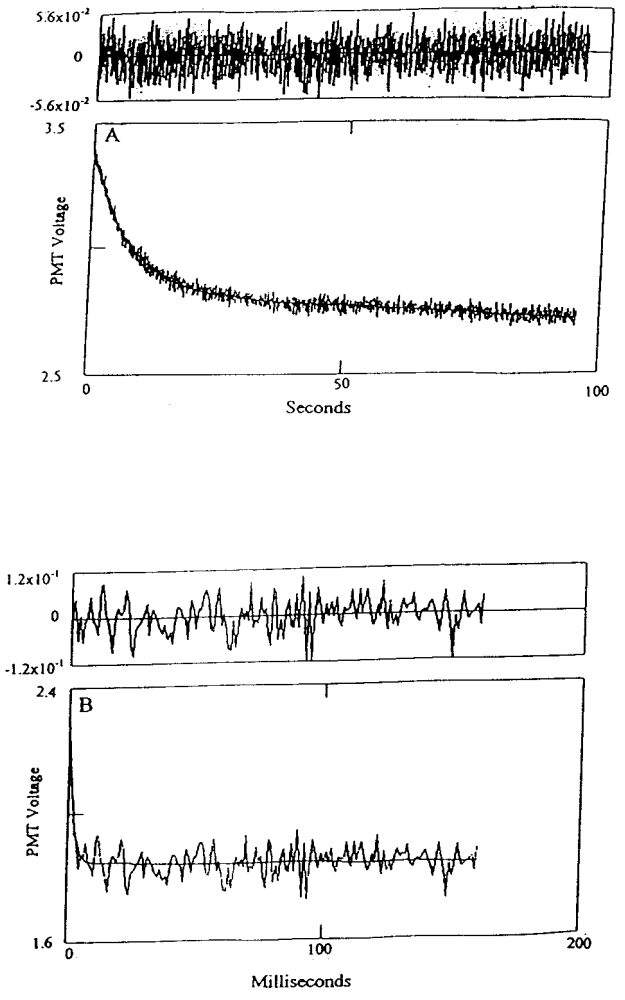

FIGURE 1 (*A*) Fluorescence intensity (as photomultiplier tube output voltages) decay after the addition of 4 μM adriamycin (final concentration) to POPC LUVs. PPDPC ($X = 0.03$) was included in the vesicles as a donor for resonance energy transfer. The final concentration of phospholipid was 25 μM in 5 mM HEPES, 0.1 mM EDTA, pH 7.4. The cuvette was thermostatted at 30°C. The time span of the measurement was 96 s, and a total of 1000 spectra were recorded. (*B*) A representative measurement in the 250-ms time domain and the fast fluorescence decay. The matrix lipid was POPC with $X_{PG} = 0.15$, including PPDPG ($X = 0.03$) as a fluorescence donor. The upper small panels show the residuals after the data were fitted by the equation $I = A \cdot e^{(-k1 \cdot t)} + B \cdot e^{(-k2 \cdot t)}$ (see text for further details).

Importantly, although the decrease in the fluorescence intensity correlates with the number of adriamycin molecules bound to the liposomes, it is not possible to determine the actual number of drug molecules bound to the vesicles. This is due to the fact that at low surface occupancy, the number of donors quenched by each bound acceptor is large. However, upon increasing surface occupancy by adriamycin, the decrement in emission intensity versus the number of additional drug molecules bound diminishes in a progressive manner. Quantitation of resonance energy transfer processes of this type has been shown to be inherently complex (Drake et al., 1991). Yet, although the above drawback necessarily prevents the construction of an accurate kinetic model and calculation of the true rate constants, it does not compromise in any way the data and the qualitative conclusions of this study.

Steady-state fluorescence measurements

DPPC:PPDPC (97:3, mol/mol) and DPPC:PPDPG (97:3, mol/mol) LUVs for steady-state measurements were prepared as described above. The final lipid concentration was 10 μM. The fluorescence emission spectra were recorded with a Perkin-Elmer LS50B spectrofluorometer with a magnetically stirred cuvette compartment thermostatted with a circulating water bath (WK4DS; Colora Messtechnik Gmbh, Lorch, Germany). The instrument is connected to a Pentium 133-MHz PC, and the data were analyzed with dedicated software from Perkin-Elmer. An excited-state pyrene molecule relaxes to ground state by emitting photons with a maximum at ~378 nm (I_m) and the exact peak intensity and spectral fine structure depending on the solvent polarity. During its lifetime, excited-state pyrene can form a characteristic short-lived complex excimer with a ground-state pyrene. This complex relaxes back to ground state by emitting quanta as a broad and featureless band centered at ~480 nm (I_e). In the absence of possible quantum mechanical effects, the excimer-to-monomer ratio (I_e/I_m) depends on the rate of collisions between pyrenes. Consequently, for pyrene-containing phospholipid analogs, the value of I_e/I_m reflects the lateral diffusion as well as the local concentration of the fluorophore in the membrane (for a brief recent review see Kinnunen et al., 1993). The excitation and emission bandpasses were set at 2.5 and 5 nm, respectively. Excitation was at 344 nm, and pyrene monomer and excimer emissions were recorded at ~378 nm and ~480 nm, respectively. The average heating rate in the temperature scanning measurements was 0.1°C/min.

Differential scanning calorimetry

LUVs were prepared as above, with the exception that after extrusion the samples were kept at +4°C overnight before the heat capacity scans were recorded at a rate of 0.5°C/min. Gel→liquid-crystalline phase transition temperatures of DPPC, DPPC:PPDPC (97:3, mol/mol), and DPPC:PPDPG (97:3, mol/mol) LUVs were recorded by VP-differential scanning calorimetry (VP-DSC) (Microcal, Northampton, MA). This was done and the data were analyzed using the routines of the software provided with the instrument. The final lipid concentration in the DSC cuvette was 0.2 mM. Deviation of the heat capacity from the baseline was taken as the beginning of the transition. Likewise, the point where the enthalpy returned to the baseline was taken as the end of the transition. The endotherms were deconvoluted assuming the components to be Gaussian. It must be stressed, however, that this choice for distribution profiles is arbitrary and stems from the uncertainties regarding the detailed transition mechanisms. Yet, as long as the latter remain poorly understood, the assumption of Gaussian distribution is commonly used (e.g., McMullen et al., 1993). The significance of deconvoluted DSC data must therefore be viewed with caution and can only provide circumstantial support to results from other kinds of measurements. Importantly, the components derived cannot be attributed to physical processes such as phase separation or microdomains.

