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ARTICLE

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The importance of cholesterol in maintenance of P-glycoprotein activity and its membrane perturbing influence

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Abstract In tumour cell lines that display multidrug resistance, expression of P-glycoprotein (P-gp) alters many aspects of biomembrane organization in addition to its well-characterized drug transport activity. We have developed a reconstitution system to directly investigate the effect of purified P-gp on the biophysical properties of lipid bilayers. Using a mixed detergent system it was possible to efficiently reconstitute P-gp at lipid:protein ratios as low as 2.5 (w/w) by removal of detergent using adsorption to SM-2 BioBeads. P-gp was able to alter many biophysical parameters associated with lipid organization within bilayers. For example, the changes in overall fluidity and excimer formation by lipid analogues indicate modified packing organization of bilayer constituents. Surprisingly, given its role in conferring drug resistance, P-gp insertion into bilayers also caused significantly increased permeability to aqueous compounds, also reflecting a modified phospholipid environment. Translocation of various phospholipid species between leaflets of the bilayer was increased in the presence of P-gp; however, the effect was not dependent on ATP hydrolysis by the protein. Physiological concentrations of cholesterol modified P-gp function and the degree to which it perturbed bilayer organization. The basal ATPase activity of P-gp was increased in a dose-dependent fashion by the incorporation of cholesterol in PC:PE liposomes. In addition, the degree to which the modulator verapamil was able to stimulate this basal ATPase activity was reduced by the presence of cholesterol in proteoliposomes. However, the potency of verapamil was unaltered, suggesting a specific effect, not simply caused by lower drug penetration into the cholesterol containing bilayers. In summary, P-gp is able to cause perturbation in the organization of bilayer constituents. Cholesterol imparted "stability" to this perturbation of bilayer organization by P-gp and moreover this led to altered protein function.

Keywords P-glycoprotein · Multidrug resistance · Membrane biophysics · Reconstitution · Cholesterol

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Introduction

The multiple drug resistance phenotype observed in tumour cells is often associated with altered plasma membrane permeability to anticancer agents (Dalton et al. 1989; Woodhouse and Ferry 1995). P-glycoprotein (P-gp), a member of the ATP-binding-cassette (ABC) transporter superfamily (Higgins 1992), confers this type of resistance in many cancer cells by acting as an ATP-dependent drug efflux pump, thereby maintaining low intracellular concentrations of chemotherapeutic agents (Gottesman and Pastan 1993).

Early characterizations of multidrug resistant (MDR) cancer cells expressing P-gp consistently demonstrated altered biophysical properties of the plasma membrane. Amongst the alterations are ultrastructural modifications such as the appearance of intramembranous particles on the cell surface (Arsenault et al. 1988; Garcia-Segura et al. 1990). These particles were present in proportion to the level of drug resistance. Alterations in overall fluidity have also been observed in cell lines selected for drug resistance (Dalton et al. 1989; Montoudan et al. 1986; Sinicrope et al. 1992), reflecting an increase in the rotational and translational movement

of phospholipids in the bilayer. The decrease in surface hydrophobicity of drug resistant cells also suggests aberrant packing of phospholipid species within the bilayer (Callaghan et al. 1992). Membrane alterations in MDR cells are also manifested in a macroscopic fashion, as exhibited by the significantly increased rates of fluid-phase endocytosis and the number of endosomal particles (Callaghan et al. 1992; Sehested et al. 1987). In addition to displaying these distinct biophysical properties, MDR tumour cells expressing P-gp also have altered plasma membrane lipid composition (Alon et al. 1991). Interestingly, a number of indirect studies have implicated P-gp as a relatively non-specific lipid translocase and this activity may contribute to organization of phospholipid distribution between hemileaflets of a bilayer (Alburob and Gumbleton 1999; Bosch et al. 1997; van Helvoort et al. 1996).

What are the functional consequences of the reported changes in lipid composition and membrane biophysical properties in MDR cells? The modified membranes appear to play a role in resistance, since agents that alter the state of membrane fluidity and modify the phospholipid composition have been demonstrated to compromise the degree of drug resistance (Callaghan et al. 1993; Drori et al. 1995). However, cells expressing the MDR phenotype display an increased fragility to membrane perturbants in comparison with their parental drug sensitive cells. This phenomenon, known as collateral sensitivity, occurs in response to factors such as mild detergents, many hydrophobic drugs and the drug vehicle cremophor EL (Riehm and Biedler 1972; Woodcock et al. 1992). Therefore, it appears that whilst the alterations in membrane properties facilitate the MDR phenotype, resistant tumour cells are paradoxically vulnerable to stress on the bilayer.

How do the alterations in membrane lipid environment modify the degree of cellular resistance to chemotherapy? First, most agents that interact with the MDR efflux pump P-gp share the common property of hydrophobicity (Zamora et al. 1988) and there is strong evidence that drugs interact with P-gp via the lipid phase (Homolya et al. 1993; Raviv et al. 1990). Thus any alterations in membrane biophysical properties would also be expected to influence the sub-cellular distribution of drugs and their access to P-gp. An altered membrane biophysical environment may also affect the MDR phenotype by a direct effect on P-gp activity. The activity of P-gp requires interaction with a defined lipid environment, purification in the absence of exogenous lipids inactivates P-gp (Callaghan et al. 1997) and the addition of various phospholipids restores or increases activity to protein that has previously been stripped of lipids (Lerner-Marmarosh et al. 1999; Sharom et al. 1995). A wide spectrum of lipid-induced effects has been observed on the ability of purified solubilized P-gp to hydrolyse ATP and bind substrates (Romsicki and Sharom 1999; Saeki et al. 1992; Sharom et al. 1995; Urbatsch and Senior 1995). Finally, phospholipid addition also modifies communication between the substrate binding and nucleotide hydrolysing domains in solubilized P-gp (Romsicki and Sharom 1999).

To date it remains unclear whether the alterations in membrane biophysical properties directly result from P-gp expression, are caused by the activity of P-gp or derive as a consequence of prolonged exposure to chemotherapeutic agents in resistant cell lines. In this study we have developed a reproducible and efficient system for reconstituting P-gp into defined lipid vesicles over a wide range of lipid:protein ratios. This defined system was used to directly examine the influence of P-gp on several membrane biophysical properties. Overall, P-gp led to significant alterations in the packing, distribution and structural organization of bilayer constituents, most of which were attenuated by cholesterol. Moreover, the effects of cholesterol directly affected the activity of P-gp and its interaction with drugs. These findings may have important consequences, given the propensity of P-gp to localize within cholesterol-rich domains in biological membranes.

Materials and methods

Materials

Egg phosphatidylcholine (PC), bovine liver phosphatidylethanolamine (PE), cholesterol, verapamil hydrochloride and disodium adenosine triphosphate (Na_2ATP) were obtained from Sigma (Poole, UK). Soya bean asolectin (50% pure) was obtained from Fluka. 1,2-Dipalmitoylphosphatidyl[^{3}H]methylcholine (50–62 mCi mmol^{-1}) was purchased from Amersham-Pharmacia (Amersham, UK). The detergents octyl-, decyl- and dodecyl-β-D-maltoside were obtained from Calbiochem (Nottingham, UK) and were of at least 95% purity. SM2-BioBeads and Econo-Pac Q anion exchange chromatography cartridges were purchased from BioRad (Hemel Hempstead, UK). (1,4-Diphenylhexatrienyl)trimethylammonium (TMA-DPH), 1-pyrenedecanoic acid (PDA), 1-hexadecanoyl-2-(1-pyrenehexanoyl)glycerophosphocholine (pyrene-PC) and the lipids 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-1-hexadecanoyl-*sn*-glycerophosphocholine (NBD-C_6-PC), NBD-C_6-PE and NBD-C_6-ceramide were purchased from Molecular Probes (Leiden, NL). The anti-P-gp monoclonal antibody C219 was purchased from CIS (High Wycombe, UK).

