

P-glycoprotein retains function when reconstituted into a sphingolipid- and cholesterol-rich environment

Szabolcs Modok, Catherine Heyward, and Richard Callaghan¹

Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, United Kingdom

Abstract P-glycoprotein (P-gp) appears to be associated within specialized raftlike membrane microdomains. The activity of P-gp is sensitive to its lipid environment, and a functional association in raft microdomains will require that P-gp retains activity in the microenvironment. Purified hamster P-gp was reconstituted in liposomes comprising sphingomyelin and cholesterol, both highly enriched in membrane microdomains and known to impart a liquid-ordered phase to bilayers. The activity of P-gp was compared with that of proteoliposomes composed of crude egg phosphatidylcholine (unsaturated) or dipalmitoyl phosphatidylcholine (saturated) in the presence or absence of cholesterol. The maximal rate of ATP hydrolysis was not significantly altered by the nature of the lipid species. However, the potencies of nicardipine and XR9576 to modulate the ATPase activity of P-gp were increased in the sphingolipid-based proteoliposomes. The drug-P-gp interaction was investigated by measurement of the rates of [³H]XR9576 association and dissociation from the transporter. The lipid environment of P-gp did not affect these kinetic parameters of drug binding. In summary, P-gp retains function in liquid-ordered cholesterol and sphingolipid model membranes in which the communication between the transmembrane and the nucleotide binding domains after drug binding to the protein is more efficient.—Modok, S., C. Heyward, and R. Callaghan. P-glycoprotein retains function when reconstituted into a sphingolipid- and cholesterol-rich environment. *J. Lipid Res.* 2004. 45: 1910–1918.

Supplementary key words membrane microdomain • lipid raft • liquid-ordered phase • ATP binding cassette transporter • drug binding

The phenomenon of resistance to chemotherapy displayed by cancer cells is a significant clinical problem, and the underlying mechanisms are numerous. Resistance pathways are either inherent to the three-dimensional arrangement of the tissue or acquired after exposure to anticancer drugs. The plasma membrane plays a major role in resistance to chemotherapy by preventing the accumulation of drugs at efficacious concentrations. The princi-

pal mechanism is via active extrusion of anticancer drugs by transporters belonging to the ATP binding cassette (ABC) superfamily. The best-described and most widely observed ABC transporter in cancer tissues is P-glycoprotein (P-gp), whose expression is associated with a reduced rate of remission and poor prognosis. P-gp is an unusual transporter in that it recognizes an extremely broad range of drug substrates that are neither chemically nor functionally related. As a consequence, it is classified as a “multidrug” transporter, and the ability to recognize a range of drugs is attributable to the presence of multiple allosterically linked binding sites (1).

The only common characteristic of drugs transported by P-gp is hydrophobicity, and several lines of evidence have demonstrated that the binding sites on the protein are in the intrabilayer region (2–5). Over the last 20 years, a consistent, if unexplained, observation has been that P-gp and its membrane environment display a complex and interwoven relationship [for review, see ref. (6)]. For example, the incorporation of P-gp into bilayers is known to alter lipid-packing properties (7), to produce ultrastructural changes in the membrane (8, 9), and has even been suggested to mediate the transbilayer translocation of specific phospholipids (7, 10, 11). More recent observations have proposed that P-gp is localized to detergent-resistant low density membrane microdomains (12) and caveolae (13, 14). In addition, the major lipid species (e.g., sphingolipids and cholesterol) found in caveolae and rafts display increased levels in several P-gp-containing drug-resistant cell lines (14–17). Do these correlative observations indicate a physiological or functional role for the localization of P-gp to membrane microdomains? Addressing this question remains the subject of intense scientific research.

The association between P-gp and membrane microdomains can only be important in drug resistance if the protein retains function in this lipid environment. Microdomains in rafts or caveolae are characterized by the pres-

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¹ To whom correspondence should be addressed.
e-mail: richard.callaghan@ndcls.ox.ac.uk

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ence of high levels of cholesterol, sphingolipids, and glycosphingolipids (18, 19). Moreover, cholesterol and high-melting-point lipids, such as sphingomyelin (SM), are well established to generate the low-density, detergent-resistant, rigid membrane environment known as a liquid-ordered (l_o) phase (19–21). The l_o phase is characterized by the tight packing of saturated acyl chains resembling the solid (l_s) phase but still allows relatively rapid lateral motility of lipids similar to the liquid-crystalline (l_c) phase (22). The presence of P-gp in a l_o phase may affect its function, and therefore its ability to confer drug resistance, by two major mechanisms. First, P-gp moves between several conformationally dependent transition states during a transport or catalytic cycle (23–27), and the l_o domain inherent to the sphingolipid- and cholesterol-rich environment may retard this (18). Second, the drug-P-gp interaction that is known to occur within the lipid bilayer (2, 4, 5) may be modified as a result of altered drug partition into l_o microdomains.

The focus of the present article was to determine whether P-gp could retain function in the l_o phase generated by a sphingolipid- and cholesterol-rich environment. To achieve this, P-gp was reconstituted into liposomes comprising SM, egg phosphatidylcholine (PC), or the saturated dipalmitoyl phosphatidylcholine (DPPC) in the presence or absence of high cholesterol (CH) content. P-gp was thereby exposed to l_c (PC, PC:CH), l_s (SM, DPPC), and l_o (DPPC:CH, SM:CH) phase lipid environments. At a high cholesterol-phospholipid ratio there is no lateral phase separation in the liposomes (28), providing a homogenous bilayer environment for P-gp.