RESULTS

Dependence on the content of acidic phospholipid in liposomes

Two different binding sites for adriamycin in liposomes and the requirement for acidic phospholipid for the high-affinity membrane association of this drug are well established (Karczmar and Tritton, 1979; Goormagtigh et al., 1980b; Goormaghtigh and Ruysschaert, 1984; Henry et al., 1985; Nicolay et al., 1988; de Wolf et al., 1991; Mustonen and Kinnunen, 1991). Accordingly, it was of interest to determine if these binding sites and the dependence on the content of acidic phospholipids in membranes could also be verified by kinetic measurements. For this purpose POPC was used as a matrix while the content of POPG in the liposomes was progressively increased. Resonance energy transfer between pyrene emission and adriamycin absorption was utilized to monitor the membrane association of the latter (Mustonen and Kinnunen, 1991). The measurement temperature was 30°C, so as to have the liposomes in the liquid-crystalline (fluid) state. For liposomes consisting of the zwitterionic POPC and including the pyrene-derivatized phosphatidylcholine analog PPDPC as the fluorescent donor ($X_{PG} = 0$, i.e., with no net charge), the fluorescence decay was single exponential, with a half-time of ~2 s (Fig. 2 *A*). As the mole fraction of PG was increased to 0.04, a second, fast component with $t_{1/2} \approx 2$ ms became evident. The amplitude of the fast process increased progressively as X_{PG} was increased to 0.25, after which saturation was reached (Fig. 2 *C*). Concomitantly with the increase in the amplitude of the fast process, the half-time of the slow process increased (Fig. 2 *A*). Interestingly, $t_{1/2}$ for the fast component varies in a highly irregular manner as a function of X_{PG} (Fig. 2 *B*). More specifically, increasing X_{PG} from 0.05 to 0.065 causes $t_{1/2}$ reach a peak. This peak in $t_{1/2}$ is followed by enhanced binding at X_{PG} within 0.07–0.09, whereafter a larger value for $t_{1/2}$ at $X_{PG} = 0.10$ is again evident.

To obtain further insight into the nature of the binding process, the content of PG in LUVs was kept constant at $X_{PG} = 0.25$, and ionic strength was varied. In keeping with an electrostatic interaction, an increase in [NaCl] up to 50 mM diminished the amplitude of the fast process, and at 100 mM NaCl this interaction was abolished completely (Fig. 3 *B*). The half-time of the fast process was nearly independent of [NaCl] and varied between 2 and 4 ms (Fig. 3 *A*). Instead, $t_{1/2}$ for the slower component varied between 4 and 10 s, and this decay process became faster as [NaCl] was increased to 200 mM.

Ca^{2+} ions bind to acidic phospholipids and are known to have a strong influence on their phase behavior and organization (Galla and Sackmann, 1975; Hartmann et al., 1977; Eklund et al., 1988, 1991). Binding of adriamycin to LUVs ($X_{PG} = 0.25$) was subsequently measured at increasing [$CaCl_2$]. At [Ca^{2+}] from 0 to 150 μM, two-exponential fluorescence decay was observed. The fast component had a $t_{1/2}$ between 2 and 4 ms, and it was not observed when [Ca^{2+}] exceeded 150 μM, thus lending further support to this process being due to an electrostatic attraction. The amplitude of the fast process as a function of [$CaCl_2$] clearly revealed a nonlinear dependence (data not shown). More specifically, when [$CaCl_2$] was increased from 0 to 25 μM, RFI decreased from 0.76 to 0.25, whereas a further increase in [$CaCl_2$] up to 150 μM had a smaller effect, decreasing RFI from 0.25 to 0.11. Increasing [Ca^{2+}] also caused a minor decrease in $t_{1/2}$ for the slower component, from 12 to 8 s (data not shown). The possibility that vesicle aggregation would have influenced the measured binding can be excluded, as the threshold concentration for the Ca^{2+}-induced aggregation in the literature has been observed to be ~1 mM (Eklund et al., 1991), which is five times the highest [Ca^{2+}] used in the present experiments.