1-Palmitoyl-2-(5-doxylpentanoyl)-*sn*-glycero-3-phosphocholine [(0,2)-doxylPC], the corresponding phosphatidylethanolamine derivative [(0,2)-doxylPE] and the two dialkyl derivatives dialkyl-(0,2)-doxylPC and dialkyl-(0,2)-doxylPE were synthesized as described previously (Fellmann et al. 1994). Spin-labeled glucosylceramide (1-*O*-β-D-glucopyranosyl-*N*-acylsphingosine), GlcCer, was obtained by acylating the corresponding glycosphingosines with 4-doxylpentanoic acid as described (Belleau and Malek 1968). Detailed synthesis of the latter spin label will be given elsewhere (Fellmann et al. in preparation).

Cell culture, membrane preparation and purification

The MDR cell line CH^{r}B30 was grown in α-MEM with 10% fetal calf serum as previously described (Kartner et al. 1983). Selection pressure to maintain resistance was achieved using a supplement of 30 μg mL^{-1} colchicine. Plasma membranes were prepared from the CH^{r}B30 cell lines as previously described using nitrogen cavitation to disrupt cells and sucrose density centrifugation to harvest the light membrane fraction (Lever 1977). Membrane preparations

were stored at –70 °C in 0.25 M sucrose, 10 mM Tris.HCl (pH 7.4) buffer for up to 6 months.

P-gp was enriched to approximately 60% purity by anion exchange chromatography using minor modifications of an established procedure (Callaghan et al. 1997). Prior to chromatography, P-gp was solubilized in 1% (w/v) dodecyl-maltoside with 0.4% (w/v) crude lipid mixture (asolectin) in buffer containing 5 mM PIPES at pH 7.1. The soluble protein was passed down a 5 mL bed volume anion-exchange column (Econo-Pac HighQ). Chromatography buffers contained 0.1% (w/v) dodecyl-maltoside to maintain protein solubility. Proteins were eluted with a gradient of NaCl and fractions containing P-gp were concentrated to approximately 1 mg mL^{-1} protein using 50 kDa membranes in an Amicon Stirred Cell concentrator.

Liposome preparation and solubilization

PC and PE in the ratio (w/w) 9:1 were dried onto glass test-tubes from chloroform suspensions. Cholesterol was added to the phospholipids to proportions of 5, 10, 20 or 30% (w/w) of total lipid. [^{3}H]-PC (0.1 μCi per 10 mg lipid) was also added to allow monitoring of total phospholipid as appropriate in subsequent analyses. The films were resuspended at a concentration of 10 mg mL^{-1} in buffer A (150 mM NaCl, 1 mM EDTA, 50 mM Tris.HCl, pH 7.4) by vigorous mixing. The lipid suspensions were subjected to five freeze-thaw cycles and large unilamellar liposomes (LUVs) were produced by subsequent extrusion through 200 nm filters as previously described (Callaghan et al. 1997).

To determine the amount of detergent required to saturate or solubilize liposomes, 2–5 μL aliquots of concentrated detergent were sequentially added to the LUVs (1–10 mg mL^{-1}) in a total volume of 500 μL buffer A. The turbidity of samples was defined as the optical density of solutions at 750 nm. Complete solubilization of the liposomes was deemed to have occurred when the solutions were optically clear ($A_{750} < 0.01$ a.u.).

Reconstitution with SM-2 BioBeads

Reconstitution was achieved by removal from protein/lipid/detergent mixtures of detergent using SM-2 BioBeads. The BioBeads were pretreated with successive washes in water, 50% methanol/water, 100% methanol, 50% methanol/water and, finally, several washes of water. Unilamellar liposomes were solubilized or saturated with the appropriate amount of detergent (see above), which was added slowly and with constant stirring of sample. Following detergent addition, the amount of lipid required to give a specific lipid:protein ratio was added to an aliquot of solubilized P-gp. The volume ratio of detergent/lipid mixture to protein was maintained at 2:1 to dilute the protein-associated dodecyl-maltoside below its CMC in the final mixture. The detergent/lipid/protein mixture was allowed to equilibrate for 40 min at room temperature prior to BioBead additions. The BioBead additions were repeated at 40-min intervals and the solutions mixed by slow rotation.

Sucrose density centrifugation

The relative migration of protein and lipid through sucrose density gradients was used to determine the efficiency of reconstitution. Following addition of BioBeads the proteoliposomes (10 μg protein) were diluted to a volume of 200 μL and mixed with an equal volume of 60% sucrose in buffer A containing 0.05% Triton X-100. Layered over this fraction were 400 μL each of 20, 10, 5 and 0% sucrose in buffer A. The samples were centrifuged at 150,000×*g* for 8–10 h to reach sedimentation equilibrium. Fractions of 200 μL were collected from the top of the gradient following centrifugation. Protein was precipitated from 150 μL aliquots of each fraction with trichloroacetic acid and resuspended in 20 μL of 4% SDS, 0.2 M Tris.HCl (pH 10), 0.15 M NaOH and 5 μL Lammelli sample buffer (5×). These samples were electrophoresed through 8% polyacrylamide gels and subsequently transferred to nitrocellulose membranes as described previously (Callaghan et al. 1997). P-gp location was determined through Western immunoblotting procedures using the C219 monoclonal antibody and a chemiluminescence detection system or with silver staining. A further aliquot (50 μL) was used to detect the presence of phospholipid by liquid scintillation counting for [^{3}H]-PC.

ATPase assays of purified P-gp

The basal and drug-stimulated ATPase activity of P-gp was measured using a colorimetric assay to follow release of inorganic phosphate, based on a modification of the assay first described by Chifflet et al. (1988). Membrane vesicles (1 μg) were incubated in ATPase buffer [150 mM NH_4Cl, 50 mM Tris.HCl (pH 7.4), 5 mM $MgSO_4$, 0.02% NaN_3] in the presence of 0–4 mM Na_2ATP for a 20-min period at 37 °C. The calcium channel blocker and P-gp modulator verapamil was used to stimulate ATPase activity. Drug effects on basal activity were measured in the presence of 2 mM Na_2ATP by adding compounds from concentrated stocks in DMSO or ethanol (final concentration of solvent not exceeding 1% v/v).

ATPase activity (nmol min^{-1} mg^{-1} membrane protein) was plotted as a function of drug concentration and fitted, using non-linear regression, by the general dose-response equation below (De Lean et al. 1978):

$$Y = (Y_{max} - Y_{min})/\{1 + ([B]/EC_{50})^n\} + Y_{min} \quad (1)$$

where Y_{max} = maximal activity, Y_{min} = minimum activity, [B] = drug concentration (M), EC_{50} = concentration of drug which causes a 50% change in response, and n = slope factor.

Liposome leakiness assay

The leakiness of liposomes was determined by measuring the permeability to dithionite (McIntyre and Sleight 1991). Unilamellar vesicles were prepared (see Liposome preparation and solubilization above) containing 1% (mol/mol) fluorescent lipid, NBD-C_6-PC. These liposomes displayed symmetric distribution of NBD-C_6-PC between the inner and outer leaflets of the bilayer. Fluorescence of the NBD-C_6-PC containing liposomes was measured in a Hitachi F2500 fluorimeter using an excitation wavelength of 470 ± 2 nm and an emission wavelength of 530 ± 2 nm. The reducing agent dithionite, which irreversibly quenches the fluorescence of NBD-containing compounds, was added to liposomes from a 0.5 M stock in 0.1 M Tris, pH 10. The fluorescence decay was monitored continuously and the liposomes were permeabilized with the addition of 0.05% (v/v) Triton X-100 to completely quench NBD-C_6-PC fluorescence.

Liposome fluidity measurements

Bilayer fluidity was determined from the steady-state polarization of the fluorophore TMA-DPH according to previously published methods (Spiegel et al. 1981). TMA-DPH was added from a 500 μM stock in DMSO to 50 μg lipid to give a final molar lipid:probe ratio of 125:1, and incubated at 37 °C for 10 min to ensure complete incorporation into the bilayer. Excitation and emission wavelengths were set at 360 ± 5 and 430 ± 5 nm, respectively. Polarization of the probe was calculated using the equation:

$$P = (I_{||} - I_{\perp})/(I_{||} + I_{\perp}) \quad (2)$$

where I = fluorescence intensity through polarizers orientated vertically ($I_{||}$) or horizontally ($I_{\perp}$) to the vertical plane of polarization of the exciting light.