The function of P-gp was assessed by measurement of *i*) basal and drug-modified ATP hydrolysis, and *ii*) the kinetic binding characteristics of the P-gp inhibitor XR9576. The protein retained full function regardless of the precise lipid composition in the proteoliposomes. However, the potencies of a stimulator and an inhibitor drug to modify ATPase activity were significantly greater in the l_o phase encountered in SM- and cholesterol-rich proteoliposomes. The initial interaction of the drug with the transporter was not altered by the different lipid bilayer phases. Therefore, we conclude that the l_o phase bilayer of rafts does not restrict the function of the integral membrane protein P-gp. In fact, it provides optimal conditions for crucial drug-induced conformational changes.

MATERIALS AND METHODS

Materials

PC [melting temperature (T_m) \approx 10°C] and DPPC (T_m = 41°C) were purchased from Avanti Polar Lipids (Alabaster, AL). Chicken egg yolk SM (T_m \approx 37°C), cholesterol, Na₂ATP, nocardipine hydrochloride, and sodium orthovanadate were obtained from Sigma-Aldrich (Poole, UK). [³H]XR9576 (27 Ci/mmol) and the nonradioactive parent compound were provided by Xenova Ltd. (Slough, UK). [³H]PC (83 Ci/mmol) and [¹⁴C]cholesterol (53 mCi/mmol) were purchased from Amersham Pharmacia Biotech (Amersham, UK). Econo-Pac Q anion-exchange chromatography cartridges (5 ml) and SM2 BioBeads were from Bio-Rad (Hemel-Hempstead, UK). The nonionic deter-

gents dodecyl- β -D-maltoside and decyl- β -D-maltoside were obtained from CN BioSciences (Nottingham, UK). All other materials were of at least analytical grade.

Cell culture

The P-gp-expressing, drug-resistant Chinese Hamster Ovary cell line CH^FB30 was grown in α -minimal essential medium supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin (100 IU/ml and 100 mg/ml, respectively). The medium was supplemented with 30 μ g/ml colchicine to maintain selection pressure for P-gp expression (29).

Purification and reconstitution of P-gp

Nitrogen cavitation was used to disrupt CH^FB30 cells, and sucrose gradient centrifugation was used to harvest the plasma membrane fraction as previously described (29). P-gp was solubilized from these membranes using 1% (w/v) dodecyl- β -D-maltoside with 0.4% (w/v) crude asolectin lipids (45% PC) in buffer containing 5 mM PIPES, pH 7.1, and purified using anion-exchange chromatography as previously reported (29), subject to minor modifications (7).

Reconstitution of P-gp was achieved using selective detergent extraction from a protein/lipid/decyl- β -D-maltoside mixture by SM2 BioBeads, as previously described (29). The reconstitution system has previously been fully characterized with respect to efficiency, lipid recovery, liposome size, and morphology (29). Unilamellar liposomes were prepared for each lipid composition before detergent/protein addition using the extrusion technique previously described (29). The lipid-protein ratio for reconstitution was 2:1 (w/w) to reduce the possible interference to binding assays described below. Before extrusion, all lipid mixtures were heated to 65°C for 15 min, thereby ensuring homogeneous incorporation of high T_m lipids and cholesterol (22). The efficacy of reconstitution was determined by sucrose density centrifugation, and lipid migration was detected by virtue of the inclusion of trace amounts of [³H]PC and/or [¹⁴C]cholesterol in the reconstitution mixture.

ATPase activity of P-gp

The fundamental characteristics of P-gp-mediated ATPase activity (V_{max} and K_m) were determined using a colorimetric assay for the detection of liberated inorganic phosphate (P_i) (30). The proteoliposomes (1 μ g of protein) were incubated in 50 mM Tris buffer, pH 7.4, at 37°C for 25 min in the presence of 0–2 mM Na₂ATP as previously described (29), and ATPase activity was determined in the presence or absence of 50 μ M nocardipine, which was added from a concentrated stock in DMSO such that the final concentration of organic solvent did not exceed 1% (v/v).

The potency of drugs to modify the rate of ATP hydrolysis was determined by incubating proteoliposomes (1 μ g of protein) with a fixed concentration of Na₂ATP (2 mM). Nocardipine stimulation of ATP hydrolysis was determined by the addition of drug in the range 3×10^{-8} to 3×10^{-3} M from concentrated stocks in DMSO. Inhibition by XR9576 (3×10^{-9} to 3×10^{-4} M, stock in DMSO) and sodium orthovanadate (3×10^{-8} to 3×10^{-3} M, stock in water) was measured by the addition of these compounds to proteoliposomes in the presence of 50 μ M nocardipine.

Kinetic drug binding assay

A rapid filtration procedure was used to measure the binding of [³H]XR9576 to P-gp based on previously published methods (31). The proteoliposomes (1 μ g of protein) were incubated at 20°C with [³H]XR9576 (20–25 nM) in 100 μ l of 10 mM Tris buffer, pH 7.4, and for measurement of the dissociation kinetics, an extended incubation time of 4 h was used to ensure that bind-

ing equilibrium was achieved. The dissociation of radioligand was initiated by dilution of the samples with 5 ml of buffer, because the 50-fold reduction in ligand concentration results in negligible association. Samples were left for 0, 1, 2, 3, 5, 10, 15, 30, 60, 90, or 120 min before light vacuum filtration through GF/F filters in a manifold box to separate bound and free ligand. Association kinetics was determined under similar conditions, except for the initial period to reach equilibrium. The proteoliposomes (1 μg) were incubated with 20–25 nM [³H]XR9576 for 0, 1, 2, 5, 10, 15, 20, 60, 120, 180, or 240 min at 20°C before filtration.

