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Detachment of Cytochrome *c* by Cationic Drugs from Membranes Containing Acidic Phospholipids: Comparison of Lidocaine, Propranolol, and Gentamycin

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ABSTRACT

A large number of pharmaceutically active compounds have a high affinity to acidic phospholipids; good examples are the cationic compounds lidocaine, propranolol, and gentamycin. These drugs influenced the lipid dynamics of liposomes composed of phosphatidylcholine and the acidic phosphatidylglycerol, as judged by the excimer/monomer emission intensity ratio for a pyrene-labeled phospholipid analog, as well as by polarization of DPH fluorescence. When the mole fraction X of PG (X_{PG}) was 0.20, lidocaine increased membrane fluidity. The opposite was true for propranolol, which caused the formation of pyrene lipid-enriched microdomains. Gentamycin had no apparent effect. At $X_{PG} = 1.00$, all these drugs rigidified membrane. Subsequently, we investigated the detachment of a cationic peripheral membrane protein, cytochrome *c* (cyt *c*), by these compounds from liposomes. This was accomplished by monitoring resonance energy transfer from a pyrene-labeled phospholipid to the heme of cyt *c*. The efficiency of the above compounds to dissociate cyt *c* varied considerably. In brief, significantly lower concentrations of gentamycin than propranolol or lidocaine were required for half-maximal dissociation of cyt *c* from liposomes, although the final extent of protein detachment by gentamycin was less complete. ATP augmented the dissociation of cyt *c* from membranes by lidocaine and propranolol. Stopped-flow measurements also revealed that the half-times differed for the release of cyt *c* from the membranes. Our results are likely to reflect differences in the contributions of the electrostatic interactions and hydrophobicity to the drug/lipid interaction and comply with two different acidic phospholipid binding sites in cyt *c*.

A large number of drugs of diverse chemical structure and with a range of pharmacological effects bind avidly to lipid bilayers. Prominent examples are provided by the anticancer drug doxorubicin (Mustonen and Kinnunen, 1993; Praet and Ruysschaert, 1993), the aminoglucosidic antibiotic gentamycin (Brasseur *et al*., 1984; Chung *et al*., 1985; Kubo *et al*., 1986; Gurnani *et al*., 1995), the β-adrenergic drug propranolol (Schlieper and Steiner, 1983; Hanpft and Mohr, 1985; Kubo *et al*., 1986; Albertini *et al*., 1990), and local anesthetics such as lidocaine (Davio and Low, 1981; Schlieper and Steiner, 1983; Hanpft and Mohr, 1985; Ueda *et al*., 1994). The ability of propanolol and lidocaine to penetrate into membranes and to disorder the hydrocarbon core has been found to correlate with anesthetic potency of these drugs (Ueda *et al*., 1994). However, for all the above compounds, the significance of their lipid-binding properties to their pharmacological mechanisms of action remain uncertain. Not excluding other sites of action, these compounds could, in principle, interfere with the lipid/protein reactions of integral membrane proteins. Likewise, they also may detach peripheral proteins from membrane surfaces, as demonstrated for vinculin and the membrane-partitioning drug chlorpromazine (Ito *et al*., 1983).

Peripheral, lipid-associating proteins are abundant in all cell types and are involved in diverse cellular functions, such as signal transmission, blood coagulation, and mitochondrial respiration (for a recent review, see Kinnunen *et al*., 1994). A well established example is provided by protein kinase C (Newton, 1993). Unlike integral proteins, the association of peripheral proteins to membranes can be controlled reversibly, thus offering excellent means for rapid and efficient regulation of their functions due to attachment and detachment from lipids. Although the molecular level details of peripheral lipid/protein interactions still are understood

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ABBREVIATIONS: cyt *c*, cytochrome *c*; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; PG, phosphatidylglycerol; I_e/I_m, ratio of excimer and monomer fluorescence; LUV, large unilamellar vesicle; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PPDPG, 1-palmitoyl-2[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphatidylglycerol; RET, resonance energy transfer; RFI, relative fluorescence intensity; X_{lipid}, mole fraction of the indicated lipid.

poorly, it is obvious that they also may provide for potential sites for therapeutic intervention. In other words, it should be possible to design compounds for interfering with the membrane binding of specific target proteins.

Cyt *c* is a well-characterized peripheral protein of the inner mitochondrial membrane, with a high affinity to acidic phospholipids (for a review, see Kinnunen *et al*., 1994). Intriguing recent results show that cyt *c* also is centrally involved in programmed cell death, apoptosis (Kluck *et al*., 1997; Yang *et al*., 1997). We used cyt *c* as a model to characterize the role of electrostatics in the regulation of its binding to acidic phospholipids. Cyt *c* is particularly well suited for *in vitro* studies in that quenching of pyrene monomer fluorescence due to resonance energy transfer from this aromatic hydrocarbon to the heme of cyt *c* allows facile measurement of the attachment of this protein to membranes containing pyrene-labeled lipids (Mustonen *et al*., 1987). Membrane association of cyt *c* is controlled by ionic strength and pH (Rytömaa *et al*., 1992), and the mode of interaction of cyt *c* with liposomes is strongly dependent on their content of acidic phospholipids (Rytömaa *et al*., 1992; Rytömaa and Kinnunen, 1994, 1995, 1996). More specifically, we have been able to recognize two acidic phospholipid-binding sites in cyt *c*, with distinctly different characteristics, as follows. In brief, cyt *c* binds to deprotonated PG electrostatically via its A site, whereas binding to protonated PG occurs via the C site of cyt *c* and is likely to involve hydrogen bonding. The former predominates at $X_{PG} = 0.20$ and is effectively reversed by ATP, whereas the latter is effective at $X_{PG} = 1.00$ and is insensitive to ATP. We previously demonstrated dissociation of cyt *c* from liposomes by other cationic ligands, adrenocorticotropic hormone, poly-Lys, myristoylated basic peptide KRTLR, histone H_1 (Rytömaa and Kinnunen, 1996), and the cationic amphiphile sphingosine (Mustonen and Kinnunen, 1993).

We have undertaken efforts to elucidate the mechanisms governing competition between lipid-binding drugs and peripheral membrane proteins. As a first step, we compared the detachment of cyt *c* from membranes containing acidic phospholipids by three cationic drugs: lidocaine, propranolol, and gentamycin (Fig. 1). Our results demonstrate clear differences in the interference by these compounds with lipid/cyt *c* interactions. The importance of membrane lipid composition and, in particular, the importance of the content of acidic phospholipids are identified as critical determinants for the detachment of cyt *c* from membranes by these drugs.

Experimental Procedures

Materials. Horse heart cyt *c* (type VI, oxidized form), egg PC, egg PG, lidocaine, propranolol, gentamycin, HEPES, and EDTA were from Sigma Chemie (Deisenhofen, Germany). DPH was from EGA Chemie (Steinheim, Germany). POPG and POPC were from Avanti Polar Lipids (Birmingham, AL). As judged from its absorption spectra, the cyt *c* used was mainly in the oxidized form. Na_2 salt of ATP was from Boehringer-Mannheim (Mannheim, Germany). PPDPG was purchased from K&V Bioware (Espoo, Finland). No impurities were detected in these lipids with thin layer chromatography on silicic acid using chloroform/methanol/water/ammonia (65:20:2:2, v/v/v/v) as the solvent system and examination of the plates for pyrene fluorescence or after iodine staining. The concentrations of the nonfluorescent phospholipids were determined by phosphorus assay and that of PPDPG was determined spectrophotometrically at 342 nm using 42,000 cm^{-1} as the molar extinction coefficient for pyrene. Water used in the experiments was freshly deionized in a Milli RO/Milli Q (Millipore, Bedford, MA) filtering system.

Preparation of liposomes. Lipids were dissolved and mixed in chloroform to obtain the desired compositions. The fluorescent lipid analog PPDPG was present at an X of 0.01 in steady state measurements and at X = 0.03 in stopped-flow measurements, and the content of the other lipids was varied as indicated. In fluorescence anisotropy measurements, X = 0.002 of DPH was incorporated into liposomes. After mixing of the lipids, the solvent was removed under a stream of nitrogen. The lipid residue subsequently was maintained under reduced pressure for ≥2 hr and then hydrated in 20 mM HEPES/0.1 mM EDTA at room temperature to yield a lipid concentration of 1 mM. The pH of the buffer was adjusted to 7.0 with 5 M NaOH. To obtain unilamellar vesicles, the hydrated lipid mixtures were extruded with a LiposoFast small-volume homogenizer (Avestin, Ottawa, Canada). Samples were subjected to 19 passes through two polycarbonate filters (100-nm pore size; Nucleopore, Pleasanton, CA). Minimal exposure of the lipids to light was ensured throughout the procedure. Subsequently, the liposome solution was divided into proper aliquots and diluted with the above buffer. The final lipid concentration used in the experiments was 25 μM.

