Research Paper

P-Glycoprotein in Proteoliposomes with Low Residual Detergent: The Effects of Cholesterol

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Purpose. There is evidence that cholesterol affects the ATPase and transport functions of P-glycoprotein (P-gp). To study the influence of cholesterol on P-gp in a well defined lipid environment, we reconstituted P-gp in egg phosphatidylcholine (PhC) and PhC/cholesterol proteoliposomes with negligible residual amounts of detergents.

Materials and methods. P-gp proteoliposomes were prepared by continuous dialysis from micelles consisting of P-gp, lipids, sodium dodecyl sulfate and cholate. Basal and modulator-induced ATPase activities were studied in an established enzyme assay. Modulator affinities to P-gp and to the lipid bilayers were determined by equilibrium dialysis.

Results. In the absence of cholesterol the basal ATPase activity was six fold lower than in the presence of 20 or 40% cholesterol, and no P-gp binding and ATPase induction was detected for the tested modulators verapamil and progesterone. In proteoliposomes containing 20 and 40% cholesterol, respectively, the modulators showed significant P-gp binding and ATPase activation. The concentration of the modulators for half maximal activation of the ATPase was higher with 40% than with 20% cholesterol.

Conclusions. Cholesterol influences P-gp in three ways: (a) it enhances its basal ATPase activity, (b) it renders P-gp sensitive towards the modulators verapamil and progesterone and (c) it affects the modulator concentration at half maximal ATPase activation.

KEY WORDS: ATPase activity; binding; cholesterol; P-glycoprotein; verapamil.

INTRODUCTION

The multi-drug resistance protein P-glycoprotein (P-gp) is an efflux transporter for a wide variety of drug compounds and cytotoxins. The 170 kDa ATP binding cassette (ABC) protein contains 12 transmembrane domains and two cytosolic ATP-binding sites. It is encoded by the MDR1 (ABCB1) gene and is expressed under physiological conditions in tissues with excretory functions, such as the small intestine, liver and kidney, and at blood-tissue barriers such as the blood-brain barrier, blood-testis barrier and blood-placenta barrier. It is typically but not exclusively localized in the apical plasma membrane domain of P-gp expressing cells (1). P-gp gets frequently upregulated in tumor cells upon treatment with cytotoxic drugs, causing multi-drug resistance by expelling a wide palette of chemotherapeutics from these cells. For reviews see (1–3).

The transported substrates have no common structural features which would point to a well defined binding pocket in the transporter and which would allow to clearly distinguish them from non-substrates. As P-gp substrates are relatively lipophilic, it is assumed that the binding to the transporter occurs from within the lipid bilayer (2). Several studies on drug–P-gp interactions give indeed evidence for drug binding sites within the transmembrane domains of the protein (2,4–6). This is in line with the observations that the transport and ATPase activities of P-gp are strongly dependent on the lipid environment (7). P-gp may contain various binding sites for different compounds or display one common binding pocket of high flexibility to enable an induced fit mechanism (8). In addition to the model of direct drug binding to the transporter, indirect drug/P-gp interactions *via* the lipid bilayer or a pH gradient were discussed (9).

P-gp hydrolyses ATP in the absence of known substrates when reconstituted in a lipid bilayer environment; this is defined as the basal ATPase activity. The ATPase activity is significantly enhanced in the presence of many transported substrates and of so called modulators. The latter activate the P-gp ATPase activity without undergoing a significant P-gp related net transport. This phenomenon may occur for compounds which are translocated by P-gp but have a high

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ABBREVIATIONS: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate; DTT, Dithiothreitol; DMPC, Di-myristoyl phosphatidylcholine; DPPC, Di-palmitoyl phosphatidylcholine; PBS, Phosphate buffered saline; TNE, Tris/NaCl/EDTA buffer; P-gp, P-glycoprotein; PhC, Phosphatidylcholine; SDS, Sodium dodecyl sulfate; SUBS, Standardized universal buffer solution.

passive permeation rate across the membrane, exceeding the transport efficiency of the protein (10).

P-gp is enriched in detergent-insoluble membrane domains, the so called rafts, which contain relatively high amounts of cholesterol and sphingolipids (11–13). Cholesterol plays a crucial role for the function of P-gp as an ATPase and a transport system. In cell culture, the sterol controls P-gp functions and P-gp localization (11–18). In general, the ATPase activity of P-gp decreased upon depletion of cells from cholesterol and increased upon repletion or saturation of the cells with cholesterol.