Data analysis

The Michaelis-Menten parameters of maximal velocity (V_{max}) and ATP affinity (K_m) were obtained from plots of the ATPase activity as a function of nucleotide concentration by nonlinear regression of the following equation:

$$v = \frac{(V_{max} \times [S])}{(K_m + [S])} \quad (Eq. 1)$$

where v = enzyme activity (nmol P_i/min/mg), V_{max} = maximal ATPase activity (nmol P_i/min/mg), K_m = Michaelis-Menten constant for ATP (mM), and $[S]$ = substrate concentration (mM). The potencies of drugs to alter ATPase activity were obtained from plots of the rate of ATP hydrolysis as a function of drug concentration by nonlinear regression of the general dose-response equation:

$$v = V_{min} + \frac{(V_{max} - V_{min})}{(1 + 10^{\log EC_{50} - L})} \quad (Eq. 2)$$

where v = response (nmol P_i/min/mg), V_{min} = minimal response, V_{max} = maximal response, EC_{50} = ligand concentration producing 50% of maximal response (M), and L = logarithm to base 10 of drug concentration. The association of [³H]XR9576 to P-gp-containing proteoliposomes was determined from plots of the amount bound (pmol/mg protein) as a function of time and the observed association rate determined by nonlinear regression of the two-phase exponential association equation:

$$B_d = A(1 - e^{-k_{obs\alpha}t}) + B(1 - e^{-k_{obs\beta}t}) \quad (Eq. 3)$$

where B_d = amount bound at time t (pmol/mg), A = amount bound in the first exponential phase (pmol/mg), B = amount bound in the second exponential phase (pmol/mg), $k_{obs\alpha}$ = observed association rate for the initial phase (min⁻¹), $k_{obs\beta}$ = observed association rate for the second phase (min⁻¹), and t = time (min). Equation 4 describes the relationship between the observed association rate (k_{obs}) and the association (k_{on}) and dissociation (k_{off}) rate constants, where $[X]$ is the drug concentration.

$$k_{obs} = [X] \times k_{on} + k_{off} \quad (Eq. 4)$$

Dissociation rate constants were determined from plots of the radioligand amount bound (pmol/mg) as a function of time and the two-phase exponential decay equation fitted:

$$B_d = Ae^{-k_{off\alpha}t} + Be^{-k_{off\beta}t} \quad (Eq. 5)$$

where B_d = amount bound at time t (pmol/mg), A = amount displaced in the first exponential phase (pmol/mg), B = amount displaced in the second exponential phase (pmol/mg), $k_{off\alpha}$ = dissociation rate constant for the initial phase (min⁻¹), $k_{off\beta}$ = dissociation rate constant for the second phase (min⁻¹),

and t = time (min). All regression analyses were done using Prism3.0 software (GraphPad), data are presented as means ± SEM, and comparisons of means were performed with one-way ANOVA. Tukey's posthoc test was used to compare pairs in ANOVA, and $P < 0.05$ was considered statistically significant.

RESULTS

The influence of a series of lipid environments on overall P-gp function

Purified, detergent-solubilized P-gp was reconstituted into liposomes composed of glycerophospholipids or sphingophospholipids in the presence or absence of 45% (mol/mol) cholesterol. The reconstitution procedure involving detergent adsorption onto solid phase has previously been adapted to P-gp (7), and efficient reconstitution was achieved for each lipid mixture in the present experiments (data not shown). **Fig. 1A** is a representative example showing the overall function of P-gp, as assessed by ATPase activity, at a range of nucleotide concentrations in proteoliposomes containing PC. Data obtained in each composition of lipid, and for the detergent-solubilized state, are summarized in **Table 1**. The data demonstrate that even in mixed detergent-phospholipid-P-gp micelles in the absence of any drug, ATP hydrolysis was measurable. ATP hydrolysis in the absence of any drug is known as "basal" ATPase activity, and in the case of detergent-solubilized P-gp, the maximal rate of hydrolysis was stimulated from the basal value of 21 ± 1 nmol P_i/min/mg by a factor of 6.5-fold to 137 ± 9 nmol P_i/min/mg in the presence of nifedipine. This compound is a well-established allosteric modulator of P-gp function (1), although it is unclear whether the compound is transported. Furthermore, the affinity of P-gp for ATP was also marginally affected by nifedipine, except in SM:CH and DPPC:CH proteoliposomes. In the latter, the K_{mATP} of the unstimulated P-gp was already higher than observed with other vesicle compositions. The K_{mATP} of P-gp both in the presence and absence of nifedipine was higher in the DPPC:CH compared with the other proteoliposomes. In general, the K_m of an enzyme for a substrate is a combined measure of many events during the catalytic cycle. However, the rate-limiting step in catalysis is usually the dissociation of the product, ADP, from P-gp (32), and it may be possible for nifedipine to influence this.