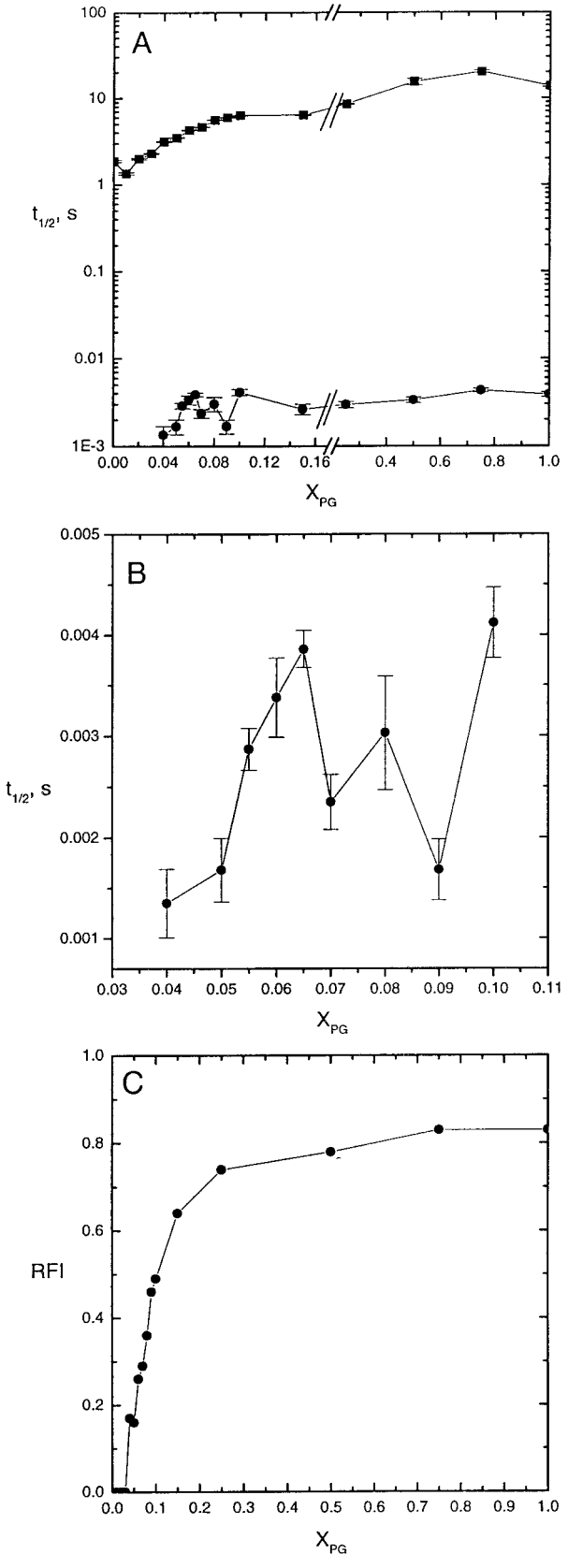

FIGURE 2 (*A*) The dependence of the binding of adriamycin to LUVs on the content of acidic phospholipid. At $X_{PG} = 0$ the fluorescent probe was PPDPC ($X = 0.03$). When X_{PG} was 0.01, the fluorescent probes were PPDPC ($X = 0.02$) and PPDPG ($X = 0.01$). At $X_{PG} = 0.02$ the mole fractions of the probes were $X_{PPDPC} = 0.01$ and $X_{PPDPG} = 0.02$. When X_{PG} was ≥ 0.03, only PPDPG was used ($X = 0.03$). The slow component (■) was obtained from 96-s measurements, and the fast component (●) from 250-ms measurements. The final concentration of phospholipid was 25 μM, and that of adriamycin was 4 μM. The temperature was 30°C. The buffer was 5 mM HEPES, 0.1 mM EDTA, pH 7.4. Error bars indicate mean error range for the data points and were obtained from the fitting program. The data points shown are averages from three measurements. Please note that the values for $t_{1/2}$ are given on a logarithmic scale. *B* demonstrates better the irregular dependence of $t_{1/2}$ of the fast component at low X_{PG}. (*C*) The relative amplitudes of the rapid fluorescence decay as a function of X_{PG}.

Effects of lateral heterogeneity accompanying the main transition of DPPC/PPDPG LUVs

Exclusion of the pyrene-containing phospholipid analog, PPDPC, from gel-state matrix consisting of DPPC has been shown to result in the formation of microdomains enriched in the fluorescent probe (Somerharju et al., 1985; Hresko et al., 1986). To explore the feasibility of a similar mechanism controlling the lateral distribution of an acidic phospholipid, we included PPDPG at $X = 0.03$ in a matrix of DPPC. As shown above, with this content of the acidic phospholipid in a fluid bilayer, only the slow binding process is observed, i.e., the surface density of PG in LUVs is too low to yield the fast, electrostatically driven membrane association of adriamycin. By DSC the DPPC/PPDPG LUVs exhibited a single endotherm with a peak centered at 40.4°C, thus verifying that this content of PPDPG did not suffice to abolish the main transition of DPPC (see below). In keeping with the predicted segregation of PPDPG into microdomains, the fast component in adriamycin binding was observed at $T < 38$°C. Likewise, above $T_m = 40.4$°C, the fast component was absent, thus confirming PPDPG to be effectively dispersed in the membrane so as to have the surface negative charge density below the critical threshold required for the fast, electrostatically driven binding of the drug to liposomes. Interestingly, there was an intermediate temperature range between 34 and 38.5°C, where the decay of fluorescence was double exponential (Fig. 4 *A*). The amplitude of the fast fluorescence decay (half-times ranging between 1 and 4 ms) decreased linearly with increasing temperature from 30 to 34°C, whereafter RFI decreased more steeply, approaching zero at ~38°C (Fig. 4 *B*). Increasing the temperature from 37 to 38.5°C prolonged $t_{1/2}$ for the fast component, and at $T \geq 39$°C only the slow binding process was observed. Notably, the latter component is due to the nonelectrostatic attraction, i.e., hydrophobicity. This notion is supported by measurements similar to those above, but here the zwitterionic PPDPC instead of the negatively charged PPDPG is used. These measurements with the zwitterionic liposomes also confirmed that the rapid fluorescence decay ($t_{1/2} \approx 1$–4 ms) between 30 and 38°C for the PPDPG-containing LUVs results from an electrostatic interaction between adriamycin and the acidic phospholipid.