Pyrene excimer formation

The packing of lipids within bilayers was determined by measuring the formation of pyrene excimers in LUVs. PDA in the range

1–10 μM was added stepwise to 50 μg lipid in 1 mL buffer A from a 1 mM stock in DMSO. Full incorporation of the probe was achieved within 2 min of addition to the buffer. Pyrene-PC was used in the concentration range 0.01–4 μM from an ethanol stock solution and required 2 h for complete incorporation into the liposomes. The excitation wavelength was 341 ± 2 nm and the emission spectra recorded between 350 and 500 nm. The monomer emission peak was found at 377 nm and the excimer peak at 470 nm.

Lipid translocation by P-gp

The first series of investigations involved a fluorescence-quenching assay. NBD-C_6-lipids (1 mol%) were added to P-gp-containing proteoliposomes composed of PC:PE:cholesterol (72:8:20). These proteoliposomes were incubated at 37 °C in the presence or absence of 5 mM Mg^{2+}-ATP for periods indicated. Following incubation, aliquots of 50 μg lipid were diluted to 1 mL with buffer A and 0.5 mM dithionite was added to selectively quench the fluorescence of lipids located in the outer leaflet of the proteoliposomes. Fluorescence intensity was measured as described in "Liposome leakiness assay" above. These investigations necessitated the use of mixtures containing 20% (w/w) cholesterol to counteract the inherent permeability of P-gp-containing proteoliposomes to dithionite.

The second assay to measure lipid translocation involves the use of an electron paramagnetic resonance (EPR) technique with doxyl-labeled phosholipid. The assay does not require the use of quenching agents and is therefore applicable to liposomes devoid of cholesterol. Short-chain spin-labeled phospholipids were added to P-gp-containing proteoliposomes to a concentration of 5 mol%. EPR spectra were detected using a Bruker 200D X-band spectrometer. This high local concentration of probe gave rise to significant line broadening of the spectra. Should a fraction of the spin-labeled phospholipids re-orientate, the signal component produced by this dilution of probe in the inner leaflet will significantly alter the line shape of the overall EPR spectrum. The principles associated with this technique have been described elsewhere (Devaux and McConnell 1972). A quantitative analysis of the change in line shape resulting from the dilution of the probe from the outer to the inner leaflet will be given elsewhere (Traikia and Devaux, manuscript in preparation). Following incorporation of spin-labeled lipid, the proteoliposomes were incubated at 37 °C for specified times in the presence or absence of 5 mM MgATP. At appropriate times, spectra were recorded at 22 °C for aliquots corresponding to 50 μg lipid. Diether lipids as well as sphingolipids were used rather than diester lipids to avoid any artifact associated with possible hydrolysis of the short *sn*-2 spin-labeled acyl chain (Fellmann et al. 2000).

Results

Detergent solubilization of liposomes

Perturbation of lipid bilayers, usually by detergent, is an essential step in the successful reconstitution of membrane proteins. Usually, this perturbation involves the complete solubilization of lipid bilayers to form mixed micelles of detergent and lipid. We characterized the solubilization of large unilamellar liposomes comprising (9:1) PC:PE by octyl- (CMC = 22 mM), decyl- (CMC = 2 mM) and dodecyl-maltoside (CMC = 0.5 mM). Liposomes (1–10 mg mL^{-1}) were treated with increasing detergent concentrations and the effects on turbidity for 1, 2 and 4 mg mL^{-1} lipid are shown in Fig. 1. At low concentrations of each detergent there was an increase in turbidity (OD_{750}), due presumably to detergent-mediated fusion of liposomes. A plateau of turbidity was reached and defined as the concentration of detergent that saturated the lipid bilayer. As the detergent concentration was further increased, rapid solubilization of the liposomes occurred; complete solubilization was defined as the point at which the

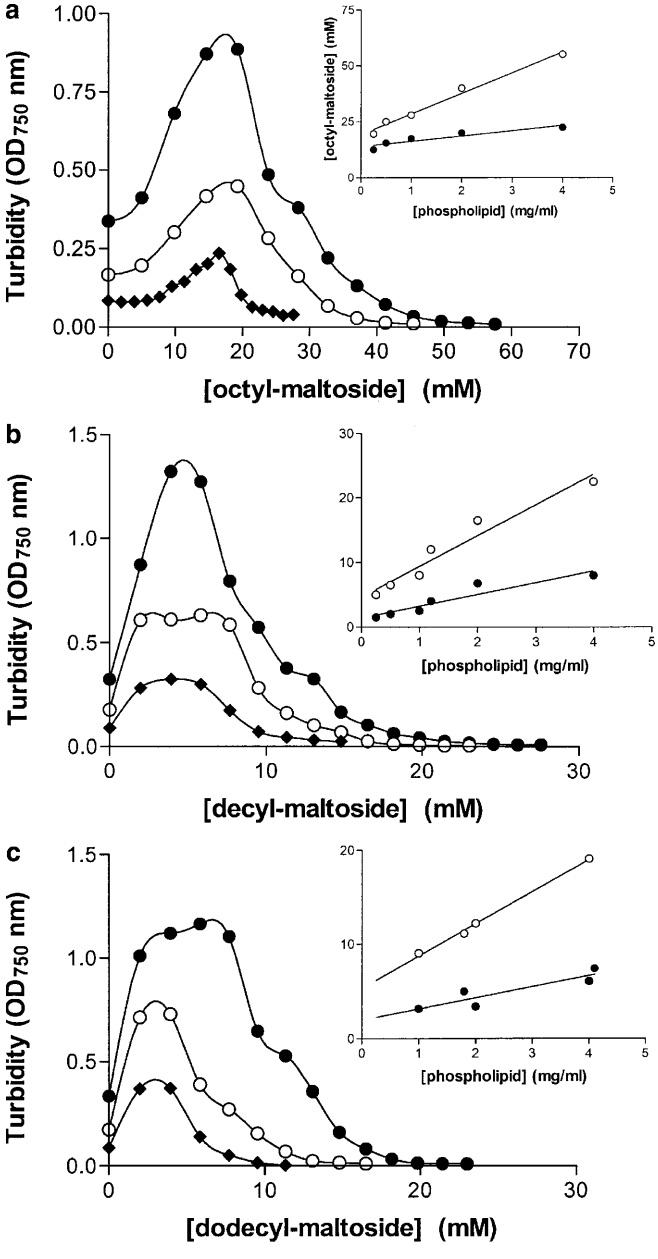

Fig. 1a–c Liposome solubilization by alkyl-maltoside detergents. The turbidity of a series of unilamellar vesicle (d = 200 nm) concentrations [only 1(■), 2 (○) and 4 (●) mg mL^{-1} displayed for clarity] was measured as a function of **a** octyl-maltoside, **b** decyl-maltoside or **c** dodecyl-maltoside concentration. Turbidity was defined as the optical density at 750 nm for the lipid suspensions as described in Materials and methods. The *inset* to each figure is a secondary plot of the detergent concentration required to either saturate (●) or solubilize (○) the vesicles as a function of lipid concentration

solutions were optically clear (absorbance < 0.01 a.u.). The concentration of detergent required to either saturate ($[det]_{sat}$) or solubilize ($[det]_{sol}$) the liposomes was plotted as a function of lipid concentration (Fig. 1 insets). In each case a linear relationship defined as [detergent] = $D_w + R$[lipid] was observed. The value of D_w is the monomeric concentration of detergent and the value of R is the ratio of detergent to lipid that is required to saturate (R_{sat}) or solubilize (R_{sol}) the liposomes. Detergent to lipid ratios of 2.4, 1.8 and 1.2 were observed to saturate the PC:PE liposomes for octyl-, decyl- and dodecyl-maltoside, respectively. As expected, higher values of R_{sol} were required to completely solubilize the liposomes with octyl- ($R = 9.2$), decyl ($R = 4.8$) or dodecyl-maltoside ($R = 3.4$).