Cardiolipin, either as such or as a complex with cyt *c* oxidase, is likely to provide the physiological binding site for cyt *c* in the mitochondrial inner membrane (Vik *et al*., 1981). However, binding of cyt *c* to cardiolipin has been reported to cause the formation of inverted nonlamellar membrane structures, whereas this has not been ob-

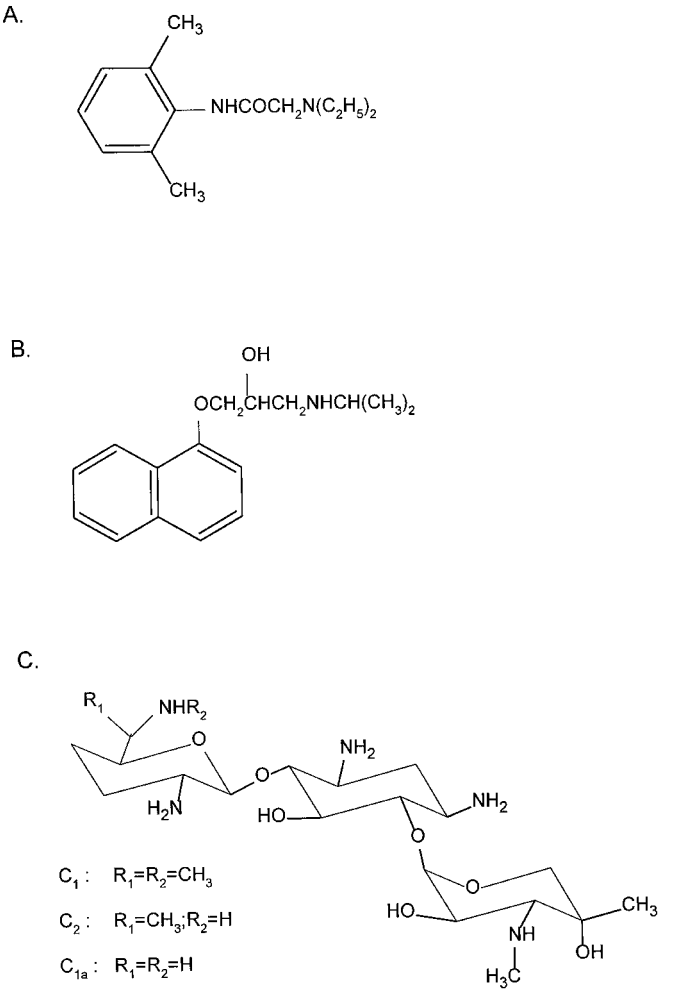

Fig. 1. Molecular structures of (A) lidocaine, (B) propranolol, and (C) gentamycin. Gentamycin is a mixture of three components: C_1, C_2, and C_{1a}.

served for PG. Accordingly, to avoid ambiguities in the interpretation of our data, the latter acidic phospholipid was chosen. Moreover, as has been noted previously, the characteristics of cardiolipin seem to be rather complex, presumably due to the vicinity of the two protonating phosphates in this molecule. To this end, except for the above, there seems to be no principal differences between different acidic phospholipids in the binding of cyt *c* (Rytömaa and Kinnunen, 1995).

Steady state fluorescence spectroscopy. The lipid binding and detachment of cyt *c* were assessed as described previously (Mustonen *et al.*, 1987; Rytömaa *et al.*, 1992; Rytömaa and Kinnunen, 1994, 1995) by monitoring resonance energy transfer between PPDPG and the heme of cyt *c*. Steady state fluorescence measurements were carried out with a Perkin Elmer LS-50B spectrofluorometer. The instrument was operated and data collected and analyzed using the dedicated software provided by Perkin-Elmer Cetus (Norwalk, CT). Pyrene was excited at 344 nm, and monomer and excimer emission was detected at 398 and 480 nm, respectively. In fluorescence measurements assessing RET between PPDPG and cyt *c*, bandpasses of 2.5 and 4.0 nm were used for excitation and emission, respectively. All measurements were carried out at 25°. Low concentrations of both lipids and protein ensure neglible interference due to inner filter effect (Lakowicz, 1983). Likewise, at the low probe concentrations used, the magnitude of the signals rising from probe superlattices (Kinnunen *et al.*, 1987) is insignificant compared with those caused by the drugs. Similarly, the magnitude of changes due to RET greatly exceeds the reduction in I_m due to the excimer formation. Furthermore, the rate of RET is much faster than excimer formation requiring collisional encounters after lateral diffusion, thus increasing the probability of the former process. To avoid nonequilibrium effects, we waited ~ 2 min after each addition of drug or protein before measuring the emission intensity. The fluorescence intensity values given have been corrected for decrease due to dilution. The advantages and limitations of the use of pyrene-labeled lipids in energy transfer measurements have been discussed elsewhere (Kaihovaara *et al.*, 1991; Rytömaa *et al.*, 1992; Mustonen and Kinnunen, 1993; Kinnunen *et al.*, 1993). More detailed description of the experimental procedures can be found in our previous publications (Mustonen *et al.*, 1987; Rytömaa *et al.*, 1992; Rytömaa and Kinnunen, 1994, 1995).

In fluorescence anisotropy measurements, polarizers were inserted into both the excitation and emission light paths, and 350 and 450 nm were used as excitation and emission wavelengths, respectively, with the corresponding bandwidths of 2.5 and 15 nm. Fluorescence anisotropy *r* was calculated according to Lakowicz (1983) with the equation

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

Measurements on possible effects of cyt *c* on fluorescence anisotropy of DPH would be ambiquous because of the strong overlap of their absorption spectra. Nevertheless, it is very unlikely that in this case, membrane dynamics would change without having an effect on I_e/I_m, at least for the lipids in direct contact with cyt *c*. However, because of quenching, this cannot be measured (Kaihovaara *et al.*, 1991). Changes in *r* and I_e/I_m have been correlated in another system by measuring the membrane binding of a protein containing no quenching moiety [i.e., histone H1 (Rytömaa and Kinnunen, 1996)].

Stopped-flow fluorescence spectroscopy. The binding and dissociation of cyt *c* were measured with a stopped-flow spectrofluorometer (Olis RSM 1000F; On-Line Instruments, Bogart, GA) equipped with a rapid scanning emission monochromator and a water-cooled 450-W xenon lamp. The temperature of the capillary cuvette compartment and the reactants was controlled with a circulating waterbath. The fluorescence traces were analyzed by the dedicated software provided by Olis. Excitation was at 344 nm, whereas emission spectra were recorded in the wavelength range of 365–515 nm. LUVs composed of POPG and POPC in the indicated stoichiometries and with PPDPG included as a fluorescent marker were used. Concentrations of the drugs and the protein were high enough so as to result in saturating responses in steady state measurements. Values given for the half-times represent average values from at least three separate measurements.

Results

Effects of lidocaine, propranolol, and gentamycin on lipid dynamics. To allow for an unambiquous interpretation of the data on the dissociation of cyt *c* from LUVs by these drugs, we first assessed the changes in pyrene fluorescence due to their binding to PPDPG containing liposomes in the absence of cyt *c*. These experiments were conducted at both X_{PG} = 0.20 and 1.00 so as to compare further their effects on the A and C site lipid association of cyt *c*, respectively.