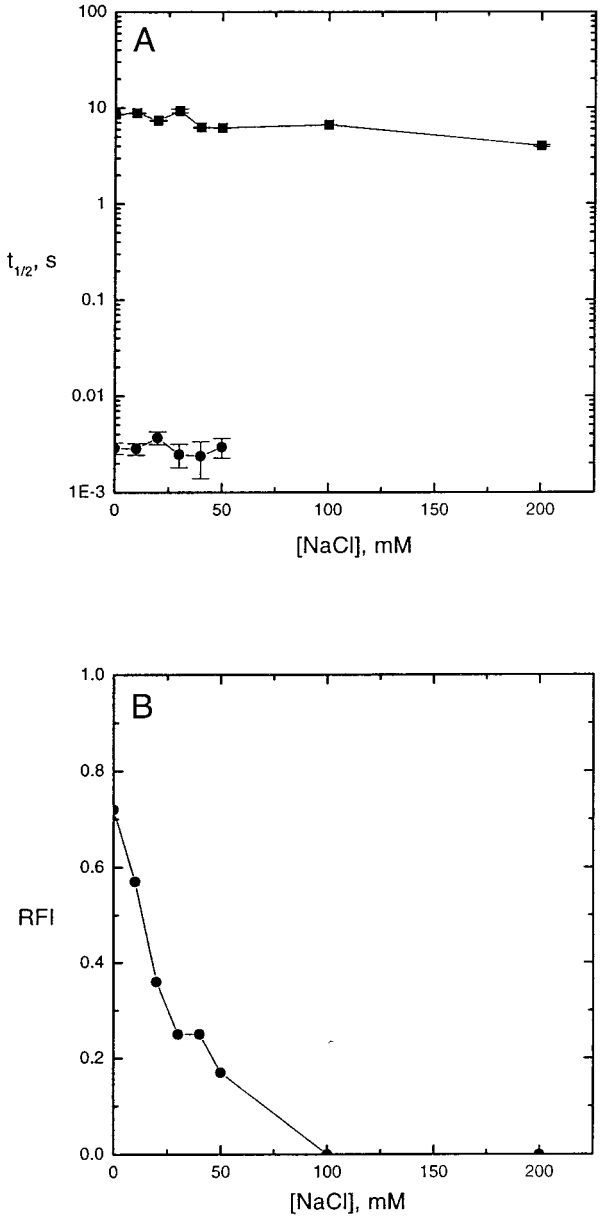

FIGURE 3 Effect of NaCl on the kinetics of binding of adriamycin to POPC/POPG LUVs (total $X_{PG} = 0.25$) with PPDPG $X = 0.03$. (*A*) The fast (●) and slow (■) components obtained from 250-ms and 96-s measurements, respectively. The total lipid concentration was 25 μM, and that of adriamycin was 4 μM. The temperature was 30°C; the buffer was 5 mM HEPES, 0.1 mM EDTA, pH 7.4, with the indicated concentrations of NaCl. Error bars are as in Fig. 2. (*B*) The relative amplitudes of the rapid fluorescence decay, obtained from the same measurements as the data in *A*.

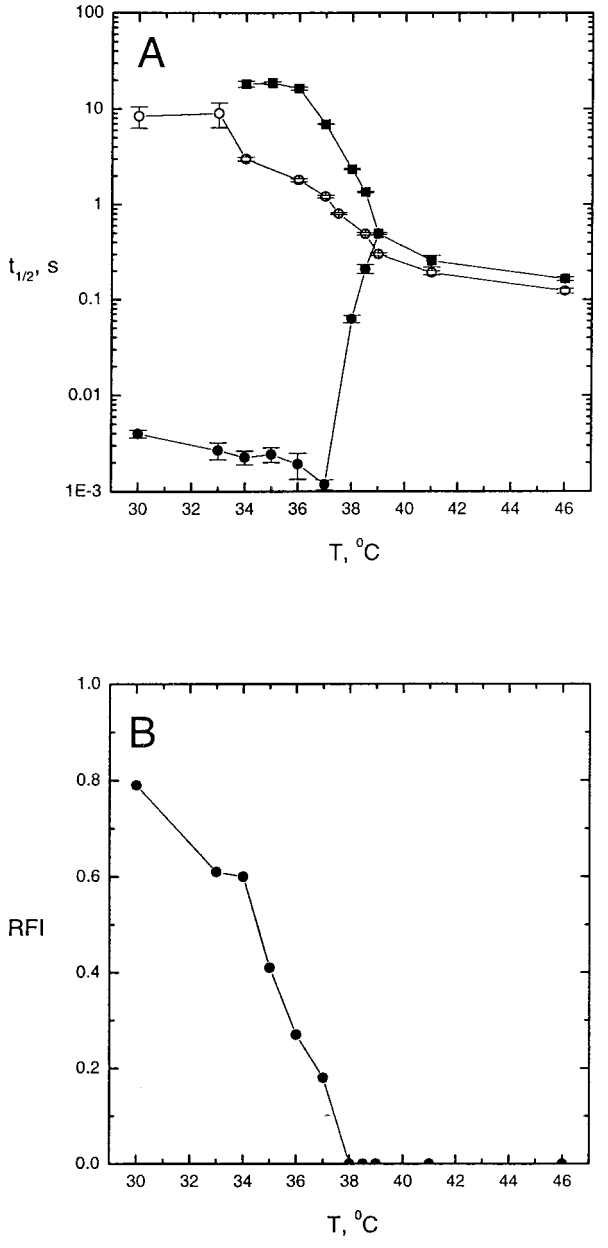

FIGURE 4 Dependence of the binding of adriamycin to LUVs on the main transition of DPPC. LUVs were composed of DPPC:PPDPG (●,■) (97:3, mol/mol). The buffer was 5 mM HEPES, 0.1 mM EDTA, pH 7.4. The final lipid concentration was 25 μM, and that of adriamycin was 4 μM. The temperature was varied as indicated. For the sake of clarity, the values for $t_{1/2}$ are given on a logarithmic scale. Half-times for the components are compiled in *A*, and the amplitude of the fast component of the fluorescence decay for DPPC:PPDPG LUVs is given in *B*.