Reconstitution of P-gp into liposomes saturated by alkyl-maltosides

To achieve successful reconstitution of P-gp, the liposomes (PC:PE 9:1) were saturated with the appropriate amount of either octyl-, decyl- or dodecyl-maltoside and incubated with P-gp at lipid to protein ratios between 2.5 and 40 (w/w). Saturating rather than solubilizing concentrations of detergent were chosen in order to minimize the detrimental effects of detergent on P-gp. At least five additions of SM2-BioBeads at 20, 40, 80, 120 or 240 mg mL^{-1} were used to remove detergent. The efficiency of reconstitution was assessed by migration of lipid and protein on sucrose gradients and the data obtained using a lipid:protein ratio of 10 and BioBead additions at 40 mg mL^{-1} are shown in Fig. 2. P-gp migrates as a diffuse band of 180–190 kDa. The data in Fig. 2a also demonstrate the degree of P-gp purification obtained following anion exchange chromatography. In the case of decyl- (Fig. 2a) and octyl-maltoside (Fig. 2b), there was complete and homogeneous reconstitution of P-gp, indicated by the co-migration of protein and lipid through the sucrose gradient. The total amount of BioBeads added was significantly greater than that required to adsorb the total amount of decyl-maltoside (Rigaud et al. 1998). At BioBead concentrations of 20 mg mL^{-1} the number of additions was increased to ensure complete reconstitution and removal of detergent. At high BioBead concentrations (120 and 240 mg mL^{-1}), significant amounts of P-gp were not incorporated into the liposomes; using 120 mg mL^{-1} BioBead additions, the 30% sucrose fraction contained protein-rich (greater than 90%), lipid-deficient (10–15% [^{3}H]-PC) aggregates (data not shown). Rapid detergent removal (240 mg mL^{-1} BioBeads) produced complete

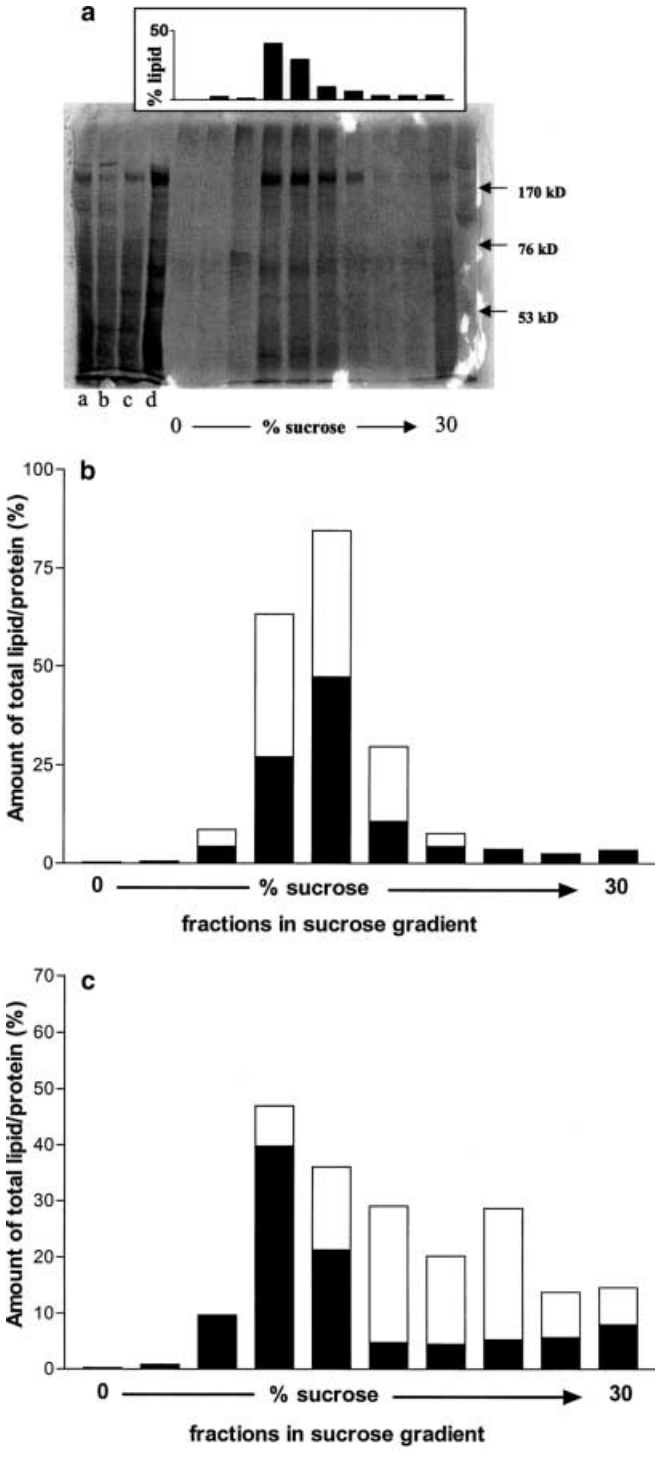

Fig. 2a–c Purification and reconstitution of P-gp. **a** SDS-PAGE analysis of P-gp in native membranes (lane *a*), the detergent insoluble fraction (lane *b*), the detergent soluble fraction (lane *c*), following anion exchange chromatography (lane *d*) and in fractions throughout a sucrose density gradient (0–30%) following reconstitution. Proteins (2 μg) were separated using 8% SDS-PAGE and detected using a silver stain kit. Molecular weights are indicated with *arrows*. The *histogram* shows the distribution of [^{3}H]-PC through the sucrose gradient. **b**, **c** Total phospholipid (■) and protein (□) distributions throughout sucrose density gradients of P-gp reconstituted from liposomes saturated with the detergents octyl- (**b**) or dodecyl-maltoside (**c**). Phospholipid distribution was detected by the presence of [^{3}H]-PC and protein levels quantitated by densitometry of Western immunoblots on each fraction of the sucrose gradient. The intensities indicate amounts per fraction expressed as a percentage of total phospholipid or protein loaded onto the gradient

separation of protein aggregates in 30% sucrose from liposomes that migrated to 10% sucrose. The different results with different BioBead concentrations reflect an intimate relationship between P-gp insertion and the rate of detergent removal, similar to that previously described for other membrane proteins. Similar results were obtained at lipid:protein ratios of 2.5, 5, 20 and 40 (w/w) (data not shown).

The use of dodecyl-maltoside did not result in efficient reconstitution of P-gp. The profile obtained at a lipid:protein ratio of 10 (w/w) and saturating dodecyl-maltoside concentrations is shown in Fig. 2c. Similar profiles were also consistently observed at lipid:protein ratios of up to 40 (w/w) and also with solubilizing detergent concentrations (data not shown). Partial association of P-gp with lipids was observed; however, the absence of homogeneous co-migration of the two species indicated unsuccessful reconstitution. Increasing the number of BioBead additions did not alter the profile and therefore the protein-rich structures do not simply result from inadequate detergent removal. Varying the rate of detergent removal by BioBeads (20–240 mg mL^{-1}) also did not produce homogenous incorporation of P-gp into the liposomes. It is unclear why P-gp has a greater propensity to aggregate in lipid-depleted structures when dodecyl-maltoside is used to solubilize or saturate liposomes. Successful reconstitution was only observed when using a lipid:protein ratio of 100 (w/w); however, liposomes with such a low protein density are not amenable to biophysical or structural studies.