At X_{PG} = 0.20, increasing lidocaine concentration progressively augmented excimer formation by the pyrene-labeled lipid, and at ~10 mM, saturation was reached with a ~ 7% increase in I_e/I_m (Fig. 2A). More pronounced effect on I_e/I_m, an increase by 42%, was observed at 20 mM propranolol. A further increase in propranolol concentration up to the highest concentration studied, 34 mM, enhanced I_e/I_m linearly

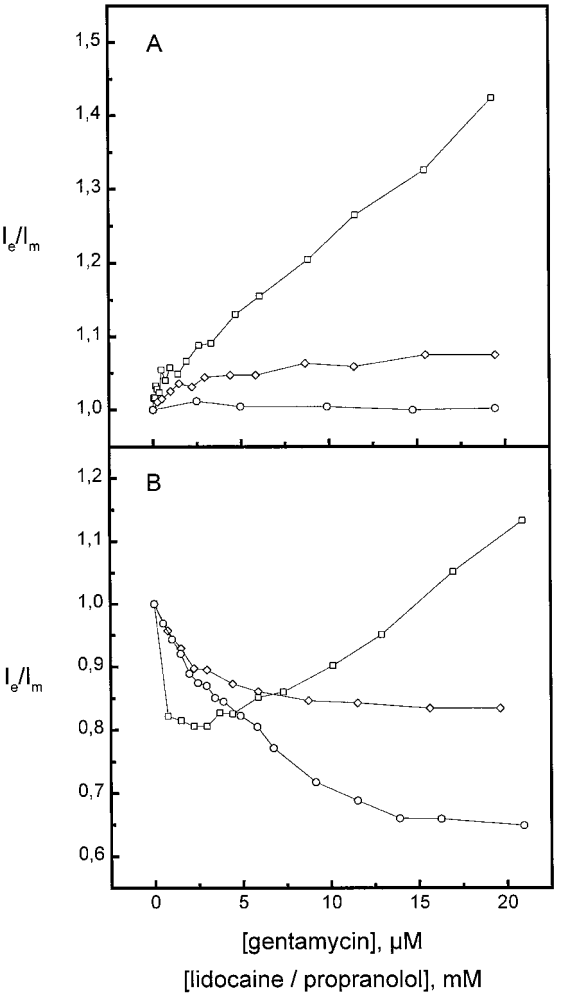

Fig. 2. The effects of lidocaine (◇), propranolol (□), and gentamycin (○) on I_e/I_m of pyrene fluorescence at X_{PG} = 0.20 (A) and 1.00 (B). Total phospholipid concentration was 25 μM. Values have been normalized to 1.0 in the absence of drugs. The aqueous phase was 20 mM HEPES/0.1 mM EDTA, pH 7.0.

(data not shown). Under these conditions, gentamycin (up to 63 μM) caused no changes in I_e/I_m.

For intermolecular excimer forming probes such as PPDPG, changes in I_e/I_m can be due to altered lateral diffusion, changes in the lateral distribution of the fluorescent probe, or both. To distinguish between these possibilities, we measured the corresponding changes in fluorescence anisotropy (r) for the rod-like hydrophobic probe DPH incorporated into liposomes (Fig. 3). In general, an increase in anisotropy can be expected to mirror rigidification of the membrane, which in turn attenuates lateral diffusion of lipids. The latter should be evident as decreased I_e/I_m. Accordingly, under conditions in which both r and I_e/I_m increase, the latter parameter is likely to reflect lateral enrichment of the pyrene-labeled lipid (Rytömaa and Kinnunen, 1996). At $X_{PG} = 0.20$, increased I_e/I_m is caused by lidocaine, whereas the opposite is true for r, thus indicating an increase in membrane-free volume to be due to this drug. Accordingly, it can be concluded that lidocaine under these conditions increases the rate of lipid lateral diffusion. In contrast, for propranolol, an increase in I_e/I_m is paralleled by an increase in r, thus revealing the fluorescent lipid PPDPG to become enriched into microdomains. Because gentamycin has no effect on I_e/I_m and the changes in r are maximally ≈5%, no correlation was observed in this case.

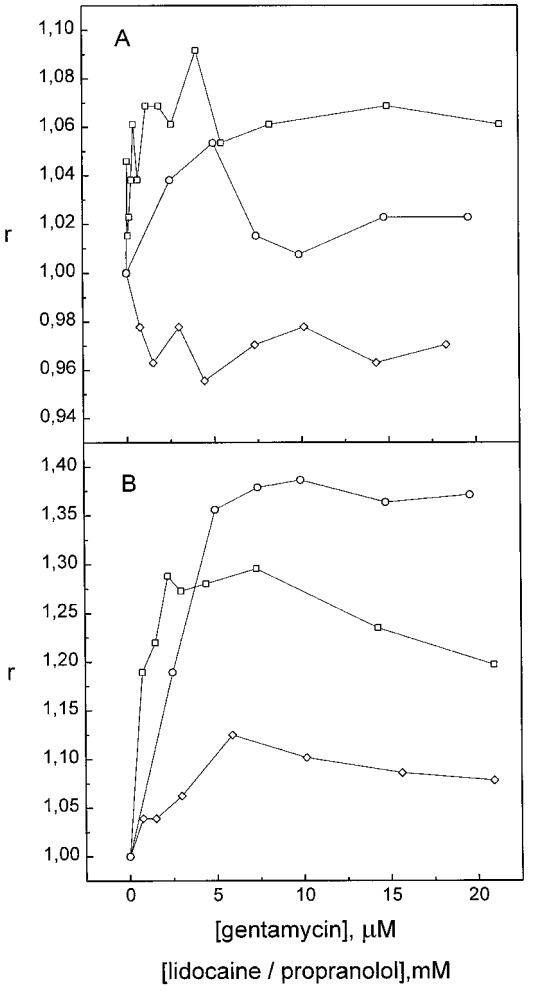

Fig. 3. The effects of lidocaine (◇), propranolol (□), and gentamycin (○) on fluorescence anisotropy r of DPH at $X_{PG} = 0.20$ (A) and 1.00 (B). Values have been normalized to 1.0 measured in the absence of the drugs.

At $X_{PG} = 1.00$, the effects of these drugs on I_e/I_m were strikingly different (Fig. 2B). A decrement in I_e/I_m by ~15% was observed for 15 mM lidocaine. A ≈20% decrease in I_e/I_m was first caused by 3 mM propranolol. However, this decrement was followed by a subsequent linear increase, similar to the effect of this drug at $X_{PG} = 0.20$. Interestingly, also at $X_{PG} = 1.00$, gentamycin decreased I_e/I_m by ~ 35%. The latter effect was evident at a 20 μM concentration.

We proceeded to study the effects of these drugs on DPH anisotropy at $X_{PG} = 1.00$ (Fig. 3B). At $X_{PG} = 1.00$ and at low concentrations, all three drugs increased r. Accordingly, the attenuation of excimer formation is at least partly caused by diminished lateral diffusion caused by these drugs. However, at a lidocaine concentration of >6 mM, anisotropy decreases, thus indicating that the observed further decrease in I_e/I_m results from lateral enrichment of PPDPG. The same pattern also was evident for gentamycin, which in concentrations of >6 μM has no effect on r. At a propranolol concentration of >3 mM, the increase in I_e/I_m is accompanied by decreased DPH anisotropy. However, this decrement in r is not as pronounced as the increase evident at lower propranolol concentrations (i.e., <3 mM), thus indicating that the increment in I_e/I_m caused by this drug is only partly due to an augmented lateral diffusion of PPDPG.

Binding of cyt *c* to liposomes. Resonance energy transfer from the pyrene-labeled phospholipid analog PPDPG to cyt c bound to liposomes containing acidic phospholipids results in a progressive decrease in RFI until an apparent saturation is reached at [cyt c] ≈ 1.0 μM (Fig. 4). Notably, at $X_{PG} = 0.20$, the apparent affinity of the A site of cyt c for acidic phospholipids exceeds that of the C site measured at $X_{PG} = 1.00$, and significantly lower protein concentrations produce a similar extent of quenching under the former conditions. Accordingly, at $X_{PG} = 0.20$ and 1.00, half-maximal quenching was evident at 0.15 and 0.35 μM cyt c, respectively.