To verify changes in the lateral enrichment of the pyrene-labeled probe into microdomains in the course of the main transition, we then measured I_e/I_m for DPPC/PPDPG alloy as a function of temperature (Fig. 5). For DPPC/PPDPG LUVs, values of I_e/I_m increased with temperature up to ~34°C, with a plateau between 34 and 37°C. Above ~37°C I_e/I_m begins to decrease in a progressive manner, reaching a minimum at ~41°C, after which I_e/I_m in fluid-state LUVs increases linearly as a function of temperature. For comparison we also carried out the measurements under identical conditions for the neutral pyrene-labeled probe containing alloy, DPPC/PPDPC (Fig. 5). For the latter LUVs the I_e/I_m value increases linearly below T_m, reaching a maximum at ~40°C, in accordance with previous studies (Somerharju et al., 1985; Marcie and Lentz, 1986; Hresko et al., 1986). A sharp decline in I_e/I_m is observed at T_m with a minimum at ~41°C, after which I_e/I_m in the liquid crystalline state

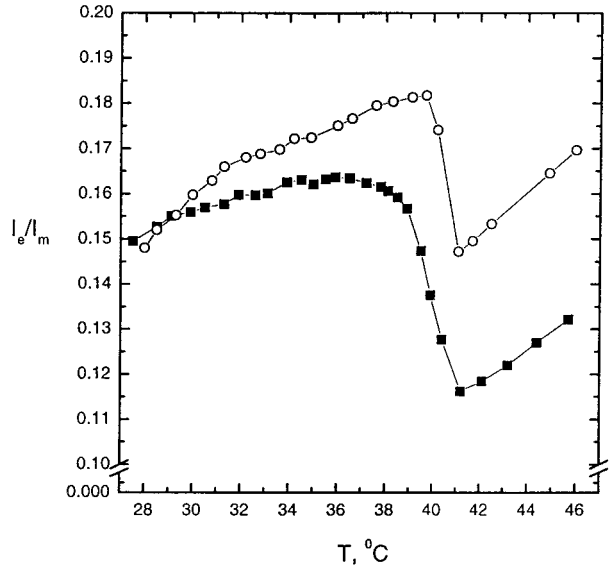

FIGURE 5 Steady-state fluorescence measurements of I_e/I_m for DPPC:PPDPG (97:3, mol/mol, ■) and DPPC:PPDPC (97:3, mol/mol, ○) LUVs as a function of temperature. The total lipid concentration was 10 μM in 5 mM HEPES, 0.1 mM EDTA, pH 7.4.

increases linearly with temperature. In keeping with electrostatic repulsion between the PPDPG probes augmenting the exclusion of this substitutional impurity from the gel state DPPC matrix and in general aiding the dispersion of this probe, the values for I_e/I_m measured for PPDPG at temperatures above ~30°C were clearly less than those for the zwitterionic PPDPC.

To elucidate the correlation between fluorescence measurements as a function of temperature and the thermally induced main phase transition of the LUVs, used we performed high-sensitivity DSC measurements for DPPC, DPPC:PPDPC (97:3, mol/mol), and DPPC:PPDPG (97:3, mol/mol) LUVs (Fig. 6). The numerical data from the DSC measurements, i.e., peak onset, offset, and maximum values of both experimental and deconvoluted data, are compiled in Table 1. For pure DPPC, deconvolution with one Gaussian peak was not satisfactory, whereas two overlapping Gaussian peaks yielded very good fit with the scan. For the binary mixtures three Gaussian peaks were necessary to reproduce the measured heat capacity scans and gave profiles indistinguishable from the experimental data.

DISCUSSION

Confirming the importance of electrostatic attraction between the cationic adriamycin and acidic phospholipids, the rate of the membrane association of this drug was profoundly increased upon the introduction of the negatively charged lipid into fluid POPC LUVs. More specifically, for POPC vesicles the half-time for fluorescence decay was ~2 s, whereas as X_{PG} was increased to ≥0.04-s, a fast decay with $t_{1/2}$ in the range of 2–4 ms became evident. Although exact values cannot be calculated from the resonance energy transfer data, this difference in the values for $t_{1/2}$ readily translates into a ~1000-fold enhancement in the binding rate in the presence of the acidic lipid. Reversal of the fast component in the fluorescene decay by increasing [NaCl] and [$CaCl_2$] provides unequivocal evidence for this fast process being mediated by electrostatic attraction. With increasing X_{PG}, $t_{1/2}$ for the slow hydrophobic binding increased from 2 s to 18 s. This is likely to reflect the decrease in the content of adriamycin in the solution, which occurs as a progressively larger fraction of the drug becomes rapidly associated with liposomes by the fast electrostatic mechanism. Charge neutralization in the membrane by the bound adriamycin also readily diminishes the electrostatic attraction between the surface and the drug in the solution. Experiments using different [Adr] demonstrated $t_{1/2}$ for the slow component to be concentration dependent, i.e., to decrease with increasing [Adr] (data not shown). Adriamycin has been shown to penetrate through the lipid bilayers into the liposomes (Praet et al., 1993). Translocation of adriamycin is, however, very slow, requiring 5–10 min (Praet et al., 1993). Accordingly, it is unlikely that this process would contribute to the fluorescence changes reported here. The strong dependence of the fast electrostatic-driven association of adriamycin on X_{PG} allows us to use this process to probe the initial organization of the liposome surface prevailing before the binding of adriamycin has commenced. At this point it is relevant to note that we did not study the effects of adriamycin either on the membrane organization or on the transitional behavior of the lipids. Instead, we assessed the effects of changes in membrane organization on the kinetics of binding of adriamycin, with the main emphasis on the fast electrostatic association of the drug with liposomes. More specifically, we measured the half-times for fluorescence intensity decays due to resonance energy transfer (RET), resulting from the electrostatically driven membrane association of drug, as further determined by the membrane content (X_{PG}), as well as by the initial distribution of the acidic phospholipid, PG, in the membrane. The latter also appears to be strongly dependent on the phase state of the DPPC matrix. After the binding process is complete and the system has reached a new equilibrium, the distribution of PG should be distinctly different from that in the initial state, assessed here. Likewise, the phase behavior of the liposomes has been shown to be altered by the associated drug (Goldman et al., 1978; Constantinides et al., 1986).