Cholesterol increases the maximal ATPase activity of reconstituted P-gp

The basal ATPase activity of solubilized P-gp, partially purified by anion exchange chromatography, displayed maximal activity (V_{max}) of 16 ± 2 nmol min^{-1} mg^{-1} ($n = 8$) and an affinity for ATP (K_m) of 0.31 ± 0.09 mM. The V_{max} was stimulated by 50 μM verapamil to 83 ± 11 nmol min^{-1} mg^{-1}, while the K_m for ATP was unchanged (0.35 ± 0.05 mM). P-gp that was reconstituted into PC:PE (9:1) liposomes displayed basal ATPase activity with a $V_{max} = 24 \pm 4$ nmol min^{-1} mg^{-1} ($n = 6$), which was stimulated 5-fold by 50 μM verapamil ($V_{max} = 137 \pm 31$ nmol min^{-1} mg^{-1}, $n = 6$; $P < 0.05$). The affinity for ATP (K_m) of either basal or drug-stimulated activity of P-gp was identical to that of solubilized P-gp. Increasing the proportion of cholesterol in the liposomes caused an increase in basal activity, which reached a maximum of 49 ± 9 nmol min^{-1} mg^{-1} ($n = 4$, $P < 0.05$) at 30% cholesterol (Fig. 3a). The V_{max} of drug-stimulated ATPase activity also increased, reaching a value of 190 ± 40 nmol min^{-1} mg^{-1} at 30% cholesterol. The K_m for ATP was unaffected by the cholesterol content of the proteoliposomes. Despite the overall increase in stimulated ATPase activity, the degree of stimulation by verapamil was reduced from 5.2-fold in liposomes devoid of cholesterol to 3-fold in those containing 30% cholesterol (Fig. 3). In addition, the potency of verapamil to stimulate ATPase activity was similar in liposomes containing 0% ($EC_{50} = 3.19 \pm 0.15$ μM), 20% ($EC_{50} = 2.45 \pm 0.24$ μM) or 30% ($EC_{50} = 3.64 \pm 0.80$ μM) cholesterol (Fig. 3b). Therefore the altered magnitude of stimulation appeared to be a direct effect of cholesterol on P-gp rather than simply an alteration in the concentration of verapamil that partitioned into the bilayer.

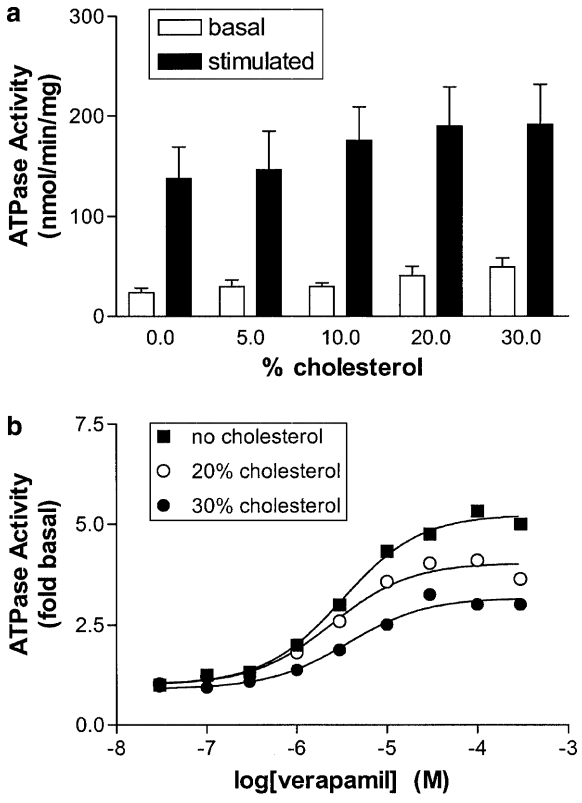

Fig. 3a, b The effect of cholesterol on the activity of reconstituted P-gp. **a** The maximal (V_{max}) basal and verapamil (50 μM) stimulated ATPase activity (nmol P_i min^{-1} mg^{-1}) was determined for P-gp reconstituted into liposomes containing 0, 5, 10, 20 or 30% (w/w) cholesterol. The V_{max} was determined from activities measured with 1 μg protein at 37 °C and ATP concentration in the range 0–4 mM. Values are the mean ± SEM of values obtained from at least five independent preparations. **b** ATPase activity was measured at each level of cholesterol incorporation (only 0, 20 and 30% shown for clarity) as a function of verapamil concentration. The ATP concentration was 2 mM and the activity was expressed as fold increase over the activity in absence of verapamil. The relationships were fitted by the general dose-response curve using non-linear regression

Effects of cholesterol, P-gp and temperature on membrane fluidity

TMA-DPH readily partitions into lipid bilayers and the polarization of its fluorescence may be used as a measure of averaged rotational and translational lipid movement within membranes (i.e. fluidity). The steady-state

polarization of TMA-DPH was determined in liposomes and P-gp-containing proteoliposomes, both with varying cholesterol content and at a range of temperatures (Table 1). The probe concentration was < 1 mol% and proteoliposomes with a lipid:protein ratio of 10 (w/w) were used. As expected, an increase in the cholesterol content from 0 to 30% (w/v) in liposomes was characterized by a concomitant rise in the polarization of TMA-DPH from 0.299 ± 0.007 to 0.375 ± 0.005 at 20 °C, indicating a significant reduction in the bilayer fluidity. Increasing the temperature caused a decrease in TMA-DPH polarization (which was more pronounced at higher cholesterol levels) and the magnitude of this effect was less than that caused by cholesterol. A decrease in bilayer fluidity as a function of cholesterol content was observed at all temperatures measured.

The steady-state polarization of TMA-DPH fluorescence was examined in proteoliposomes to determine the effect of P-gp incorporation on bilayer fluidity. Decreased polarization was observed at all temperatures and cholesterol concentrations following P-gp insertion into the bilayer (Table 1). This decreased polarization corresponds to an increased fluidity; however, the incorporation of P-gp did not modify the relationship between fluidity and temperature or cholesterol content. The magnitude of the effect caused by P-gp incorporation on overall lipid movement within a bilayer was in keeping for a lipid:protein ratio of 10 (w/w), which corresponds to a molar ratio of approximately 1800.

The influences of cholesterol and P-gp on pyrene excimer formation in liposomes

P-gp incorporation into a bilayer may manifest more significantly in measurements of local perturbation of amphiphile packing. Therefore, the ability of pyrene-containing amphiphiles to produce excimer fluorescence was used to probe such localized properties in liposomes and P-gp-containing proteoliposomes. Lipid mixtures that contained 0 or 20% cholesterol were used in each case. The ratio of excimer to monomer fluorescence intensity was determined for a range of PDA or pyrene-PC concentrations added to LUVs or proteoliposomes and the data are shown in Fig. 4. Neither PDA nor pyrene-PC displayed fluorescence in aqueous solution and thus their incorporation into lipid bilayers was followed by the increase in monomer fluorescence intensity. PDA was rapidly incorporated into bilayers and reached saturable levels within 2 min at 37 °C, whilst pyrene-PC incorporated slowly and required 2 h to saturate. There was no difference in the total amount of either PDA or pyrene-PC that could incorporate into liposomes containing 20% cholesterol.

The formation of excimers increased as a function of PDA concentration (Fig. 4a), regardless of the type of liposome, reflecting the increased probability of monomer-monomer contact required to produce the excimer signal. Incorporation of (20%) cholesterol resulted in lower amounts of excimer produced at each probe concentration in the liposomes. Furthermore, P-gp-containing proteoliposomes also displayed significantly lower excimer formation compared with liposomes. This effect was observed irrespective of the initial probe concentration, and whether or not the bilayers contained cholesterol. While cholesterol caused large effects in liposomes, its incorporation into P-gp-containing liposomes did not have a further, statistically significant, influence. This differential effect of cholesterol on liposomes, compared with proteoliposomes, is perhaps indicative of altered PDA distribution in bilayers containing P-gp.