Interestingly, at $X_{PG} = 1.00$ and in the presence of 5 mM ATP, the binding of cyt c to liposomes resembles that measured at $X_{PG} = 0.20$ in the absence of ATP, which is in keeping with an ATP-induced conformational change in cyt c enhancing the efficiency of RET (Rytömaa *et al.*, 1992). High affinity binding sites for ATP have been described in cyt c (Corthésy and Wallace, 1986). At $X_{PG} = 0.20$, the A site-mediated binding of cyt c to deprotonated acidic phospholipids is prevented by ATP. This reversal is readily rationalized in terms of the acidic phospholipids and ATP competing for the same cationic binding site or sites in cyt c (Rytömaa and Kinnunen, 1994; Tuominen *et al.*, 1997). At $X_{PG} = 0.20$ and in the presence of 5 mM ATP, the extent of quenching by cyt c was significantly diminished, from 75% to 20%. Increasing X_{PG} to 0.75 progressively increased the extent of quenching caused by 1.0 μM cyt c. Likewise, the extent of dissociation of cyt c from membrane by 5 mM ATP was attenuated with increasing X_{PG} (Fig. 4C). In keeping with our previous data (Rytömaa and Kinnunen, 1994), ATP did not affect the binding of cyt c to LUVs via the C site at $X_{PG} = 1.00$ (Fig. 4B). Instead, the apparent lipid affinity of cyt c at $X_{PG} = 1.00$ was enhanced by ATP, presumably due to a conformational change in cyt c induced by the nucleotide (Rytömaa *et al.*, 1992).

A and C site-mediated association of cyt c to LUVs subsequently were studied using stopped-flow spectrofluorometry.

The fluorescence decay curves resulting from the binding of cyt *c* to liposomes are two-exponential (Fig. 5A), with approximately equal amplitudes for the two components. On increasing X_{PG} from 0.20 to 1.00, the half-time of the faster component remains at ~5 msec, whereas the half-time of the slower component increases from 19 to 30 msec. Dissociation of cyt *c* by ATP at X_{PG} = 0.20 was complete within <3 msec and thus inaccessible to measurement with our instrument with a dead-time of ~5 msec. The sensitivity to the liposome composition of this interaction is demonstrated by the slightly different values measured by Subramanian *et al.* (1998).

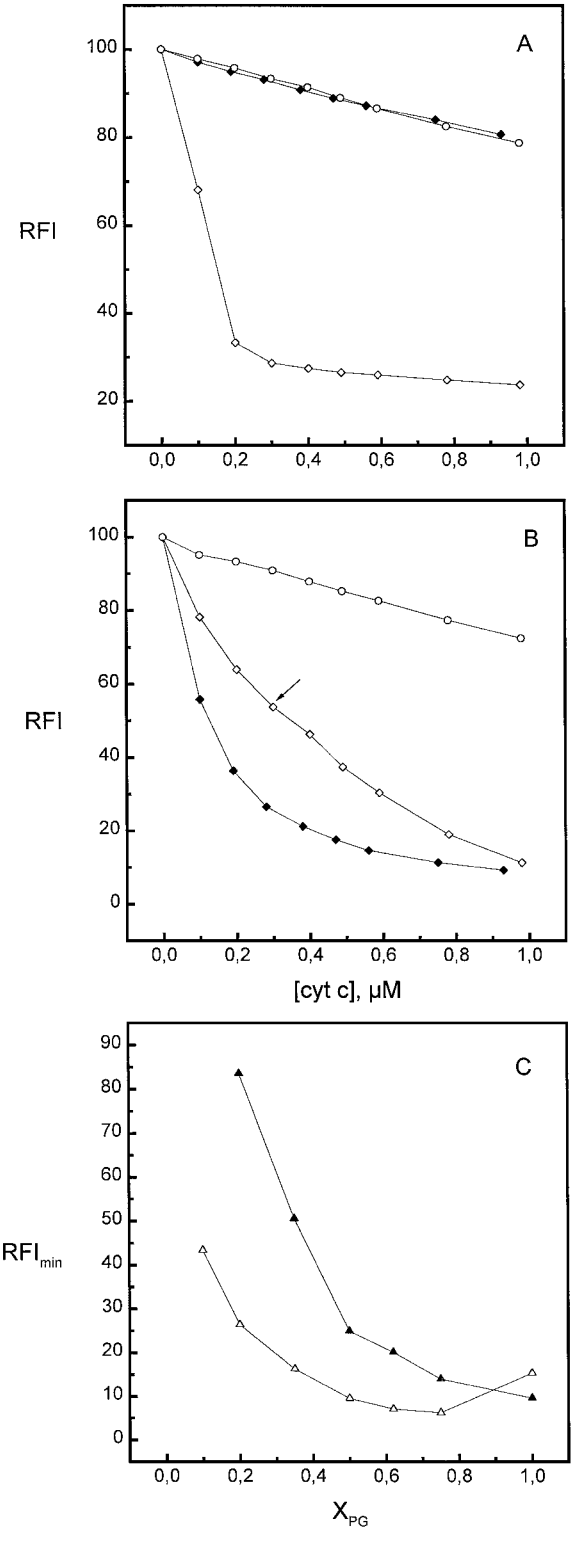

Fig. 4. Binding of cyt *c* to LUVs at X_{PG} = 0.20 (A) and 1.00 (B) in both the absence (◇) and presence of 5 mM ATP (◆). ○, Binding of cyt *c* in the presence of 60 and 6 μM gentamycin at X_{PG} = 0.20 and 1.00, respectively. C, Maximal extent of quenching by cyt *c* in the absence (△) and presence (▲) of 5 mM ATP as a function of X_{PG}.

Dissociation of cyt *c* from liposomes by lidocaine. Electrostatic interactions are critically involved in the binding of cyt *c* to acidic phospholipids. Accordingly, it could be readily anticipated that similar to the effect of sphingosine (Mustonen *et al.*, 1993), cationic, membrane-partitioning drugs should interfere with the lipid binding of cyt *c* and eventually dissociate this protein from liposomes. In the experimental system used in the current study, this should be evident as an increase in pyrene RFI (i.e., diminished RET), measured after the addition of increasing concentration of the drugs.

The detachment of cyt *c* from liposomes by lidocaine is illustrated in Fig. 6. To compare the ability of this drug to dissociate cyt *c* from liposomes, bound to the vesicles via its A or C site, these experiments were conducted at X_{PG} = 0.20 and 1.00, respectively. Lidocaine in a concentration of 8 mM reversed the A site interaction of cyt *c* with acidic phospholipids, with a maximum of ~80% recovery of the initial fluorescence intensity (Fig. 6A). At X_{PG} = 1.00, lidocaine concentrations up to 110 mM increase RFI from 8 to a maximum of 35 (Fig. 6B). Compared with the increase in I_m resulting from the dissociation of cyt *c* from membrane, the changes in RFI caused by lidocaine alone are small enough to allow the detachment of cyt *c* to be distinguished from drug-induced changes in I_m. Notably, because of the opposing signals caused by the binding of the drug to and the release of cyt *c* from the liposomes, it is clear that the magnitude of the recovery of fluorescence resulting from the detachment of cyt *c* represents a minimal estimate.

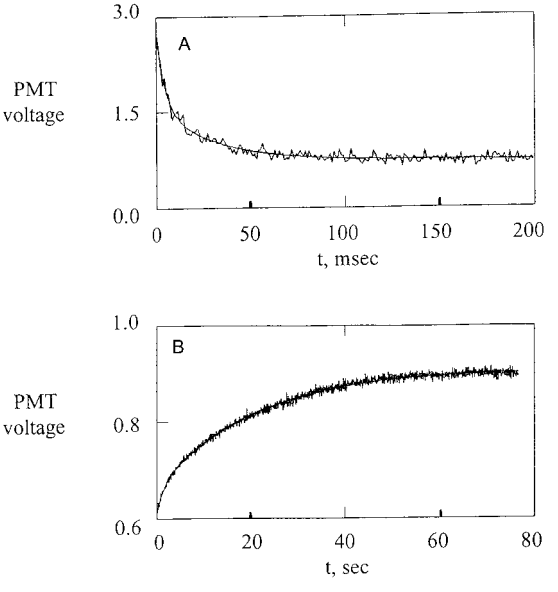

Fig. 5. Time-resolved binding of cyt *c* to liposomes (A) and dissociation by gentamycin at X_{PG} = 1.00 (B). Final concentrations of cyt *c*, phospholipids, and gentamycin in the mixing chamber were 1, 25, and 8 μM, respectively. Buffer was 20 mM HEPES/0.1 mM EDTA, pH 7.0. Temperature was maintained at 25°. *PMT*, photomultiplier.