An interesting feature of the dependence of $t_{1/2}$ versus X_{PG} is that the fast component is absent when $X_{PG} < 0.04$. This is somewhat counterintuitive, as an increase in the net negative surface charge is readily anticipated to enhance the rate of membrane binding of the cationic drug. Lack of the fast decay at $X_{PG} < 0.04$ is not apparent, as the amplitude of this process does not extrapolate to zero at $X_{PG} = 0$. Likewise, equally intriguing is the highly irregular dependence of $t_{1/2}$ on X_{PG} within $0 < X_{PG} < 0.10$, shown in Fig. 2 *B*. A likely explanation for the appearance of the fast decay above $X_{PG} \approx 0.04$ could be the formation of super-

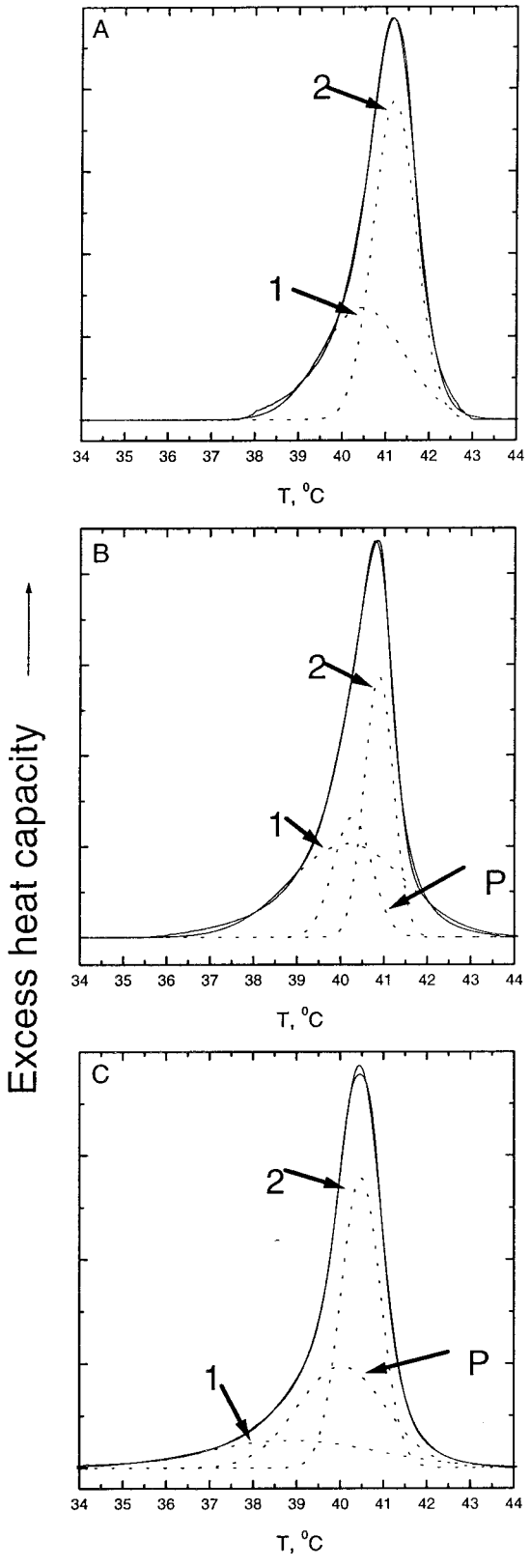

FIGURE 6 DSC profiles (raw and deconvoluted) for the main transition of DPPC (*A*), DPPC:PPDPC (97:3, mol/mol) (*B*), and DPPC:PPDPG (97:3, mol/mol) (*C*) LUVs. The total lipid concentration was 0.2 mM in 5 mM HEPES, 0.1 mM EDTA, pH 7.4. The measured enthalpy peaks were deconvoluted by using either two (DPPC) or three (DPPC:PPDPC, DPPC:PPDPG) overlapping Gaussian endotherms.

TABLE 1 Compilation of the measured and deconvoluted DSC endotherms for the LUVs

	DPPC	DPPC/PPDPC 97:3, mol/mol	DPPC/PPDPG 97:3, mol/mol
T_m	41.2°C	40.9°C	40.4°C
T_1	37.7°C	35.6°C	34.0°C
T_2	43.0°C		
Peak 1			
T_{max}	40.5°C	40.2°C	38.9°C
T_1	37.7°C	36.2°C	34.0°C
T_2	43.4°C	43.8°C	43.5°C
Peak 2			
T_{max}	41.2°C	40.9°C	40.5°C
T_1	39.6°C	39.8°C	38.9°C
T_2	42.8°C	42.0°C	42.0°C
Peak P			
T_{max}		40.3°C	40.0°C
T_1		39.0°C	36.9°C
T_2		41.6°C	43.3°C

Numerical data for the main transitions are those illustrated in Fig. 6. The maximum in heat capacity in each curve is marked as T_m (experimental data) or T_{max} (in deconvoluted peaks). The deviation from the baseline is denoted as T_1 and return to the baseline as T_2. The numbering of the peaks is identical to that used in Fig. 6. See text for details.

lattices of the acidic phospholipid in the liquid crystalline membranes. In brief, because of coulombic repulsion the distribution of the deprotonated PGs can be readily expected to have a free energy minimum when the separating distances are at maximum. This condition is fulfilled when the acidic phospholipids are distributed as a hexagonal superlattice in the bilayer. As a consequence, for $X_{PG} < 0.04$ the increase in the coulombic attraction of the drug to the bilayer due to the acidic phospholipid would remain below the counteracting factors, i.e., entropy and hydrophilicity of the drug, which retard the partitioning of adriamycin into the bilayer. However, as X_{PG} is increased, denser superlattices of PG are formed, and the magnitude of the electrostatic attraction between the drug and the membrane surface exceeds the above energy barriers. Yet the effective net negative surface charge density does not increase monotonously with X_{PG}, but is a step function. As a consequence, there is a sharp threshold in X_{PG} above which the coulombic attraction of the drug is greatly enhanced. This is shown in Fig. 7 *A*, which illustrates the regular distribution of PG at $X = 0.037$. At this content of PG, maximum separation of charges is achieved when PG molecules are each separated by five rows of phosphatidylcholine (PC). However, when X_{PG} exceeds 0.037, the added PG molecules must be accommodated in the membrane at interstitial sites within the original superlattice (Fig. 7 *B*). Accordingly, there is a sharp local increment in net negative surface charge density. A further increase in X_{PG} necessitates the formation of a denser lattice. A maximum in $t_{1/2}$ is observed at $X_{PG} = 0.065$. This is in accordance with a previously predicted hexagonal superlattice (Virtanen et al., 1988) yielding the