Pyrene-PC, like PDA, displayed a dose-dependent ability to form excimers in liposomes and proteoliposomes (Fig. 4b). In contrast to the effects on PDA, the incorporation of 20% cholesterol into bilayers did not alter the excimer:monomer ratio of pyrene-PC in liposomes at any concentration. This ''cholesterol insensitivity'' may reflect a different packing arrangement of pyrene-PC compared with PDA. The reconstitution of P-gp into liposomes was able to cause a statistically significant decrease in pyrene-PC excimer formation in the bilayer. This indicates that pyrene-PC, like PDA, has a distribution or packing within bilayers that is modified

Table 1 The effects of P-gp and cholesterol incorporation into liposomes on polarization of TMA-DPH at various temperatures. TMA-DPH was incorporated into 50 μg lipid vesicles at a probe:lipid ratio of 1:125. Spectra were recorded at an excitation wavelength of 360 ± 5 nm and the emission wavelength set at 430 ± 5 nm. Data represent mean ± SEM from three independent preparations and the values of the polarization determined as described in Materials and methods

% Cholesterol		0%	5%	10%	20%	30%
20 °C	LUV	0.299 ± 0.007	0.316 ± 0.012	0.325 ± 0.015	0.347 ± 0.007	0.375 ± 0.005
	PL	0.283 ± 0.008	0.295 ± 0.011	0.319 ± 0.003	0.350 ± 0.007	0.359 ± 0.005
25 °C	LUV	0.301 ± 0.023	0.313 ± 0.012	0.327 ± 0.019	0.343 ± 0.009	0.361 ± 0.005
	PL	0.280 ± 0.004	0.293 ± 0.010	0.315 ± 0.001	0.333 ± 0.006	0.351 ± 0.003
30 °C	LUV	0.311 ± 0.015	0.311 ± 0.014	0.318 ± 0.011	0.344 ± 0.006	0.361 ± 0.006
	PL	0.276 ± 0.006	0.284 ± 0.009	0.305 ± 0.004	0.330 ± 0.010	0.343 ± 0.005
35 °C	LUV	0.304 ± 0.012	0.300 ± 0.017	0.308 ± 0.005	0.328 ± 0.006	0.353 ± 0.004
	PL	0.273 ± 0.009	0.282 ± 0.009	0.300 ± 0.004	0.314 ± 0.009	0.340 ± 0.005
40 °C	LUV	0.291 ± 0.004	0.298 ± 0.013	0.307 ± 0.005	0.331 ± 0.009	0.348 ± 0.002
	PL	0.272 ± 0.005	0.278 ± 0.008	0.296 ± 0.004	0.310 ± 0.008	0.332 ± 0.010

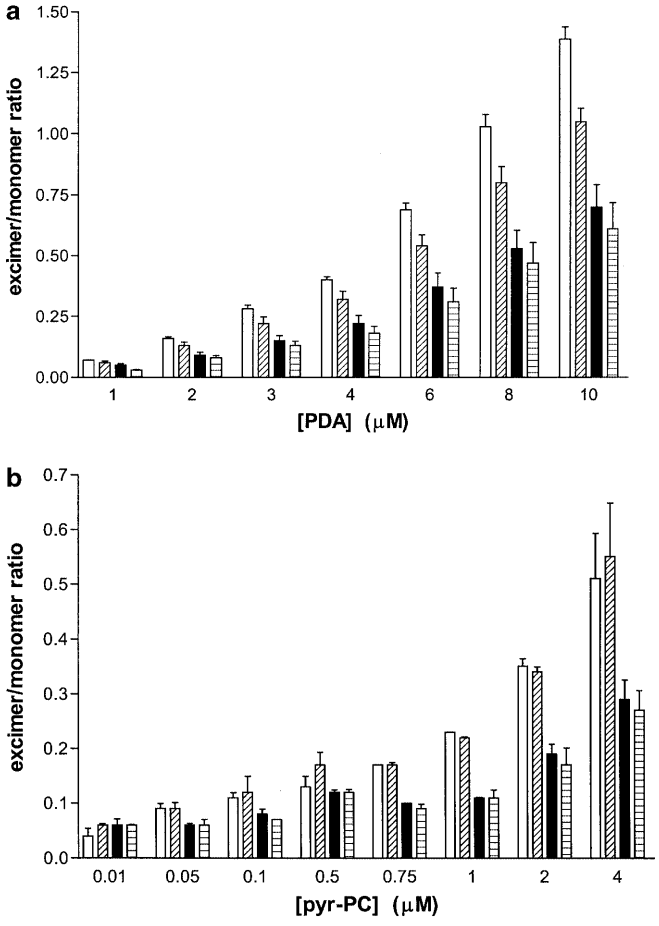

Fig. 4a, b The effects of P-gp and cholesterol on excimer formation of PDA and pyrene-PC. **a** PDA was incorporated into 50 μg lipid in the concentration range 1–10 μM. Emission spectra were recorded between 350–600 nm using an excitation wavelength of 341 ± 2 nm. The excimer to monomer ratio was determined from the respective emission intensities at 470 nm and 377 nm and the values represent mean ± SEM determined from three independent preparations. Values were determined for PC:PE LUVs (*open bars*), PC:PE:cholesterol LUVs (*diagonal hatched bars*), P-gp-containing PC:PE liposomes (*solid bars*) and P-gp-containing PC:PE:cholesterol liposomes (*horizontal hatched bars*). **b** Pyrene-PC was added to 50 μg lipid at concentrations between 0.01–4 μM and the emission spectra were recorded as described above. Values were determined for PC:PE LUVs (*open bars*), PC:PE:cholesterol LUVs (*diagonal hatched bars*), P-gp-containing PC:PE liposomes (*solid bars*) and PC:PE:cholesterol liposomes (*horizontal hatched bars*)

in the presence of P-gp. As was the case with PDA, cholesterol incorporation did not affect the excimer:monomer ratio in proteoliposomes.

Modulation of P-gp-mediated increases in liposome permeability by cholesterol

The above data demonstrate that P-gp causes a direct change in the organization or distribution of amphiphiles within the lipid bilayer, yet it is unclear whether this effect is related to its role in multiple drug resistance. The major role of P-gp in MDR cells is to selectively

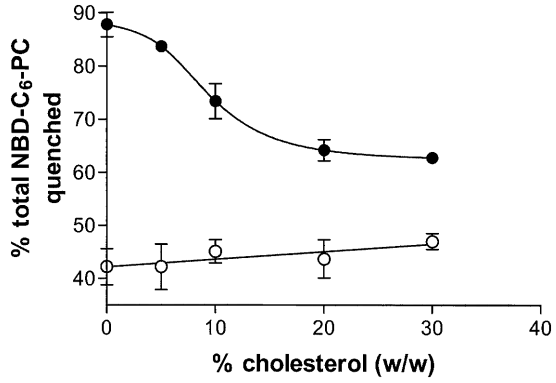

Fig. 5 The effects of cholesterol on the permeability to dithionite of P-gp-containing liposomes. The fluorescence of liposomes (50 μg lipid) containing 1 mol% NBD-C_6-PC was measured at excitation and emission wavelengths of 470 ± 2 nm and 570 ± 2 nm, respectively. Dithionite (0.5 mM) was added to completely quench the fluorescence of NBD-C_6-PC located in the external leaflet of liposomes. The final amount of NBD-C_6-PC fluorescence quenched by dithionite was plotted as a function of cholesterol content in LUVs (○) and proteoliposomes (●). Data represent the mean ± SEM of three independent preparations

alter the permeability of the plasma membrane to chemotherapeutic agents. Investigations were carried out to determine the perturbing effect of P-gp incorporation on overall bilayer permeability using dithionite. Liposomes with varying cholesterol content were produced with a symmetrical bilayer distribution of the fluorescent short-chain lipid probe NBD-C_6-PC. The fluorescence of NBD-C_6-PC in the outer leaflet may be irreversibly quenched by the membrane impermeant compound dithionite (McIntyre and Sleight 1991). The maximal effect of dithionite in liposomes labeled in both leaflets was observed at 0.5–1 mM and total quenching of the probe in symmetric vesicles was only achieved after complete disruption of the liposomes using 0.5% Triton X-100 (data not shown).