ATP augments the detachment of cyt *c* by lidocaine at X_{PG} = 0.20, and lower drug concentration suffices in reducing RET between PPDPG and cyt *c* (Fig. 6A). Likewise, also at X_{PG} = 1.00, ATP decreases the concentration of lidocaine required for the detachment of cyt *c* from liposomes. However, low concentrations of lidocaine (up to ~10 mM) added subsequently to cyt *c* actually decrease RFI at X_{PG} = 1.00. In the presence of ATP, this phenomenon was less pronounced.

Data from measurements similar to those illustrated in Fig. 6, A and B, were subsequently collected so as to quantify $[lidocaine]_{50}$ versus X_{PG} (i.e., drug concentrations required for half-maximal reversal of the quenching of pyrene fluorescence by cyt *c* at different values of X_{PG}). On increasing X_{PG} from 0.20 to 1.00, $[lidocaine]_{50}$ increases ~20-fold in the absence and ~50-fold in the presence of 5 mM ATP (Fig. 6C). The extent of maximal detachment of cyt *c* from LUVs as a function of X_{PG} is illustrated in Fig. 6D. The ability of lidocaine to detach cyt *c* from membrane is strongly reduced when $X_{PG} \geq 0.50$, thus indicating a change in the nature of either cyt *c*/phospholipid or lidocaine/phospholipid interaction, or both, at this liposome composition. This change is likely to arise from different lipid packing below and above this mole fraction of the acidic phospholipid. At $X_{PG} \leq 0.75$, with the A site binding contributing to the cyt *c*/lipid interaction, ATP increases the maximal extent of recovery of RFI by lidocaine.

We then proceeded to study the dissociation of cyt *c* from liposomes by lidocaine using stopped-flow fluorescence measurements. At X_{PG} = 0.20, a two-exponentional increase in RFI was observed, with half-times of 6.5 msec and 14.5 sec and of approximately equal amplitudes (Table 1). At X_{PG} = 1.00, however, the magnitude of the increase in RFI due to the dissociation of cyt *c* by lidocaine was too small to be amenable to more detailed analysis.

Dissociation of cyt *c* from liposomes by propranolol. The effects of propranolol were investigated under conditions identical to those used for lidocaine (Fig. 7). More than 75% of the initial fluorescence is recovered by this drug at $X_{PG} \leq$ 0.50 and 1.00 (Fig. 7D). However, the efficiency of this drug to detach cyt *c* has a shallow minimum when $0.50 \leq X_{PG} \leq$ 0.75. Similar to lidocaine, propranolol dissociates cyt *c* more efficiently in the presence of ATP. More specifically, at X_{PG} = 0.20, almost complete recovery of RFI is evident in the presence of 5 mM ATP, whereas an 80% recovery is observed when the nucleotide is absent (Fig. 7A). At X_{PG} = 1.00, ATP decreased $[propranolol]_{50}$ from 14 to 8 mM (Fig. 7B). With propranolol concentration of >15 mM, values for fluorescence intensity exceeding the initial RFI were observed. Part of the signal is likely to be due to light scattering because at high drug concentrations (≥15 mM), this sample became turbid. For reasons that remain unclear, this turbidity was not observed in the absence of ATP. Similar to lidocaine, propran-

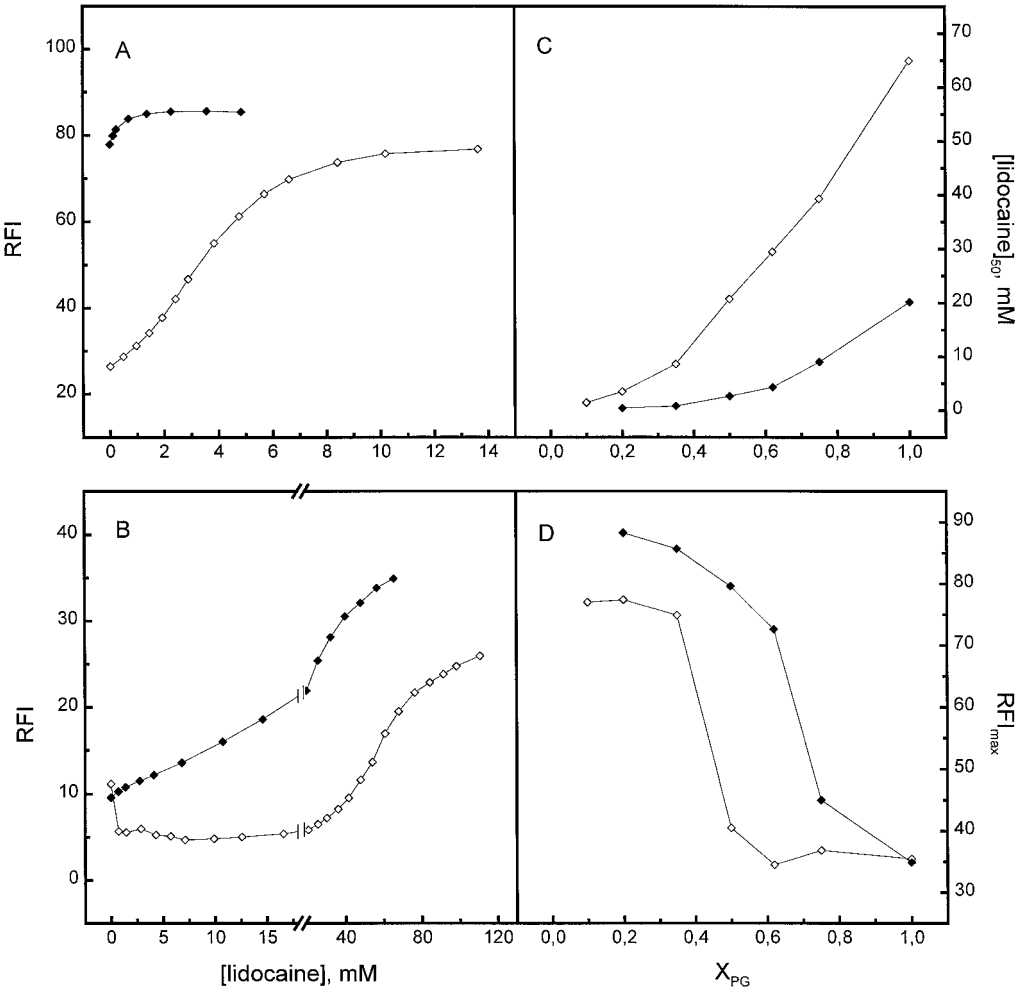

Fig. 6. Dissociation of cyt *c* from LUVs by increasing concentrations of lidocaine at X_{PG} = 0.20 (A) and 1.00 (B). *Open and solid symbols*, absence and presence of 5 mM ATP, respectively. C, Lidocaine concentration producing 50% recovery of RFI. D, Maximal recovery of RFI by lidocaine at various X_{PG} values.

olol at X_{PG} = 1.00 in the absence of ATP and added subsequently to cyt *c* did not cause an increase in RFI. Instead, low drug concentrations (~0.1 mM) slightly decrease RFI. Data from measurements similar to those illustrated in Fig. 7, A and B, were subsequently collected to quantify $[propranolol]_{50}$ as a function of X_{PG} (Fig. 7C). An apparently exponential dependency $[propranolol]_{50}$ versus X_{PG} is evident in both the absence and presence of 5 mM ATP.

Under both conditions (X_{PG} = 0.20 and 1.00), the dissociation of cyt *c* by propranolol could be detected by stopped-flow. However, the process was too fast (complete within <3 msec) so as to allow for detailed analysis.

Dissociation of cyt *c* from liposomes by gentamycin. To compare the contributions of hydrophobic and electrostatic forces with the drug/membrane interactions, experiments similar to those described above for lidocaine and propranolol subsequently were carried out with gentamycin (Fig. 8). The values for $[gentamycin]_{50}$ required for half-maximal reversal of quenching were significantly lower than those of lidocaine: 9.3 and 3.2 μM at X_{PG} = 0.20 and 1.00, respectively. However, the extent of the reversal was less complete, in particular at higher contents of PG, with the final RFI varying between 30% and 75% (Fig. 8D). Similar to both lidocaine and propranolol, at X_{PG} = 1.00, low concentrations of gentamycin added subsequently to cyt *c* decreased RFI (Fig. 8B). This phenomenon was not observed in the presence of ATP. Indeed, ATP increased the final RFI at all values of X_{PG}, with the highest RFI of ~80% being measured at $X_{PG} \leq 0.50$. Values for $[gentamycin]_{50}$ as a function of X_{PG} measured in both the presence and absence of ATP are shown in Fig. 8C. Interestingly, in contrast to what is observed for the two amphiphilic drugs, ATP increased $[gentamycin]_{50}$.