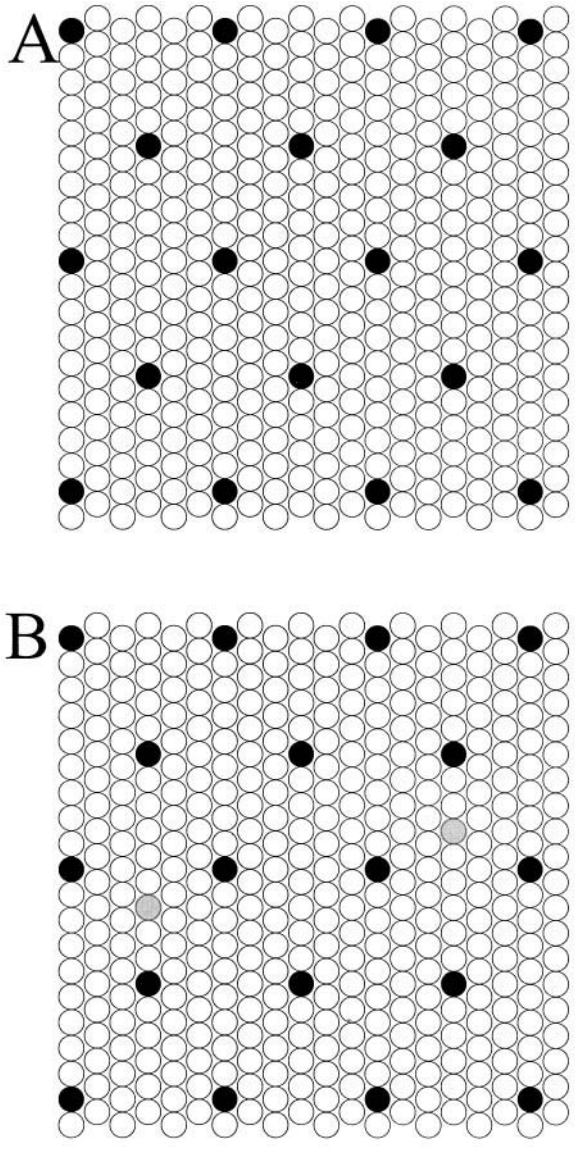

FIGURE 7 Schematic model of the hexagonal superlattices formed by PG (●) in a PC (○) matrix. (*A*) The time-averaged organization of the membrane at the first critical X_{PG}, 0.037. (*B*) Distribution of PG upon slightly exceeding $X_{PG} = 0.037$, necessitating the location of the exceeding PGs at interstitial sites (◎).

free energy minimum for this assembly. Importantly, for this lattice the average charge density is smaller than in the intermediate distribution pattern illustrated in Fig. 7 *B*. Accordingly, the rate of the membrane association of adriamycin should decrease, as observed.

Our laboratory provided the first evidence for the formation of superlattices in fluid binary alloys (Somerharju et al., 1985). These findings were subsequently confirmed and extended to other compositions (e.g., Tang and Chong, 1992; Chong et al., 1994; Tang et al., 1995). Notably, these superlattices were mainly concluded from steady-state fluorescence measurements, and the underlying repulsive potential was suggested to be steric elastic strain imposed by the bulky fluorescent moities. In the present case it is the strong coulombic repulsion that provides the main driving force. This also involves factors that complicate the prediction of the exact values of X_{PG} producing the superlattices by the acidic headgroups, as follows. In parallel with an increase in X_{PG}, the affinity of the bilayer surface for protons also increases progressively, thus decreasing the degree of the dissociation of the phosphate moiety of PG (Träuble, 1977), with a concomitant reduction in surface charge density. In addition, intermolecular hydrogen bonding between protonated and deprotonated PG headgroups becomes possible (Boggs, 1987), which will also necessarily influence the ordering in the binary lipid alloy and compete with the interaction of adriamycin with the deprotonated acidic phospholipid. The above is in keeping with the membrane binding of another cationic ligand, cytochrome *c*, becoming (at first glance paradoxically) slower with increasing X_{PG} in the liposomes (Subramanian et al., 1998). Obviously, the above processes will also affect the rate of binding of adriamycin to the liposomes. Accordingly, particularly at higher mole fractions of PG, a more complex dependence of $t_{1/2}$ versus X_{PG} would be expected. We did not pursue this problem further at this stage.

The strong dependence of the fast process on X_{PG} allowed us to use the kinetics of the membrane association of adriamycin to probe changes in the lateral distribution of PG in the course of the phospholipid main transition. Interestingly, at $T < 34°C$, only the fast binding with $t_{1/2} \approx 4$ ms was observed (Fig. 4.), thus readily revealing the formation of microdomains enriched in PG (i.e., with local $X_{PG} > 0.04$), and therefore having increased negative surface density charge. The lack of the slow hydrophobicity-driven binding is likely to reflect the tight packing of the gel-state lipid acyl chains, preventing the intercalation of adriamycin into the bilayer. At $T > 34°C$ the slow hydrophobicity driven membrane association of adriamycin also becomes evident. This is likely to result from the formation of "fluid" domains in the bilayer (Jutila and Kinnunen, 1997). The emergence of "fluid" domains in gel bulk is consistent with the enhanced hydrophobic binding of adriamycin to the electrically neutral DPPC/PPDPC LUVs (containing no acidic phospholipid) upon exceeding $T \approx 33°C$. However, as the slow binding process is observed for the DPPC/PPDPC LUVs already within the temperature range of 30–33°C, it is possible that the lack of the slow component for DPPC/PPDPG LUVs at temperatures lower than 34°C is apparent. More specifically, for the latter liposomes the slow component would not be seen, as a large fraction of the drug is already bound to the clustered acidic phospholipid, thus reducing the concentration of adriamycin remaining in the solution after the fast, electostatically driven membrane association of the drug is complete. Above $T = 34°C$ the decrease in RFI as a function of *T* also becomes augmented (Fig. 4 *B*). In other words, although *n* (the number of PG molecules encriched in clusters) diminishes when *T* approaches 34°C, as *T* exceeds this temperature, another process causing a decrease in *n* becomes effective. The interpretation of the appearance of the slow fluorescence decay