The amount of NBD-C_6-PC fluorescence accessible to quenching by dithionite was determined for LUVs and proteoliposomes containing different amounts of cholesterol and the results are displayed in Fig. 5. The amount of NBD-C_6-PC fluorescence quenched by dithionite in LUVs was unaffected by the amount of cholesterol and, as expected, was approximately half the total signal. This dithionite-inaccessible fraction represents the amount of probe found in the inner leaflet of liposomes that is impermeable to the aqueous quenching agent. In proteoliposomes devoid of cholesterol, only 12% of the NBD-C_6-PC fluorescence remained inaccessible to dithionite and reflects the increased permeability of liposomes containing reconstituted P-gp. Cholesterol incorporation in the proteoliposomes caused a dose-dependent increase in the fraction of NBD-PC not accessible to the aqueous quenching agent dithionite. However, cholesterol did not completely restore the impermeability of proteoliposomes to levels observed in LUVs. In summary, P-gp has a profound effect on

bilayer properties that leads to increased permeability and this influence may be partially circumvented by cholesterol.

The ability of P-gp to modulate short-chain phospholipid "flip-flop"

The inaccessibility to dithionite of NBD-C_6-PC found in the inner leaflet of liposomes may be used to determine the translocation of phospholipids between leaflets of a bilayer. NBD-C_6-phospholipids were added to proteoliposomes following reconstitution, thereby restricting their distribution to the outer leaflet. To determine whether P-gp mediates phospholipid "flip-flop", proteoliposomes containing an NBD-C_6-phospholipid were incubated at 37 °C for various times in the absence or presence of 5 mM MgATP. Only proteoliposomes containing 20% cholesterol were amenable to this analysis owing to the high degree of permeability found at lower cholesterol concentrations. The quenching of NBD-C_6-phospholipid probe by dithionite was measured at several time points and the fraction inaccessible to dithionite was determined. Over the 1 h incubation at 37 °C, only a small fraction ($< 10\%$) of any probe remained inaccessible owing to re-orientation to the inner leaflet (Fig. 6). In contrast, there was no measurable re-orientation of any NBD-labeled lipid in liposomes not containing P-gp (data not shown). The addition of MgATP, to activate P-gp, did not alter the amount of NBD-C_6-PC, NBD-C_6-PE or NBD-C_6-ceramide that was re-orientated in the proteoliposomes. MgATP did not affect the permeability of liposomes to dithionite since symmetric LUVs, containing NBD-C_6-PC in both leaflets, retained a 50% inaccessible fraction of probe following a 1-h incubation at 37 °C.

Experiments were carried out with spin-labeled analogues of phospholipids bearing a short *sn*-2 chain [i.e. (0,2)-doxylPC or (0,2)-doxylPE], incorporated either during (labeling on both leaflets) or after reconstitution (labeling on outer leaflet). Reduction by sodium ascorbate added externally confirmed the increased leakiness of P-gp proteoliposomes not containing cholesterol and hampered measurement of the transmembrane movement of the probes (data not shown). Therefore, the possible involvement of P-gp in active translocation of phospholipids was also measured with spin-labeled lipids using a technique not requiring quenching agents. A high concentration of spin-labeled probe (5 mol%) was added to the outer monolayer of proteoliposomes. The high local concentration gives rise to spin-spin interactions between probes that lead to line broadening and a reduced amplitude. The translocation of a small proportion of the spin-labeled lipids to the inner leaflet produces a narrow spectral component. The reduction of intensity of the outer monolayer component associated with removal of a small fraction from this leaflet produces a negligible modification of this spectral component. However, movement of the same amount of probe to the inner leaflet causes a significant increase in the intensity at certain parts of the spectrum owing to dilution of the spin-label in a fluid lipid environment. This would be evident as an increase in spectral intensity of combined spectra, although the double integral should remain constant. The shape transformation would be significantly more complicated if more than 10% of spin-label is translocated from the outer to the inner monolayer.

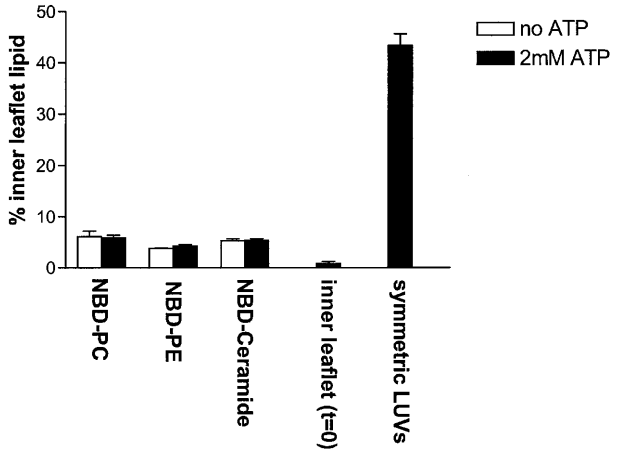

Fig. 6 The influence of P-gp on the translocation of NBD-C_6-lipids. The probes NBD-PE, NBD-PC or NBD-ceramide were added to proteoliposomes at concentrations of < 1 mol%. The samples were then allowed to incubate at 37 °C for 60 min in the presence or absence of 5 mM ATP. After incubation, dithionite (500 μM) was added to quench the fluorescence of probe lipid located in the outer leaflet. The fluorescence remaining corresponded to NBD-C_6-PC translocated to the inner leaflet. The amount of inner leaflet at zero time is also indicated. Symmetric LUVs were produced with equal NBD-C_6-PC in each leaflet, thereby providing a measure of liposome impermeability to dithionite

Figure 7 shows spectra derived from P-gp-containing proteoliposomes composed of PC:PE (9:1) with dialkyl-(0,2)-doxylPE added to a concentration of 5 mol%. This apparent progressive increase in spectral intensity over time was due to translocation of the probe from outer to inner leaflet of proteoliposomes during incubation at 37 °C. A quantitative analysis of the change in line shape based on the combination of a broad component (corresponding to outer leaflet) and a narrow component (corresponding to the inner leaflet) indicates that the spectral increase shown in Fig. 7 represents not more than 5% lipid translocated in 60 min. There was no comparable effect observed in liposomes that did not contain P-gp (data not shown). However, the inclusion of 5 mM MgATP did not significantly alter the spectral intensity from that recorded in the absence of nucleotide. Thus, although P-gp does cause a re-organization of lipids between leaflets in a bilayer, the effect is not an ATP-dependent one. Similar observations were found with the probes dialkyl-(0,2)-doxylPC, dialkyl-(0,2)-doxylSM and dialkyl-(0,2)-doxylGlcCer (data not shown).

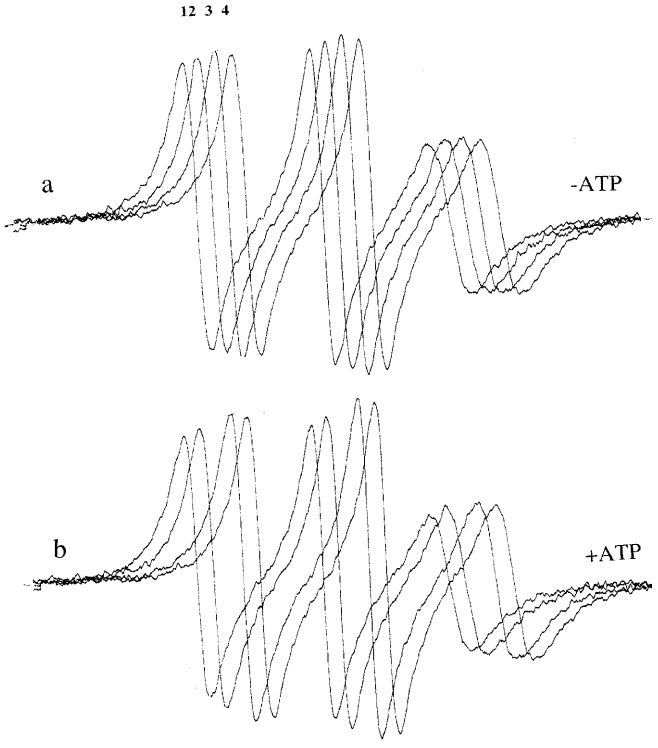

Fig. 7a, b The effect of ATP on translocation of spin-labeled lipids by P-gp. Spectra were obtained in proteoliposomes containing 5 mol% dialkyl-(0,2)-doxylPE added to the external lipid leaflet. Spectra *1*, *2*, *3* and *4* correspond to 1.5, 35, 60 and 125 min incubation, respectively. Incubation was done at 37 °C in the **a** absence or **b** presence of 5 mM MgATP. Spectra were recorded at 22 °C. For clarity of the presentation, each spectrum was shifted by a few Gauss in order to avoid superposition. There was no measurable change in line width, only the amplitude of the peaks increased slightly

Discussion

The reduced permeability of the plasma membrane to cytotoxic drugs in MDR cells relies on contributions from efflux pumps such as P-gp. However, the biophysical properties of plasma membranes in these cells are also altered and might be expected to contribute to drug resistance. In the present study we have directly characterized how P-gp alters membrane biophysical properties such as phospholipid packing, lipid translocation, bilayer fluidity and overall permeability. Cholesterol modified these "destabilizing" effects of P-gp on the bilayer at physiological concentrations. The membrane alterations occurred independent of the transport function of P-gp and may contribute to multiple drug resistance. Confirmation of a possible role was provided by the increased the rate of basal and drug-stimulated ATP hydrolysis by P-gp in proteoliposomes containing cholesterol.