Because the effects of gentamycin deviated from those of the two amphiphilic drugs, we also studied the binding of cyt *c* to liposomes subsequent to the prior additions of 60 and 6 μM gentamycin at X_{PG} = 0.20 and 1.00, respectively. Interestingly, under both conditions, only ~25% decrease in RFI was observed on increasing the cyt *c* concentration up to 1 μM (Fig. 4). Accordingly, although gentamycin lacked effect on lipid dynamics at X_{PG} = 0.20 when investigated by I_e/I_m and DPH polarization, under these conditions, this drug also must strongly bind to the liposome surface.

Stopped-flow experiments on the detachment of cyt *c* from LUVs by gentamycin revealed the recovery of fluorescence at X_{PG} = 0.20 to be a one-exponential process with a half-time of 7.9 msec (Table 1). At X_{PG} = 1.00, the dissociation became two exponential, and half-times of 1.20 and 20.8 sec with respective relative amplitudes of 83 and 17 were measured (Fig. 5B).

Discussion

The aim of the current study was to compare the efficiency of three cationic drugs, lidocaine, propranolol, and gentamycin, in displacing cyt *c* from liposomes containing acidic phospholipids. Although cyt *c* was used merely as a well-characterized model for a peripheral membrane protein, these data also could have pharmacological relevance. Both lidocaine and gentamycin have been reported to have effects on mitochondrial respiratory function. In brief, gentamycin-treated rats have been reported to have significantly declined mitochondrial cyt *c* and cytochrome oxidase concentrations (Mela-Riker *et al.*, 1986). Lidocaine has been shown to collapse transmembrane potential of mitochondria in cell cultures (Grouselle *et al.*, 1990) and to depress oxidative metabolism in porcine brain mitochondria (Haschke and Fink, 1975). Unfortunately, the effect of propranolol on respiratory function has not been studied. However, this compound has been reported to preferentially stabilize mitochondria (Kloner *et al.*, 1978), and mitochondrial inner membrane has been suggested to represent its main site of action (Johnson *et al.*, 1973).

All these drugs possess net positive charge or charges and bind avidly to membranes containing acidic phospholipids. The pK_a value of lidocaine is 7.87, and thus ~11% of the molecules are uncharged at neutral pH. This is in keeping with the decreased affinity of this drug for acidic lipids under physiological conditions (Ueda *et al.*, 1994). The pK_a value of propranolol is 9.45 (Warren *et al.*, 1974), and at neutral pH, it possesses a high affinity to acidic phospholipids (Schlieper and Steiner, 1983; Roucou *et al.*, 1995). Gentamycin has been reported to possess 3.46 positive charges at pH 7.4 (Josepovits *et al.*, 1982). Notably, these p*K* values were measured for the drug molecules in water. However, the p*K* values for membrane-bound drugs cannot be identical to those in solution and further may depend on the content of acidic phos-

TABLE 1

Compilation of the half-times for the fluorescence decays due to the binding of cyt *c* to LUVs either at X_{PG} = 0.20 or 1.00. Also shown are the corresponding values for the detachment of cyt *c* by ATP or the different cationic compounds. For two-exponential processes, estimates for the relative amplitudes of the components are in parentheses. Concentrations of the compounds used to detach cyt *c* from liposomes were those resulting in a saturating response in steady state measurements.

	X_{PG} = 0.20		X_{PG} = 1.00	
	Concentration	$t_{1/2}$	Concentration	$t_{1/2}$
		msec		*msec*
Binding of Cytochrome *c*	1 μM	5.2 (56) 19 (44)	1 μM	4.7 (50) 30 (50)
Dissociation by				
ATP	5 mM	<1[a]	[b]	
Lidocaine	6 mM	6.5 (50) 14.5×10^3 (50)	[b]	
Propranolol	0.125 mM	<1[a]	25 mM	<1[a]
Gentamycin	60 μM	7.9	8 μM	1.20×10^3 (83) 20.8×10^3 (17)

[a] Measurement limited by the dead time of the stopped-flow chamber.
[b] Insignificant dissociation.

pholipids in the bilayer. Surface pH is lower than bulk pH and decreases exponentially with increasing electrical potential of the surface. Binding of cationic molecules such as propranolol, gentamycin, or lidocaine to membranes containing acidic phospholipids decreases the negative surface charge density (Roucou *et al*., 1995), which in turn can be expected to increase the deprotonation of the acidic phospholipids (Träuble, 1976). Despite the lack of data on the net charge of membranes in the presence of the drugs or cyt *c*, the approach used in the current study allows a comparison of the abilities of these drugs to detach membrane-bound cyt *c* under identical conditions.

Both lidocaine and propranolol are amphiphilic and partially penetrate into the hydrophobic core of the membrane. The latter compound has been suggested to have two different binding sites in phospholipid membranes (Kubo *et al*., 1986; Kodavanti and Mehendele, 1990). The high affinity, low capacity binding site probably is in the surface and involves primarily electrostatic forces, whereas the low affinity, high capacity site has been proposed to reside in the interior of the lipid bilayer and mainly is due to the hydrophobicity of the drug. X-ray diffraction studies on propranolol/DPPC alloys revealed different kind of vesicles to be formed when the propranolol/DPPC molar ratio reaches 2.2 (Albertini *et al*., 1990). Accordingly, at high drug concentration, the possibility of the liposomes being transformed into smaller vesicles, perhaps micelles, must be considered. Using X-ray diffraction, Albertini *et al*. (1990) found propranolol to increase water layer thickness on DPPC membrane surface. Compared with propranolol, the affinity of lidocaine to membranes is less, and its effects on bilayers are not as pronounced (Schlieper and Steiner, 1983; Hanpft and Mohr, 1985). Ueda *et al*. (1994) demonstrated by FTIR lidocaine that hydrogen-bonded water is released from the phosphate and glycerol moieties of DPPC.

Gentamycin, a widely used aminoglycoside antibiotic, is hydrophilic, and its binding to liposomes requires acidic phospholipids (Brasseur *et al*., 1984; Chung *et al*., 1985; Kubo *et al*., 1986). The electrostatic association of gentamycin to liposomes results in charge neutralization and tightening of lipid packing (Gurnani *et al*., 1995). Due to its net positive charge of ~ 3, gentamycin should be able to complex with three negatively charged phospholipids. Minor hydrophobic interaction between gentamycin and membranes is indicated by the penetration of the drug into phospholipid monolayers (Brasseur *et al*., 1984).

Binding of these drugs to liposomes influenced lipid dynamics as judged by changes in I_e/I_m for PPDPG as well as in anisotropy of DPH. In brief, at X_{PG} = 0.20, lidocaine enhanced lipid lateral diffusion, whereas gentamycin had no effect. On the other hand, propranolol rigidified the membrane and caused lateral enrichment of PPDPG. At X_{PG} =