Reconstitution of P-gp into PC-containing liposomes caused a 3-fold increase ($P < 0.05$) in the basal ATPase activity to 62 ± 9 nmol P_i/min/mg, compared with the detergent-solubilized protein. In contrast, the V_{max} for nifedipine-stimulated ATPase activity did not increase significantly (180 ± 21 nmol P_i/min/mg) in comparison with protein in detergent suspension. Reconstitution into the PC environment did not affect the affinity for ATP ($K_m = 0.44 \pm 0.05$ mM). As a result of the comparatively larger increase in the basal activity, the overall degree of drug stimulation was reduced (2.9-fold) after reconstitution into PC-containing liposomes. The extent of stimulation produced by nifedipine was less than half the increase

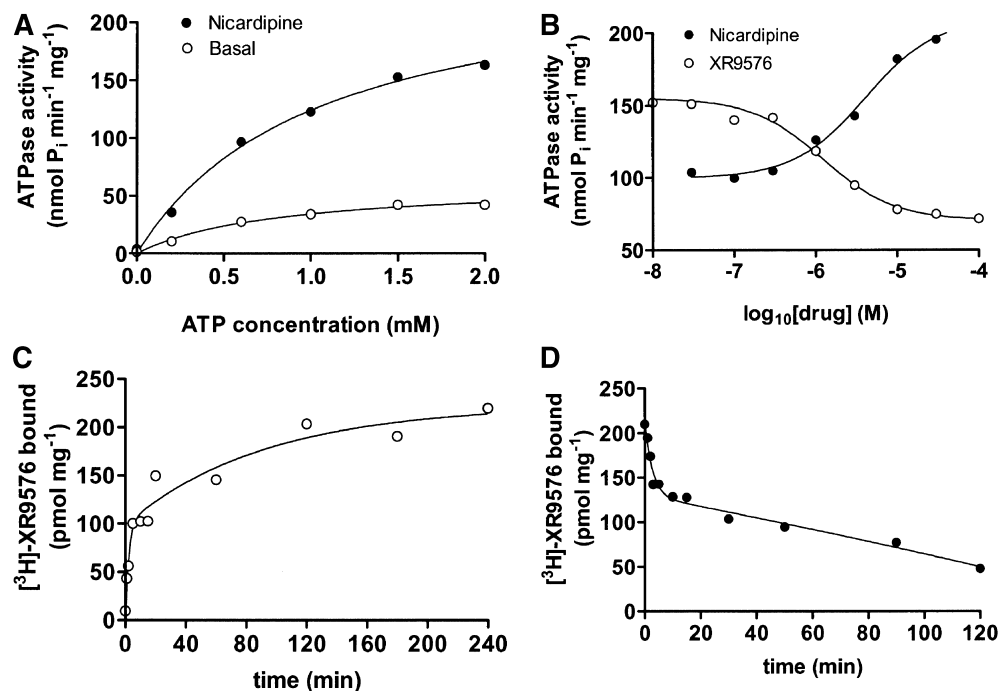


Fig. 1. Representative examples of ATPase activity and [³H]XR9576 binding parameters of P-glycoprotein (P-gp) in an egg phosphatidylcholine (PC) lipid environment. A: Basal and nicardipine-stimulated (50 μ M) ATP hydrolysis in proteoliposomes (1 μ g) was measured as a function of Mg-ATP concentrations (0–2 mM). The activity was determined at 37°C for 25 min and expressed in units of nmol inorganic phosphate (P_i)/mg/min. The K_{mATP} and V_{max} characterizing the activity were obtained by nonlinear regression of the Michaelis-Menten equation (equation 1). B: The effects of XR9576 (3×10^{-9} to 3×10^{-4} M) and nicardipine (3×10^{-8} to 3×10^{-3} M) on the stimulated (50 μ M nicardipine) and basal ATPase activity, respectively, of P-gp reconstituted into PC liposomes (1 μ g) were determined at a single Mg-ATP concentration (2 mM). Activity was expressed as nmol P_i/mg/min, and the potency (EC₅₀) was obtained by nonlinear regression of the general dose-response equation (equation 2). C: Association of [³H]XR9576 (20 nM) to P-gp in PC proteoliposomes (1 μ g) was determined over a period of 1–240 min (20°C). The observed rate constants ($k_{obs\alpha}$ and $k_{obs\beta}$) were determined by nonlinear regression of the two-phase exponential association relationship (equation 3). D: Dissociation of [³H]XR9576 (20 nM) from P-gp in PC proteoliposomes (1 μ g). The radioligand and proteoliposomes were allowed to reach equilibrium (4 h, 20°C), and dissociation was initiated by dilution up to 5 ml with washing buffer. Samples were filtered at various time intervals (1–120 min), and the dissociation rate constants ($k_{off\alpha}$ and $k_{off\beta}$) were determined by nonlinear regression of the two-phase exponential decay equation (equation 5).

observed in detergent-solubilized protein (6.5-fold). Similarly, the V_{max} for basal ATPase activity was significantly increased ($P < 0.05$) from levels observed in the detergent-solubilized state in each of the lipid compositions generated in the reconstituted proteoliposomes. The nicardipine-stimulated V_{max} values were only increased to a statistically significant level in the SM-containing compositions, compared with detergent-soluble protein.

Does the specific lipid composition of the proteoliposomes also affect the activity? Comparing the ATPase activities obtained in the various lipid compositions with those in the simple PC environment revealed that neither the basal nor the drug-stimulated values were significantly affected. In addition, the degree of stimulation produced by nicardipine was also unaffected by the lipid composition, and the range was confined to a 2.5- to 3.5-fold increase. Thus, it is clear that ATPase activity of P-gp will not be unduly affected by its location in the rigid sphingolipid- and cholesterol-rich environments.

Relative drug-induced effects on ATP hydrolysis by P-gp in different lipid environments

Although the data presented above show that the magnitude of change in ATPase activity produced by nicardipine was independent of the lipid composition in proteoliposomes, they do not examine the potency of drug effects. The issue of potency assumes significance given that overwhelming experimental evidence indicates that drugs access the protein via the lipid milieu (2, 4, 5).