In order to investigate the role of P-gp on membrane structure, an efficient reconstitution procedure was developed at low lipid:protein ratios. Reconstitution procedures have been reported for P-gp using dilution, gel filtration chromatography and dialysis techniques to remove detergent and promote protein incorporation (Callaghan et al. 1997; Ramachandra et al. 1998; Romsicki and Sharom 1999). However, these studies all employed high lipid:protein ratios (> 50 w/w) which are not appropriate for biophysical and structural studies. Therefore we developed a system using detergent removal with SM-2 BioBeads to provide rapid and highly reproducible reconstitution of P-gp under a range of conditions. The rate of detergent removal is one of the major factors determining the efficiency of reconstitution, and the use of SM2-BioBeads provides flexibility in this regard (Rigaud et al. 1997, 1998). It has previously been demonstrated that dodecyl-maltoside supports P-gp function successfully during solubilization and purification procedures (Callaghan et al. 1997; Loo and Clarke 1995). Unfortunately, P-gp could not be efficiently reconstituted at lipid:protein ratios below 50 (w/w) using dodecyl-maltoside. In contrast, a mixed system of dodecyl-maltoside solubilized P-gp and liposomes treated with decyl-maltoside (or octyl-maltoside) allowed complete and reproducible reconstitution at ratios as low as 2.5 (w/w). Another positive feature of the procedure was the ability to efficiently reconstitute at saturating rather than solubilizing detergent concentrations, thereby further reducing any possible detergent-mediated inactivation of P-gp.

Proteoliposomes were used to directly characterize the interaction between P-gp and its bilayer environment. Fluidity measurements provide an overall indication of bilayer properties since they reflect rotational and translational motions of lipid constituents (Spiegel et al. 1981). In MDR cancer cells, conflicting reports of effects on bilayer fluidity have been obtained, presumably due to the heterogeneous localization of probes throughout the cell (Callaghan et al. 1992; Regev et al. 1999; Wheeler et al. 1982). The modest effect of P-gp on overall fluidity observed in our reconstituted system is not surprising and presumably reflects a more significant alteration of lipid motion in localized annular regions of the proteoliposomes. In contrast, the incorporation of P-gp had more pronounced effects on excimer formation of pyrene-containing amphiphiles. Pyrene amphiphile excimer formation is due to localized collisional frequency and reflects the packing arrangement of these amphiphiles between phospholipid molecules in the bilayer rather than lateral diffusion as originally proposed (Barenholz et al. 1996; Blackwell et al. 1986). Cholesterol and P-gp were able to directly disrupt excimer formation by PDA within liposomes, a finding that suggests altered packing constraints within the bilayer. Both cholesterol and PDA packing in bilayers occurs between phospholipids (MacDonald et al. 1988) and their interaction may be likened to competition between the molecules for similar domains. The lack of effect by cholesterol on the phospholipid analogue pyrene-PC demonstrates different distribution of the two pyrene amphiphiles within bilayers. In contrast, P-gp modulated the overall packing arrangement within the bilayer

of both PDA and pyrene-PC, which is indicative of a more general disruption of bilayer structure than that caused by cholesterol.

A major functional consequence of altered lipid packing caused by P-gp incorporation into bilayers was the surprisingly large increase in membrane permeability. Such an effect appears contradictory to the role of P-gp in maintaining drug resistance in cancer cells and may be detrimental to cellular viability. Clearly the plasma membranes of P-gp expressing cells need to circumvent this effect. The ability of cholesterol to partially restore membrane impermeability suggests that it may facilitate in stabilizing P-gp-containing bilayers. Cholesterol is found at high concentrations in the plasma membrane of eukaryotic cells and intercalates between constituent phospholipids such that the polar β-OH moiety aligns with the glycerol backbone (New 1997). This localization confers tight packing and rigidification of lipid membranes, thereby counteracting local perturbations such as those caused by P-gp. P-gp expression in MDR cells has also been associated with elevated cholesterol levels and also increased numbers of microdomains that contain high proportions of cholesterol (Lavie et al. 1998; Yang et al. 1998).

The presence of P-gp in liposomes resulted in an increased translocation compared with that in protein-free LUVs, presumably reflecting a "destabilizing" effect on bilayers by the protein leading to this reorganization. However, there was no ATP dependence in the translocation rates observed for NBD- or spin-labeled lipid derivatives. This appears contradictory to results in the literature suggesting that NBD-phospholipids and glycolipids can be translocated by P-gp from the outer to the inner leaflet of eukaryotic cell membranes (Alburob and Gumbleton 1999; Bosch et al. 1997; van Helvoort et al. 1996). Since these results have been obtained from whole cells, the possibility that specific co-factors or distinct proteins are required to mediate lipid translocation cannot be ruled out. Alternatively, the technical limitations associated with using proteoliposomes of average diameter 200 nm may provide a more fundamental biophysical explanation for the lack of ATP-dependent translocase activity. Phospholipid translocase activity will cause a build-up of lipids at the inner leaflet of proteoliposomes, thereby generating high surface tension. The build-up of surface tension may preclude any further translocation of phospholipids between the two leaflets. Unlike cells or giant liposomes, the relatively small P-gp-containing proteoliposomes cannot produce effects such as membrane bending (Farge and Devaux 1992; Seigneuret and Devaux 1984) or endocytosis to compensate for altered surface tension. A localized build-up of phospholipids within a membrane leaflet in vivo may be involved in the generation of microdomains; however, the consequences for protein activity within them are unclear. Interestingly, the activity of membrane proteins such as mechanosensitive ion channels (Martinac et al. 1990) is modulated by the increased surface tension generated in membranes by the addition of compounds such as lyso-PC, which cannot spontaneously flip between leaflets of a bilayer.

What are the functional consequences of P-gp influencing the organization of bilayers? Several reports have indicated that substrates and inhibitors of P-gp actually access drug-binding sites on the protein via the lipid phase (Homolya et al. 1993; Raviv et al. 1990). Consequently, the formation of ordered microdomains and altered phospholipid packing arrangements near P-gp would be expected to influence drug-protein interactions and, consequently, efflux from the resistant cells. We have shown that the inclusion of cholesterol increased the ATPase activity of P-gp and modified the extent of stimulation by verapamil. Many effects of various lipid species on P-gp function have been reported and overall it appears that optimal function of the protein may be supported by lipids such as cholesterol and the non-bilayer-forming PE (Lerner-Marmarosh et al. 1999; Loo and Clarke 1995; Ramachandra et al. 1998; Shapiro and Ling 1994). The promotion of ordered lipid phases within bilayers is a characteristic effect of cholesterol incorporation (Harder and Simons 1997; New 1997; Vaz and Almieda 1993) and such domains may modulate the function of P-gp. For example, MDR cells contain increased numbers of cholesterol- and sphingolipid-rich microdomains known as caveolae (Lavie et al. 1998). Furthermore, P-gp appears to localize to these ordered microenvironments within the membrane. Future efforts will focus on reproducing these microenvironments in order to address the functional consequence of P-gp localization within them.

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