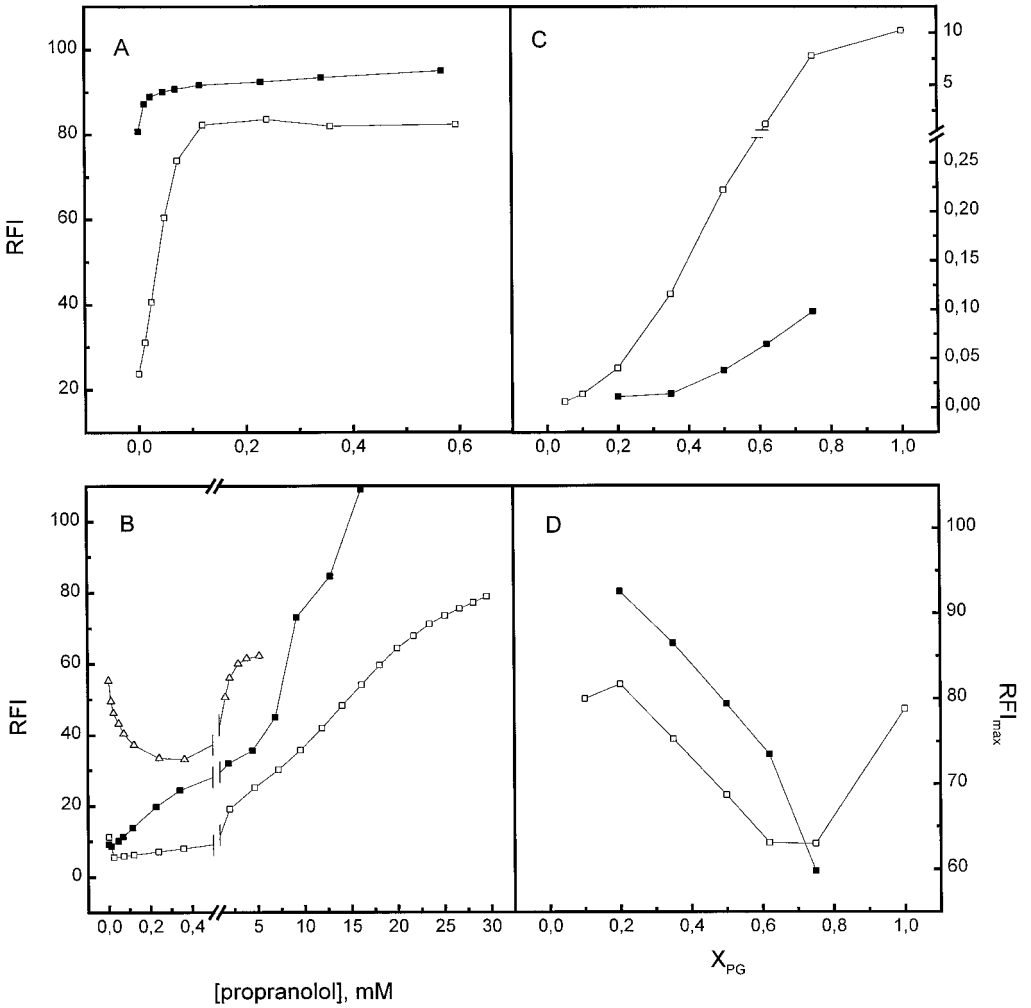

Fig. 7. Dissociation of cyt *c* from LUVs by increasing concentrations of propranolol at X_{PG} = 0.20 (A) and 1.00 (B). B, Effect of propranolol added at [cyt *c*] = 0.3 μM (△). *Arrow*, corresponding point in Fig. 4B. *Open and solid symbols*, absence and presence of 5 mM ATP, respectively. C, Propranolol concentration producing 50% recovery of RFI. D, Maximal recovery of RFI by propranolol at various X_{PG} values.

1.00, low concentrations of all the three drugs decreased lipid lateral diffusion, whereas at higher drug concentrations, lateral enrichment of PPDPG was evident. Due to its polycationic nature, gentamycin can ligand to different liposomes and cause their aggregation (Gurnani *et al.*, 1995). However, no evidence for aggregation was seen in our study. Notably, Gurnani *et al.* (1995) used lipid concentrations (10 w%) several orders of magnitude higher than those used in the current experiments (25 μM, 0.002 w%). Accordingly, it is likely that in our experiments, aggregation of acidic phospholipids by gentamycin takes place on the surface of liposomes. This would cause formation of PG-enriched domains, rigidification of bilayers, and decrease in lipid lateral diffusion, as indicated by the observed decrease in I_e/I_m (Fig. 2B). This effect weakens drastically on decreasing X_{PG}, thus revealing the affinity of gentamycin to be strongly dependent on the content of the acidic phospholipid.

At a saturating concentration (1 μM cyt *c*), the efficiency of quenching by cyt *c* of the bilayer embedded fluorescent probe decreases with increasing X_{PG} (i.e., with increasing degree of protonation of PG), thus suggesting diminished affinity of cyt *c* to membranes at X_{PG} = 1.00. In the light of the rather similar half-times for the membrane binding of cyt *c* at X_{PG} = 0.20 and 1.00, it seems unlikely that this difference should result from an altered affinity of cyt *c* for liposomes. A second alternative is that the number of cyt *c* binding sites on the liposome surface is reduced when X_{PG} is increased from 0.20 to 1.00. This explanation also seems unrealistic. The third, and most feasible, explanation is that the efficiency of RET for cyt *c* attached to liposomes via its A site at X_{PG} = 0.20 is higher than that for the C site bound cyt *c* at X_{PG} = 1.00. The rate of RET is inversely proportional to the sixth power of the distance. Therefore, particularly at close range, very small changes in the distance (<1 Å) can profoundly influence RET efficiency. Differences in the relative orientation of the oscillating dipoles of the donor and acceptor also strongly affect RET. Accordingly, the efficiency of quenching of pyrene by cyt *c* should depend on the orientation or conformation of the protein on the membrane surface. Importantly, RET is depleting excited monomers and thus reduces the formation of excimers. Accordingly, the measured I_e/I_m mirrors the state of the membrane surrounding the binding site of cyt *c* and beyond the quenching radius (>100 Å). In the concentration range studied, cyt *c* has no observable effect on I_e/I_m (data not shown).

Taking into account these differences in the lipid-binding properties of the three drugs, it was of interest to compare their ability to dissociate cyt *c* from liposomes. To correlate the capability of these compounds to release cyt *c* from membranes under conditions in which either A or C site interaction of the protein with acidic phospholipids is dominating, we determined $[drug]_{50}$ versus X_{PG} (i.e., concentrations required to produce half-maximal recovery of RFI at different values of X_{PG}). Our measurements revealed that $[lidocaine]_{50}$

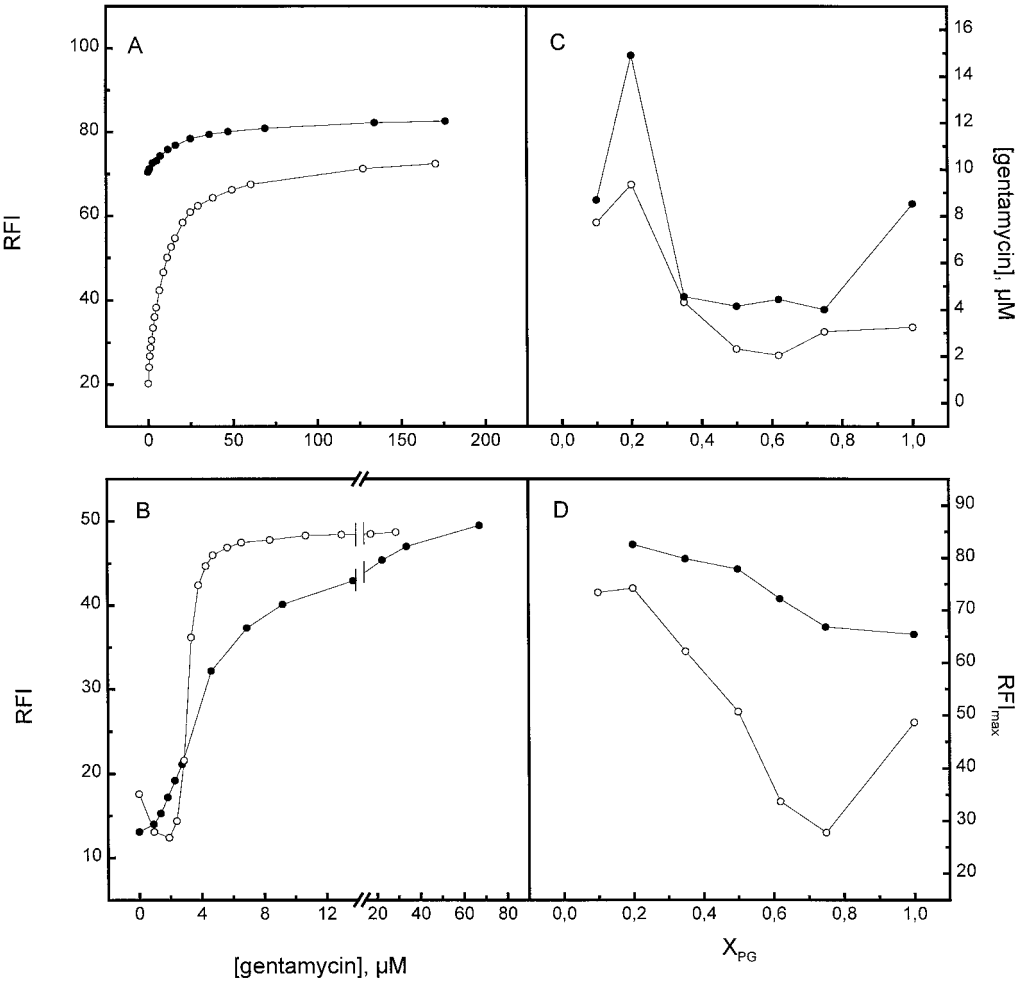

Fig. 8. Dissociation of cyt *c* from LUVs by increasing concentrations of gentamycin at X_{PG} = 0.20 (A) and 1.00 (B). ○ and ●, Absence and presence of 5 mM ATP, respectively. C, Gentamycin concentration producing 50% recovery of RFI. D, Maximal recovery of RFI by gentamycin at various X_{PG} values.