Full dose-response curves characterizing the effects of nicardipine and the specific P-gp inhibitor XR9576 on ATP hydrolysis were constructed, as shown in Fig. 1B. The potencies obtained from several independent preparations in each lipid environment are summarized in Table 2. Vanadate was also included because this metabolic inhibitor is known to bind directly to the cytosolic nucleotide binding domains. The potency of nicardipine to produce stimulation of ATPase activity was characterized by an EC₅₀ of $3.7 \pm 0.4 \mu$ M in a PC-rich lipid environ-

TABLE 1. Basal and drug-stimulated ATPase characteristics of P-gp reconstituted into various lipid environments

	Variable (Unit)	Detergent Solubilized	PC	PC:CH	SM	SM:CH	DPPC	DPPC:CH
Basal	K_m (mM)	0.43 ± 0.04	0.44 ± 0.05	0.41 ± 0.03	0.56 ± 0.05	0.63 ± 0.05	0.55 ± 0.07	0.99 ± 0.02
	V_{max} (nmol/min/mg)	21 ± 1	62 ± 9 ^a	0.76 ± 10 ^a	78 ± 11 ^a	93 ± 10 ^a	65 ± 19 ^a	104 ± 37 ^a
Stimulated	K_m (mM)	0.67 ± 0.03	0.60 ± 0.04	0.63 ± 0.05	0.76 ± 0.08	0.62 ± 0.09	0.83 ± 0.03	1.28 ± 0.27
	V_{max} (nmol/min/mg)	137 ± 9	180 ± 21	213 ± 24	278 ± 45 ^a	274 ± 49 ^a	192 ± 28	255 ± 52
Fold stimulation No.		6.5×	2.9×	2.8×	3.6×	2.9×	3.0×	2.5×
		48	20	15	13	10	3	3

CH, cholesterol; DPPC, dipalmitoyl phosphatidylcholine; PC, egg phosphatidylcholine; SM, sphingomyelin. Purified P-glycoprotein (P-gp) was reconstituted in several different lipid environments at a lipid-protein ratio (w/w) of 2:1. ATPase activity was determined in proteoliposomes (1 μg of protein) at various nucleotide concentrations (0–2 mM) by measurement of liberated inorganic phosphate. The basal activity was measured in the absence of any drug substrate, and stimulated activity was determined in the presence of 50 μM nicardipine. The maximal activity ($V_{max} \pm SEM$) and affinity constant ($K_m \pm SEM$) were determined by nonlinear regression of the Michaelis-Menten equation. The fold stimulation refers to the ratio of V_{max} in the presence compared with the absence of nicardipine. No. refers to the number of independent observations. Detergent solubilized refers to P-gp that had not been reconstituted into lipid vesicles.

^a Significantly different ($P < 0.05$) for the parameter compared with the value obtained for detergent-solubilized protein.

ment. Incorporation of 45% (mol/mol) cholesterol did not affect the potency of nicardipine; however, the reconstitution into sphingophospholipid-rich environments produced a marked change. For example, the potency of nicardipine was increased by ~3-fold to an EC_{50} of $1.3 \pm 0.3 \mu M$ ($P < 0.05$) in SM proteoliposomes. The inclusion of 45% (mol/mol) cholesterol in the SM proteoliposomes did not produce any further increase in the potency of nicardipine ($EC_{50} = 1.5 \pm 0.3 \mu M$). The PC environment obtained from extracted egg lipids contains a significant proportion of unsaturated phospholipid species, whereas the SM composition is entirely saturated. Therefore, to enable further comparison, a totally saturated DPPC mixture was also used as a host environment for P-gp. The potency of nicardipine in DPPC proteoliposomes was increased ($EC_{50} = 2.1 \pm 0.7 \mu M$) by almost 50% of the value obtained in the egg PC species; however, the difference did not reach statistical significance. Similarly, the incorporation of 45% (mol/mol) cholesterol did not have any impact on the potency of nicardipine compared with that in the DPPC liposomes.

P-gp is known to contain multiple drug binding sites, and it is conceivable that the lipid-induced changes may be specific to the binding site for the 1,4-dihydropyridine nicardipine (1). To address this issue, the potency of the P-gp inhibitor XR9576 on ATPase activity was also determined in each lipid environment in the presence of nicardipine. Previous studies have demonstrated that these two

drugs bind to different sites on P-gp, and nicardipine was shown to increase the dissociation rate of XR9576 from P-gp (1). Unlike nicardipine, the effect of XR9576 on ATP hydrolysis by P-gp was an inhibition of activity (Fig. 1B). The relative potency of XR9576 to affect P-gp in PC ($EC_{50} = 0.66 \pm 0.13 \mu M$) and PC:CH ($EC_{50} = 0.41 \pm 0.12 \mu M$) proteoliposomes was not significantly different. However, in both of the sphingolipid-containing mixtures, the potency of XR9576 to inhibit ATPase activity was significantly increased 3-fold compared with that in the purely PC environment (Table 2). Similarly, the reconstitution of P-gp into a saturated lipid environment of either DPPC or DPPC:CH also resulted in a greater potency ($P < 0.05$) for the XR9576-induced inhibition of ATP hydrolysis by P-gp (Table 2). Treatment of P-gp with vanadate also produces inhibition of ATP hydrolysis; however, the effect of this compound is produced by the generation of a stable transition complex of P-gp/ADP/vanadate. This is in contrast to XR9576, which binds to the transmembrane domains (TMDs) of P-gp. The potency of vanadate was 5-fold less than that of XR9576, with an IC_{50} value in a PC environment of $3.4 \pm 0.9 \mu M$. Vanadate is a water-soluble transition state inhibitor that acts on the nucleotide binding domains (NBDs), so as anticipated, its potency to produce inhibition was independent of the lipid environment into which P-gp was reconstituted (Table 2).