It is still not known how the sterol influences the P-gp ATPase and transport functions (1). Cholesterol may be a substrate of P-gp transport and therefore increase the ATPase activity in a similar manner as many known P-gp substrates. This is supported by several studies (19). However, the direction of the transport is not clear. Transport has been described as cell influx or translocation from the exoplasmic to the cytoplasmic membrane leaflet (20), as well as from the cytoplasmic to the exoplasmic leaflet (18).

Alternatively, cholesterol could directly interact with P-gp, enhancing its ATPase activity, without being transported. A third possibility is given by the influence of cholesterol on the characteristics of a lipid bilayer (15, 21). The sterol could thus indirectly affect the function and activity of P-gp *via* the membrane properties.

That cholesterol affects the ATPase activity of P-gp has not only been shown with P-gp expressing cells but also with proteoliposomes, i.e., after P-gp reconstitution in the lipid bilayer of lipid vesicles. Proteoliposomes have the advantage over whole cells that the transporter can be studied as an isolated protein. Indirect effects by a cellular response can be excluded. A great part of our current knowledge about the influence of the lipid environment on the efflux transporter P-gp comes from excellent work with P-gp proteoliposomes (7,14,22–30).

The work with reconstituted P-gp is hampered by the fact that most systems contain considerable amounts of residual detergents. The latter generally affect the activity of the transporter (31). In addition, mild detergents such as CHAPS do not completely delipidate the protein upon solubilization. The remaining lipids (about 50 phospholipid molecules per P-gp molecule) could affect the activity and function of the reconstituted transporter (32). We therefore established a method to prepare P-gp proteoliposomes with negligible amounts of residual detergents and lipids from the source membrane. This was achieved by the use of the strong detergents sodium dodecyl sulfate (SDS) and cholate for the solubilization and reconstitution of P-gp into egg phosphatidylcholine (PhC) and PhC/cholesterol proteoliposomes. Both detergents are quantitatively removable by dialysis due to their relatively high critical micelle concentration. The protocol was used to study the influence of cholesterol on the basal and modulator-induced ATPase activity of P-gp and on the binding of the modulators to the transporter. The investigated modulators were verapamil and progesterone.

The basal P-gp ATPase activity was about six times lower in the absence of cholesterol than in the presence of 20 or 40% (mol/mol total lipid) of the sterol. This low activity remained unchanged upon addition of the studied modulators and no specific P-gp binding of the tested compounds was observed. In proteoliposomes containing 20 and 40% cholesterol, respectively, the sterol not only enhanced the basal ATPase activity in the absence of the tested modulators, but also enabled P-gp binding and further ATPase activation by the modulators. Finally, the modulator concentrations at half maximal ATPase activation were higher at 40% than at 20% cholesterol. Based on these findings we put forward a new model for the functional interaction between cholesterol and P-gp.

MATERIALS AND METHODS

Chemicals

[N-methyl-^{3}H]-verapamil (3.145 TBq/mmol, # NET-810), [1,2,6,7-^{3}H(N)]-progesterone (3.77 TBq/mmol, # NET 381) and [2,4-^{3}H]-cholic acid (560 GBq/mmol, # CUS55054000MC) were purchased from Perkin Elmer Life Sciences (NEN, Boston MA, USA) and sodium dodecyl-1-^{14}C-sulfate (2.035 GBq/mmol, # L-2656) from Sigma (St. Louis MO, USA). Adenosine triphosphate, di-sodium (ATP) #A2383, orthovanadate, sodium #S6508, progesterone #P0130, and (±)-verapamil #V4629 were supplied by Sigma (Buchs, Switzerland), dithiothreitol (DTT) #43815, NADH #43420, phosphoenolpyruvate #79418, pyruvate kinase #83330, sodium dodecylsulfate (SDS) and 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate (CHAPS) were from Fluka (Buchs, Switzerland). Lactate dehydrogenase #127876, DNAse I and the protease inhibitor cocktail Complete® were supplied by Roche Diagnostics (Basel, Switzerland). All other chemicals were of analytical grade.

Cell Cultures and Antibodies

P388/ADR cells were obtained from the National Cancer Institute (Frederick, MD, USA). Cells were grown in suspension in RPMI1640 medium (Invitrogen Corp., Basel, Switzerland) containing 10% heat inactivated fetal bovine serum and split 1:10 every 3–4 days. The monoclonal mouse antihuman P-gp antibody C219 was supplied by DAKO Corp. (DakaCytomation, Carpinteria, CA, USA).