at $T = 34°C$ as the formation of "fluid" domains in the gel state bilayer is supported by the DSC measurements, which revealed the onset of the main phase transition endotherm at 34°C in the DPPC/PPDPG LUVs. Simultaneously with increasing T in the range from 34 to 38°C, the PG-enriched clusters seem to be diminishing either in size or number as the amplitude of the fast fluorescence decay is steeply attenuated. Yet, although the number of PG molecules in microdomains is decreasing, the net negative charge density appears to reach a weak local maximum at ~37°C, as revealed by the minimum of ~1 ms in $t_{1/2}$.

The fast fluorescence decay for DPPC:PPDPG (97:3, mol/mol) LUVs is not observed at $T > 37°C$. This temperature coincides with the plateau in I_e/I_m observed in steady-state measurements (Fig. 5), as well as with the onset of peak P in the deconvoluted DSC data for these LUVs (Fig. 6 *C* and Table 1). The apparent lack of clusters of PPDPG is revealed by the absence of the fast electrostatic component in adriamycin binding above 37°C. This is intriguing, as steady-state measurements still reveal highly efficient excimer formation in the temperature range of 38–41°C (Fig. 5). We have previously suggested PPDPC to be preferentially accommodated within the interface between gel and "fluid" domains of 1,2-myristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Jutila and Kinnunen, 1997). Similar behavior for PPDPG in the melting of DPPC matrix would be compatible with the present data. Accordingly, the characteristics of the fast component at 37°C (i.e., minimum in both $t_{1/2}$ and amplitude) would result from a change in the dimensionality of the lateral arrangement of PPDPG from 2-D (clusters) to pseudo-1-D (arrays), the latter being formed in the domain boundaries. For the latter, efficient excimer formation is still evident. Importantly, small amounts of adriamycin associated with the PPDPGs concentrated in these 1-D arrays residing within the domain boundaries will strongly diminish the effective charge density and yield a small amplitude for the process, even if the initial affinity is high. An alternative explanation is that the size of the PPDPG clusters falls progressively with T, reaching at ~37°C a size no longer having a sufficient number of charges per unit surface area to provide the site for fast binding of adriamycin. However, the latter mechanism does not explain the fact that $t_{1/2}$ has a minimum at 37°C. Furthermore, the area of "fluid" domains at 38°C, for instance, should be 75% of the total liposome surface area to reduce the concentration of the probe X_{PG} below 0.04 and, accordingly, to abolish the fast binding. This seems unlikely because the area under the nondeconvoluted endotherm up to 38°C corresponds to only ~10–15% of the total enthalpy of the transition. Accordingly, we do consider the former mechanism to be more plausible. The driving force for the enrichment of PPDPG into the boundaries between "fluid" and gel domains in the temperature range of 34–38°C can be rationalized as follows (Jutila and Kinnunen, 1997). In brief, a perturbating substitutional impurity of PPDPG is expelled from the gel-state DPPC matrix (Somerharju et al., 1985; Hresko et al., 1986). Upon the transition from gel into the liquid crystalline state, the thickness of the DPPC bilayers is reduced by ~4.5 Å, from 23 to 18.5 Å (Chapman et al., 1967; Lewis and Engelman, 1983). We have previously shown hydrophobic mismatch to promote the clustering of PPDPC in liquid crystalline bilayers of thickness <20.7 Å (Lehtonen et al., 1996). The effective length of PPDPG should equal that of PPDPC. We can thus expect PPDPG to also be expelled to some extent from the "fluid" domains below T_m. Accordingly, preferential partitioning of the probe into the domain boundary can be anticipated.

CONCLUDING REMARKS

The present stopped-flow fluorescence spectroscopy measurements demonstrate that the hydrophobic and electrostatic adriamycin-binding sites in liposomes can be distinguished by their different fluorescence decay half-times. Subsequently, this finding was exploited to study the effect of the DPPC main phase transition on the lateral distribution of a trace amount of an acidic phospholipid probe PPDPG in the bilayer. To our knowledge, this is the first observation that the binding of a drug to a membrane can be controlled by the formation of domains enriched in acidic phospholipids and with high local negative surface charge density. These measurements also provide evidence for the formation of "fluid" domains in the gel state matrix to begin at a temperature well below the peak of the main transition endotherm determined by DSC. Comparison of the stopped-flow data with values of I_e/I_m derived from steady-state fluorescence measurements provide further evidence for the enrichment of the fluorescent probe, PPDPG, in the interface separating gel-state and fluid domains in the coexistence region present in the course of the transition. These data lend further support to our previous results indicating the length of the interfacial boundary to have a maximum well below the main endotherm, in this case ~3.5° below the DSC peak at 40.4°C. Accordingly, the models and mechanisms for the phospholipid main transition may have to be revised along the lines discussed previously (Jutila and Kinnunen, 1997). More specifically, our fluorescence data indicate a lack of "fluid"/gel phase boundaries in the range from 38°C to 43.7°C. Yet, as the peak in heat capacity is observed at 40.4°C, the molecular mechanisms underlying the enthalpy changes in this temperature range and the nature of the implied "intermediate" phase pose an intriguing problem. Regarding the latter, it is possible that in this intermediate phase the chain order and lattice translational and positional order are uncoupled, like the liquid-ordered phase present in the phospholipid-cholesterol phase diagram (Vist, 1984; Ipsen et al., 1987; Vist and Davis, 1990).

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