In summary, the basal and drug-stimulated ATPase activity of P-gp was essentially similar in phosphoglycero-

TABLE 2. Potency of nicardipine, XR9576, and vanadate to modify the ATPase characteristics of P-gp reconstituted into various lipid environments

EC_{50} (μM)	PC	PC:CH	SM	SM:CH	DPPC	DPPC:CH
Nicardipine	3.7 ± 0.4 (17)	3.7 ± 0.7 (11)	1.3 ± 0.3 ^a (6)	1.5 ± 0.3 ^a (7)	2.1 ± 0.7 (3)	2.2 ± 0.5 (3)
XR9576	0.66 ± 0.13 (11)	0.41 ± 0.12 (11)	0.24 ± 0.04 ^a (10)	0.18 ± 0.04 ^a (12)	0.15 ± 0.02 ^a (3)	0.15 ± 0.03 ^a (3)
Vanadate	3.4 ± 0.9 (6)	3.6 ± 0.7 (6)	4.6 ± 0.9 (5)	4.2 ± 0.9 (6)	8.1 ± 0.9 (3)	7.1 ± 2.6 (3)

ATPase activity of P-gp reconstituted into the different lipid environments was measured using a phosphate liberation assay at an ATP concentration of 2 mM. Nicardipine and vanadate were added at a concentration range of 3×10^{-8} to 3×10^{-3} M. XR9576 (3×10^{-9} to 3×10^{-4} M) and vanadate was tested on proteoliposomes prestimulated with 50 μM nicardipine. The potencies (EC_{50}) were determined by nonlinear regression of the general dose-response relationship. EC_{50} values represent means ± SEM of independent preparations as indicated by the number values.

^a Significantly different ($P < 0.05$) for the parameter compared with the values obtained for PC proteoliposomes.

TABLE 3. Association kinetics of [³H]XR9576 binding to P-gp reconstituted into various lipid environments

Kinetics	PC	PC:CH	SM	SM:CH	DPPC	DPPC:CH
$k_{obs\alpha}$ (min ⁻¹) (n)	0.83 ± 0.29 (3)	0.86 ± 0.27 (3)	1.06 ± 0.19 (3)	0.97 ± 0.32 (4)	0.87 ± 0.21 (3)	1.16 ± 0.13 (3)
$k_{obs\beta}$ (min ⁻¹) (n)	0.018 ± 0.003 (3)	0.015 ± 0.005 (3)	0.012 ± 0.002 (3)	0.023 ± 0.010 (4)	0.015 ± 0.001 (3)	0.018 ± 0.004 (3)

[³H]XR9576 binding to reconstituted P-gp (1 μg) was measured under nonequilibrium conditions at 20°C. The apparent association rate constant (k_{obs}) was determined by incubating proteoliposomes with a fixed amount of [³H]XR9576 (20–25 nM) for various times up to 4 h before rapid filtration. The rate constants ($k_{obs\alpha}$ for aqueous-to-bilayer distribution and $k_{obs\beta}$ for bilayer-to-P-gp distribution) at this concentration of [³H]XR9576 were determined by nonlinear regression of the two-phase exponential association equation. All values represent means ± SEM obtained from several (n) independent preparations.

lipid- or sphingolipid-based lipid environments. However, the potency of drugs to modify ATP hydrolysis displayed a dependence on the nature of the lipid milieu. It is likely that the underlying cause was alterations in either *i*) the initial drug interaction with P-gp or *ii*) the induction of conformational changes between drug and nucleotide binding domains after binding events.

The kinetics of [³H]XR9576 binding to P-gp proteoliposomes

Rather than simply measuring equilibrium binding parameters such as overall capacity and dissociation constant (K_d), the component apparent association rate constant (k_{obs}) and the dissociation rate constant (k_{off}) were determined. These two parameters are likely to be influenced by the lipid milieu, and specific effects on either would not necessarily be reported by equilibrium data ($K_d = k_{on}/k_{off}$). The rapid filtration assay measures the total amount of drug ($[X]$) associated with the proteoliposomes, and this will consist of both specific high-affinity binding to P-gp ($[X\text{-}P]$) and a nonspecific low-affinity intercalation within the lipid phase ($[X]_l$). Ligand added to tubes containing membranes will be in a rapid equilibrium between the aqueous $[X]_a$ and intrabilayer $[X]_l$ environments described by the association and dissociation rate constants $k_{on\alpha}$ and $k_{off\alpha}$, respectively. Based on substantial experimental evidence, it appears that the association of drug to the protein ($[P]$) occurs directly from the lipid milieu, and in equation 6, this is described by association and dissociation constants of $k_{on\beta}$ and $k_{off\beta}$, respectively, in proteoliposomes:

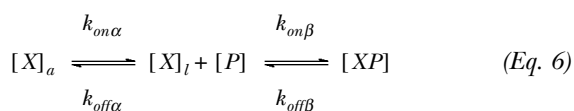


Figure 1C demonstrates the rapid initial and slower late phases of drug association with P-gp-containing liposomes

comprising PC. The rapid initial phase is proposed to represent drug intercalation into the lipid bilayer, and this was confirmed by conducting association profiles in liposomes devoid of P-gp (data not shown). The association data were fitted with equation 3 using proteoliposomes comprising different lipid species, and the observed rate constants ($k_{obs\alpha}$ and $k_{obs\beta}$) using 20–25 nM [³H]XR9576 are shown in Table 3. The initial association phase of [³H]XR9576 (i.e., bilayer intercalation), or $k_{obs\alpha}$, did not differ between the lipid compositions used in the proteoliposomes. The corresponding $k_{obs\beta}$ values were between 40- and 50-fold slower and did not vary significantly in the different proteoliposomes used in the investigations. These results indicate that the initial association of [³H]XR9576 with P-gp and its lipid environment did not significantly vary between unsaturated/saturated PC bilayers, those comprising sphingolipids, or after the inclusion of high levels of cholesterol.

The representative data obtained for the time course of [³H]XR9576 dissociation from proteoliposomes comprising PC (Fig. 1D) also display a characteristic biphasic profile. Data obtained from multiple samples using a variety of lipid compositions in proteoliposomes are summarized in Table 4. The initial rapid dissociation (from lipid to aqueous environments) was caused by the sudden decrease in the drug concentration in the aqueous phase as a result of dilution of the reaction volume. The dissociation rates describing this phase typically displayed large standard error values, presumably because of the difficulty in obtaining an accurate determination of high kinetic rates in a filtration assay. None of the values for $k_{off\alpha}$ were significantly different as judged by ANOVA. The dissociation rate of [³H]XR9576 from P-gp was considerably slower, with $k_{off\beta}$ values that were between 50- and 130-fold lower. The dissociation rate of the [³H]XR9576/P-gp complex ($k_{off\beta}$) was not affected by the lipid composition of the proteoliposomes, as shown in Table 4. The first

TABLE 4. Dissociation kinetics of [³H]XR9576 binding to P-gp reconstituted into various lipid environments

Kinetics	PC	PC:CH	SM	SM:CH	DPPC	DPPC:CH
$k_{off\alpha}$ (min ⁻¹) (n)	0.76 ± 0.17 (5)	0.34 ± 0.10 (6)	0.84 ± 0.19 (6)	0.56 ± 0.14 (6)	0.55 ± 0.13 (3)	0.55 ± 0.18 (3)
$k_{off\beta}$ (min ⁻¹) (n)	0.0063 ± 0.0006 (5)	0.0071 ± 0.0005 (6)	0.0064 ± 0.0009 (6)	0.0085 ± 0.0014 (6)	0.0056 ± 0.0007 (3)	0.0083 ± 0.0013 (3)

[³H]XR9576 binding to reconstituted P-gp (1 μg) was measured under nonequilibrium conditions at 20°C. To determine the dissociation rate constants, the proteoliposomes were first incubated with [³H]XR9576 (20–25 nM) for 4 h to reach equilibrium. Dissociation was initiated by dilution of the samples into 5 ml of 10 mM Tris buffer, pH 7.4, and the proteoliposomes were filtered at various times subsequent. The rate constants ($k_{off\alpha}$ for lipid-to-aqueous dissociation and $k_{off\beta}$ for P-gp-to-lipid dissociation) were determined by nonlinear regression of the two-phase exponential decay equation. All values represent means ± SEM obtained from several (n) independent preparations.

phase kinetic parameters were not significantly different in various proteoliposomes. So partitioning of XR9576, and thereby the local free drug concentrations in the bilayers, must be similar for all lipid combinations studied.

Therefore, although drugs interact with P-gp via the bilayer, environments rich in unsaturated PC, sphingolipid with or without cholesterol did not affect the binding sites in the transporter.

DISCUSSION

P-gp localized within a membrane environment rich in sphingolipids, with or without cholesterol, is clearly able to maintain full function as assessed by the basal and drug-stimulated ATPase activity. However, the potency of drugs such as the allosteric modulator nicardipine or the high-affinity inhibitor XR9576 to alter the rate of nucleotide hydrolysis was increased in this environment. Several transmembrane segments have been suggested to contribute to the drug binding sites on P-gp (33), and lateral forces in bilayers are expected to influence the relative positions of these helices. Thus, one possible explanation for the altered stimulation of ATP hydrolysis is a direct modulation of the drug binding sites on P-gp. Another quite simple scenario is that the bilayer environment of proteoliposomes rich in sphingolipids/cholesterol alters the drugs membrane partition coefficient, thereby producing a higher local drug concentration, which may manifest as an "increased" potency. It has been well established that drugs access the binding sites on P-gp directly via the lipid bilayer (2, 4) and that the driving force for drug association is the free ligand concentration (34). Therefore, any preferential increase in partition of drugs into the bilayer, or an altered rate of diffusion within it, would influence the drug binding kinetics. The contribution of such changes would be demonstrable by measurement of drug association and dissociation rates. However, these kinetic components, and thereby the affinity of drug binding to P-gp, were unaltered in a sphingolipid compared with a PC environment, suggesting that the changes in the efficiency of drugs to modify ATP hydrolysis were attributable to events "downstream" of drug binding. Similarly, the ATPase activity of P-gp has previously been shown to be sensitive to the composition of the host lipid bilayer, and azidopine photolabeling experiments indicated that drug binding was not altered (35). Drugs are known to interact with the TMDs of P-gp (36–38), whereas ATP hydrolysis occurs in the cytosolic NBDs (39–41). A likely explanation for the increased potency of drugs to mediate the communication required to transmit drug binding events between the TMDs and NBDs in the sphingolipid/cholesterol-rich bilayer environment is an altered efficiency of allosteric conformational changes.