Purification of P-glycoprotein

About four·10^8 P388/ADR cells suspended in 2 ml PBS, pH 7.0 (50 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, 130 mM NaCl) containing 10 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 0.4 mg/ml DNAse I and protease inhibitor cocktail Complete® were homogenized with 50 strokes of a Dounce homogenizer at 1,000 rpm. The membrane fragments were collected by centrifugation at 200,000 g for 1 h at 4°C. The pellet containing the membrane fragments was solubilized in 2 ml 50 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.4, containing 1 mM DTT in 2% (m/v) SDS. After solubilisation the preparation was diluted with 50 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.0, containing 1 mM DTT to 1% SDS. P-gp was purified from the solubilized membrane fragments by HPLC according to (33) with some modifications. Briefly, about 4 mg protein were injected on a hydroxyapatite cartridge (Econo-Pac© CHT-II Biorad, Hercules CA, USA) and eluted with a

sodium phosphate gradient (50 to 650 mM phosphate), pH 7.0, containing 0.5% SDS and 1 mM DTT. The buffer components were incubated at 40°C and the cartridge was heated to 30°C to prevent SDS precipitation. P-gp eluted at about 530 mM phosphate and was concentrated with Vivaspin 15R Hydrosart® concentrators (mw cutoff 100,000, Sartorius, Germany) before being stored at −80°C. The concentrates contained 3–5 mg protein per 10^8 cells starting material. This purification protocol results in P-gp which is virtually free of lipids (33).

Reconstitution of P-glycoprotein

Protein-free liposomes of the final lipid composition were prepared by extrusion in PBS, pH 7.4 (34). The liposomes were subsequently dissolved by 30 min incubation with sodium cholate at 60°C at a lipid/detergent molar ratio between 0.5 (PhC/cholesterol liposomes) and 0.72 (PhC liposomes) to form micelles. Complete solubilization was confirmed by dynamic light scattering with a Zetasizer 3000 HSA (Malvern Instruments, Malvern, UK).

Purified P-gp (between 1.5 and 5 mg) in phosphate buffer containing ∼0.25% SDS (see above) was added to the micelles (between 15 and 50 mg lipids) at room temperature in a total volume between 6 and 10 ml. All subsequent steps were performed at room temperature to prevent SDS precipitation. After equilibration over night under argon the protein/lipid micelles were continuously dialysed against 20 l of PBS containing 1 mM DTT and Complete®, pH 7.4, by means of a Liposomat (Diachema, Munich, Germany) equipped with a high permeability preconditioned cellulose membrane with an mw cutoff of 10,000 and a size of $18 \times 18\, \text{cm}^2$ (Diachema). Flow rates were 0.6 ml/min on the micelle side and 2.5 ml/min on the buffer side (35). Dialysis membranes were exchanged 2–3 times during the dialysis.

To monitor detergent removal, ^{3}H-cholate and ^{14}C-SDS (20,000 Bq/ml each) were added to the micelles together with the purified P-gp. Samples were taken during the dialysis to confirm the stability of the protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as well as to determine the ATPase activity and the detergent concentration. After 96 h of continuous dialysis the proteoliposomes were immediately used for the ATPase assays. The protein content of the final proteoliposomes was determined with the DC protein assay kit from Biorad with bovine serum albumin as standard. PhC and cholesterol concentrations were determined by HPLC as described (36).

Density Centrifugation and SDS-PAGE

Proteoliposomes (2 ml) were transferred to an equal volume of 80% (w/v) sucrose in TNE (25 mM Tris, 150 mM NaCl, 5 mM EDTA, and Complete®, pH 7.4) into a centrifuge tube and overlaid with 2 ml each of 30%, 20%, 10% and 1.5 ml of 5% sucrose in TNE. After centrifugation at $200,000 \times g$ for 19 h at 4°C, proteoliposomes were visible as a turbid band in the upper part of the tube corresponding to a density range between 1.03 and 1.05 g/cm^3 as determined from the refractive indices. Fractions of 1 ml were collected and the P-gp distribution was studied by SDS-PAGE, 7.5%, and immunoblotting with the anti-P-gp-antibody C219.

ATPase Kinetic Assay

The ATPase activities (V_{app}) of the proteoliposomes were determined at 37°C, pH 7.4, in 96 well plates (Corning, flat bottom, black, #3915, Corning, Acton, MA, USA) with a Synergy HT plate reader (BioTek, Winooski VT, USA) as described (12). In brief, the ATP consumption was quantified *via* an enzyme cascade resulting in NADH oxidation and ATP recycling. The concentration of ATP was 9 mM (12). The concentration-time profile of NADH was determined from its fluorescence every 75 s. The protein content per well was between 1 and 6 μg, the total sample volume per well 200 μl.