and $[propranolol]_{50}$ increased exponentially with X_{PG} in both the absence and presence of ATP. However, in the presence of the nucleotide, the concentrations of these two amphiphilic drugs required to detach cyt *c* from liposomes were significantly lower. Our results suggest that both lidocaine and propranolol detach cyt *c* mainly as a consequence of charge neutralization of the acidic phospholipid. More specifically, two mechanisms seem to be involved, as follows. At low X_{PG}, the number of charges due to deprotonated PG in the bilayer is reduced by these drugs, and accordingly, cyt *c* is released from the surface. A similar effect is observed due to ATP, which occupies the anionic phospholipid binding A site of cyt *c*. At higher values of X_{PG}, particularly when approaching $X_{PG} = 1.00$, the situation becomes different. In liposomes, the degree of protonation of the acidic phospholipid increases with its mole fraction (i.e., with increasing electrical potential) (Träuble, 1976). To detach cyt *c* bound to the protonated PG via the C site of this protein, the cationic drugs first must bind to liposomes to decrease negative surface charge density and thus deprotonate PG. In the presence of the amphiphilic cationic drugs, the mode of interaction between cyt *c* and acidic phospholipids is altered from C site to A site binding, even at $X_{PG} = 1.00$. Simultaneously, the liposome-associated drug competes with cyt *c* for binding the anionic lipid, thus releasing the protein from the bilayer. Also at $X_{PG} = 1.00$, the A site binding is inhibited by ATP, and accordingly, in the presence of ATP, all cyt *c* associated with the bilayer via the A site is released from the membranes. Therefore, it seems feasible that the differences in $[drug]_{50}$ observed for the three compounds in the presence and absence of ATP mirror the different efficiencies of these drug to deprotonate the acidic phospholipid. For all three compounds, their ability to influence the lipid pK_a should diminish on increasing X_{PG}. This property should be more pronounced for propranolol than for lidocaine, which is in keeping with the higher partition coefficient of propranolol to membranes (Hanpft and Mohr, 1985). Electrophoresis studies revealed that compared with propranolol, ~100-fold higher concentrations of lidocaine are required to induce the same change in the ζ potential of liposomes containing acidic phospholipids (Schlieper and Steiner, 1983). In the current study at $X_{PG} = 0.20$, a ~50-fold higher concentration of lidocaine than propranolol was needed to achieve a similar extent of cyt *c* detachment from liposomes. If this also applies at $X_{PG} = 1.00$, then the concentration of lidocaine required to recover 80% of fluorescence intensity would be as high as 1.5 M, which greatly exceeds the highest drug concentrations (120 mM) used in the current experiments.

To investigate further the contribution of hydrophobicity of the two amphiphilic cationic drugs on the dissociation of cyt *c* from liposomes, we carried out similar experiments with gentamycin. This antibiotic is only very weakly hydrophobic (Brasseur *et al*., 1984), and thus electrostatic attraction provides the main driving force for its membrane association. Interestingly, the effects of gentamycin differed considerably from those of the two amphiphilic cationic compounds. Compared with lidocaine and propranolol, significantly lower concentrations of gentamycin are required to release cyt *c* from liposomes. At $X_{PG} = 0.20$, for instance, the value for $[gentamycin]_{50}$ is ~20% of $[propanolol]_{50}$. The affinity of gentamycin to liposomes seems to increase with X_{PG}. Accordingly, $[gentamycin]_{50}$ has a maximal value at $X_{PG} = 0.20$ and a broad minimal value between X_{PG} ~0.3 and ~0.7. In contrast to lidocaine and propanolol, the values for $[gentamycin]_{50}$ are higher in the presence of ATP. At $X_{PG} = 0.20$ and 1.00, the positively charged gentamycin effectively neutralizes the negative charge of the acidic phospholipids and thus blocks collisions of cyt *c* with the liposome surface. The complicated nature of the cyt *c*/membrane interaction is demonstrated by the difference in the results depending on whether gentamycin or the protein is first allowed to bind to the liposomes. At $X_{PG} = 0.20$, for instance, 60 μM gentamycin added subsequently to 1 μM cyt *c* reversed RFI value to ~70, whereas the same concentrations mixed with the liposomes in reverse order yielded RFI value of ~80. At $X_{PG} = 1.00$, the corresponding values for RFI were ~50 and ~70 (Fig. 4).

As a result of charge neutralization due to the binding of the cationic drugs to liposomes, the degree of deprotonation of PG increases and thus A site binding of cyt *c* should commence. Providing that the affinity of the drug for the membrane is sufficiently high, this should happen even at $X_{PG} = 1.00$. Accordingly, RET between pyrene and the heme of cyt *c* becomes more efficient. This is evident as a further decrease in RFI at low drug concentrations added subsequently to cyt *c*. More specifically, when 0.3 μM cyt *c* was first added to yield ~50% quenching, subsequent additional propranolol (up to 0.3 mM) further decreased fluorescence intensity (Fig. 7B). In the presence of 5 mM ATP, these drugs did not decrease RFI (i.e., alter the mode of interaction of cyt *c* with liposomes). This is expected because ATP inhibits the A-site binding of cyt *c*.

The results from stopped-flow experiments are in keeping with the above steady state measurements and support the view that (1) the mode of interaction between cyt *c* and the membrane changes drastically on changing X_{PG} and (2) the mechanisms causing the dissociation of cyt *c* from the membrane are different for the three drugs studied. More specifically, there is a profound decrease in the rate of binding of cyt *c* on increasing X_{PG} from 0.20 to 1.00. However, the amplitudes of the two-exponential fluorescence decays do not change considerably on varying X_{PG}. The two-exponential decays could result from changes in the lateral distribution of the probe on binding of cyt *c* to the outer surface of the liposomes. On the other hand, the >500-fold decrease in the rate of dissociation of cyt *c* by gentamycin at $X_{PG} = 0.20$ and 1.00 is in keeping with two different modes of binding. The only two-exponential dissociation processes were those caused by lidocaine at $X_{PG} = 0.20$ and by gentamycin at $X_{PG} = 1.00$. However, as for the other conditions, the detachment process may well be multiexponential in the submillisecond time domain, which is beyond the time resolution of our instrument.

We interpret the differences among the three compounds to reflect their varying efficiencies to promote the deprotonation of the acidic phospholipids. However, the charge of the deprotonated lipid is not neutralized by the drug, perhaps due to a higher affinity of the deprotonated PG for cyt *c* than the drug, thus keeping the protein attached to membrane surface via the A site of cyt *c*. In the presence of ATP, this interaction is blocked due to the binding of the nucleotide to the A site. At $X_{PG} = 1.00$, lidocaine is not capable of promoting the deprotonation of PG and ATP has no effect on the membrane association of cyt *c*, the latter remained bound to protonated PG via the C site. Gentamycin, instead, also

seems to effectively deprotonate PG at X_{PG} = 1.00, thus enabling ATP to displace cyt *c* rather efficiently from liposomes.

An important general conclusion that can be reached based on the current study is that competition of different cationic ligands for acidic phospholipids is a very complicated process involving contributions due to a variety of physicochemical parameters. Understanding of these processes on molecular level is far from being complete. However, thorough understanding of these processes is worth pursuing to evaluate the feasibility of therapeutic effects on cells being achieved by compounds interfering with specific lipid/protein interactions.

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