P-gp has multiple drug binding sites (1), and drugs do not behave uniformly. For example, drugs such as doxorubicin and colchicine compete for azidopine labeling but do not stimulate ATP hydrolysis, which is in contrast to verapamil or vinblastine (35). However, binding of ATP, a


nonhydrolyzable ATP analog, or vanadate trapping of NBDs invariably results in changes in the binding site (23, 35). So the coupling is tight from ATP hydrolysis to drug binding; however, communication in the opposite direction (i.e., from drug binding to ATP hydrolysis) is looser. The l_o phase in a membrane may facilitate the coupling between drug binding and ATP hydrolysis, thereby generating more efficient stimulation and inhibition of ATPase activity.

The proposed explanation requires that the bilayer lipid environment is capable of imparting forces to restrict or permit conformational changes associated with protein function. The idea that membranes merely provide a permeability barrier has long been dismissed as an oversimplification, particularly given the number of different component lipids and the observations of discrete compartments or lateral segregations in biological membranes (42, 43). In general, glycosyl phosphatidyl inositol-anchored or acylated proteins cluster into rafts, whereas transmembrane proteins are thought to be mostly excluded from l_o membrane microdomains. P-gp has been demonstrated, using structural and biochemical procedures (23–27, 44), to undergo large conformational changes that require considerable modification of the local lipid environment. Both the hydrophobic effect and the network of stable hydrogen bonding arrangements with the amphiphilic membrane species will dictate the "basal or resting" configuration of the membrane-spanning helices in P-gp. Furthermore, gross packing constraints or steric effects of membrane components surrounding P-gp also need to be overcome to undergo conformational changes. Results from the current investigation and from previous indirect observations suggest that perturbation of the environment does affect the functional characteristics of P-gp (29, 35, 45, 46). Furthermore, P-gp was shown to exert unusual high transport activity at temperatures below the T_m of the respective lipid bilayer (i.e., in gel phase membranes) (47). The subtle alterations in drug-induced conformational changes observed in the present investigation were predominant in sphingolipid-rich bilayers. What features of sphingolipid-rich membranes render them more adept at modulating the drug-P-gp interaction?

Both SM and PC contain a zwitterionic choline head group and so have a similar bilayer "packing parameter," as defined by Israelachvili, Marcelja, and Horn (48). However, natural sphingolipids tend to have fully saturated acyl chains, whereas the acyl chain at position C2 of the glycerol moiety of PC is frequently unsaturated. Stronger hydrophobic interaction between the more extended saturated acyl chains and increased hydrogen bonding capacity between the head groups favors spontaneous lateral segregation of sphingolipids from the bulk phase membrane [reviewed in ref. (49)]. The strong intermolecular forces impart high T_m values to (glyco)sphingolipids. Cholesterol helps to keep these high T_m lipids in fluid (l_o) phase by abolishing the main phase transition. In addition, cholesterol fills the spaces under the sphingolipid head groups and between the acyl chains, thus producing

tighter packing and formation of a relatively larger dipole potential in sphingolipid compared with PC membranes (50). Cholesterol has been demonstrated to provide a general rigidifying effect on bilayers and to enhance the ATPase activity of P-gp, although this was observed in a PC:phosphatidylethanolamine (PE) environment (7). In the current investigation, there was no additional effect of cholesterol on ATPase activity in PC or SM liposomes. This difference may be accounted for by the distinct properties of PE in membranes. For example, PE is excluded from cholesterol-rich membrane regions (51), and this phospholipid generally displays longer acyl chains, which are known to influence the activity of P-gp (52).

The fact that l_o phase bilayer behavior can be simulated by substituting DPPC for sphingolipids suggests that hydrophobic interaction between long saturated acyl chains might be the main driving force of phase separation, with hydrogen bonding between lipid head groups assuming a secondary role (53). DPPC has the same head group as PC but contains two saturated palmitoyl chains resembling the hydrophobic tail of SM. In DPPC:CH bilayers, as in SM:CH proteoliposomes, nicardipine and XR9576 were more potent in modulating the ATPase activity of P-gp. Further evidence from the present study for the primary importance of saturated acyl chains for modifying P-gp function is that not only the cholesterol-rich l_o phase bilayers (SM:CH, DPPC:CH) but also the l_s phase bilayers (SM and DPPC) influenced the drug potency to modulate the ATPase activity of P-gp. Moreover, P-gp has relatively long transmembrane segments (M. F. Rosenberg et al., 2004, submitted data), and the wider bilayer of l_o or even the gel phase (54) may provide a more suitable environment for these domains.

To conclude, ABC transporters are transmembrane proteins, and their TDMs undergo major conformational changes during the catalytic cycle as exemplified by P-gp (26). The bilayer surrounding the TMDs should support them as a scaffold and simultaneously enable the conformational changes to occur. P-gp becomes more active when reinserted into a bilayer from mixed detergent-phospholipid-protein micelles. Furthermore, drugs can modify the catalytic activity of P-gp more efficiently if the membrane is relatively rigid. We propose that ordered, tightly packed membrane microdomains increase the coupling between the drug binding events and ATP hydrolysis. Our observations suggest that raftlike bilayers enhance the multidrug transporter activity of P-gp. Future studies will focus on determining the precise regions of P-gp involved in functionally relevant conformational changes, which will shed light on the complex interaction with its local membrane environment. 

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