The V_{app}/concentration profiles obtained with verapamil and progesterone were fitted with Eq. 1, excluding the modulator concentrations ≥100 μM, at which an unspecific inhibition occurred (12).

$$V_{app} = V_{basal} + \frac{V'}{1 + \frac{K}{x}} \qquad (1)$$

In Eq. 1, V' denotes the difference between the activity at a modulator concentration $x \rightarrow \infty$ (neglecting the inhibitory effect at high concentrations) and the basal activity (V_{basal}) in the absence of modulator. K is the concentration of modulator at half V'. For clarity, the data are shown in plots with a logarithmic concentration scale. Data were fitted using the Microsoft Excel Solver tool.

To test the influence of CHAPS, proteoliposomes were incubated for 24 h with 400 μM of the detergent at 37°C before they were diluted as stated above to determine the ATPase activity at different verapamil concentrations. The final CHAPS concentration in the assay was 28 μM.

Binding Affinity to P-glycoprotein

The binding of verapamil and progesterone to P-gp proteoliposomes was determined at 37°C by equilibrium dialysis with 0.2 ml dialysis cells and cellulose membranes (mw cutoff 10,000, Dianorm, Munich, Germany). Total drug concentrations were between 10^{-9} and 10^{-4} M, containing 1,600 Bq/ml ^{3}H-verapamil and 2,700 Bq ^{3}H-progesterone, respectively. The protein concentration was between 0.1 and 0.5 mg/ml. Dialysis was performed in PBS, pH 7.4, containing 200 μM sodium vanadate, 10 mM MgCl$_2$ and 1.2 mM ATP (37). The dialyzed samples contained in addition ∼0.5% ethanol originating from the stock solutions of the modulators.

The dialysis was terminated after 5 h (38) and verapamil and progesterone, respectively, were quantified in both cell chambers by liquid scintillation counting. The protein and lipid concentrations in the respective chambers were determined as described above.

The binding data were transformed to Scatchard plots and the data obtained with the PhC/cholesterol proteoliposomes were further analyzed by non-linear curve fitting according to Eq. 2

$$\frac{r}{C_u} = \frac{n_1 \times K_{a1}}{1 + K_{a1} \times C_u} + \frac{n_2 \times K_{a2}}{1 + K_{a2} \times C_u} \qquad (2)$$

where r is the ratio of bound compound [M] and total protein [M] (mw 170,000, assuming 100% P-gp), C_u is the unbound

drug concentration [M], n_i is the maximal number of compound molecules bound to the binding site(s) of class i [-] and K_{ai} [M^{-1}] is the association constant between a drug molecule and a binding site of class i.

Membrane Affinity

The affinity of ^{3}H-verapamil and ^{3}H-progesterone to liposomal membranes was determined by equilibrium dialysis at 37°C (38). The pH was adjusted with a standardized universal buffer solution (SUBS, (38)) as indicated. The drug concentrations were 10^{-7} M and PhC concentrations were about 1 mg/ml. Distribution coefficients (D) were calculated according to Eq. 3 (34)

$$D = (C_{LB} - C_B) \times v_{LB}/(C_B \times v_L) + 1 \qquad (3)$$

where C_{LB} is the molar solute concentration in the liposome-containing chamber and C_B in the buffer chamber. The symbol v_{LB} denotes the sample volume of the liposome suspension, v_L the volume of the lipophilic phase, i.e., the lipid bilayers. This volume was calculated from the PhC concentrations in the dialysis cells, which were determined by HPLC (see above), and a density of 1.0 g/ml for the lipids. The pH-dependent distribution of ^{3}H-verapamil between the lipid bilayer and the aqueous phase was fitted according to Eq. 4 (34):

$$D = \alpha_N \times P^N + \alpha_I \times P^I \qquad (4)$$

where α_N and α_I are the molar fractions of neutral and ionized verapamil, respectively (pK$_a$ 8.9), and P^N and P^I are the partition coefficients of the two ionization species.

RESULTS

Characterization of P-gp Proteoliposomes

P-gp was purified from P388/ADR cells and reconstituted into PhC, PhC/cholesterol 80/20 (mol/mol) and PhC/cholesterol 60/40 liposomes, respectively, as described under Materials and Methods. The final protein concentration of a typical preparation was 0.2 mg/ml. The protein to lipid ratio (w/w) after reconstitution was between 0.10 and 0.13, corresponding to molar ratios between 4.5×10^{-4} and 5.9×10^{-4}.

As detergents can significantly affect the ATPase activity of P-gp (31,39), the detergent concentrations in the proteoliposomes were determined with radio-labeled SDS and cholate (see Materials and Methods). The residual concentrations were below the detection limit. The corresponding lipid/detergent and protein/detergent ratios are depicted in Table I. The respective detergent concentrations had no influence on the ATPase activity of rafts from P388/ADR cells (12), which display high P-gp ATPase activity (data not shown).

To test whether P-gp was stable during the 96 h of dialysis needed to reach the low detergent concentrations, samples were analyzed by SDS-PAGE and immunoblotting after 9, 24, 48 and 72 h and at the end of the dialysis, i.e., after 96 h. As shown in Fig. 1a, no decomposition products were visible on the blot and the amount of P-gp was similar at all tested time points. The ATPase activity after 19 and 48 h of dialysis was 67 and 82%, respectively, of the final activity reached after 96 h, indicating the gain in activity by the removal of the detergents (data not shown). In addition to the dialysate all eluted fractions from the hydroxyapatite cartridge were checked for P-gp decomposition products by SDS-PAGE and immunoblot. No such products were detected in several purification runs (data not shown).

To confirm the successful reconstitution of P-gp, a density gradient centrifugation of the proteolioposome preparation was performed and the density fractions were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 1b there was complete reconstitution indicated by the localization of P-gp in the lipid containing turbid low density fractions (fractions 1–4, 1.03–1.05 g/cm^3). No P-gp was detected in the high density fractions (fractions 5–9) where soluble or aggregated proteins would accumulate. This result was independent of the lipid composition.

The final P-gp proteoliposomes displayed a basal ATPase activity between 16.6 and 101.0 nmol per min per mg protein depending on the lipid composition of the proteoliposomes (see next section). To verify that the observed ATPase activity originated from P-gp, ATPase activities were determined at different concentrations of the monoclonal P-gp antibody C219, which binds to both nucleotide binding regions of P-gp and has been shown to inhibit its ATPase activity (40). C219 reduced the basal activity of the proteoliposomes to $(15.0 \pm 2.6)\%$ at 5 µg antibody and 5 µg P-gp per ml. This was independent of the lipid composition. When the proteoliposomes were incubated with 400 µM sodium ortho-vanadate, an unspecific ATPase inhibitor (12,41), the ATPase activity was decreased to a similar residual activity of $(12.4 \pm 3.7)\%$ in all proteoliposomes.

The average size and size distribution of the proteoliposomes were characterized by dynamic light scattering with a Zetasizer 3000 HSA (Malvern Instruments, Malvern, UK). All proteoliposome preparations showed an average mean hydro-dynamic diameter between 130 and 150 nm and a polydispersity index <0.4 corresponding to a size variance of <63% assuming a monomodal distribution. No signs of aggregates were observed in the correlogram.

Table I. Residual Detergent in P-gp Proteoliposomes

| Detergent	Concentration [M]	Detergent/Protein Ratio		Detergent/Lipid Ratio	
		[mg/mg]	[mol/mol]^a	[mg/mg]	[mol/mol]^a
SDS	$<1.7 \times 10^{-6}$	$<2 \times 10^{-3}$	<1.4	$<3 \times 10^{-4}$	$<7 \times 10^{-4}$
Cholate	$<4.0 \times 10^{-7}$	$<8 \times 10^{-4}$	<0.3	$<9 \times 10^{-5}$	$<2 \times 10^{-4}$

The protein concentration was 0.21 mg/ml, the lipid concentration 1.8 mg/ml.
^{a} Calculated with a molecular weight of 170,000 for P-gp and 780 for egg PhC, respectively.

Influence of Cholesterol on the Basal and Drug-induced ATPase Activity of Reconstituted P-gp

Cholesterol significantly influenced the basal ATPase activity of the proteoliposomes. In the absence of cholesterol (PhC proteoliposomes) it was (16.6 ± 2.1) nmol $\times$ min^{-1} $\times$ mg^{-1} $(n = 36)$ but reached (101.0 ± 8.1) nmol $\times$ min^{-1} $\times$ mg^{-1} $(n = 36)$ in proteoliposomes containing 20% cholesterol and (86 ± 20) nmol $\times$ min^{-1} $\times$ mg^{-1} $(n = 54)$ in proteoliposomes containing 40% cholesterol, respectively (Fig. 2a).

Next, we investigated whether cholesterol had an effect on the ATPase activity in the presence of P-gp modulators. Verapamil and progesterone are known to enhance the
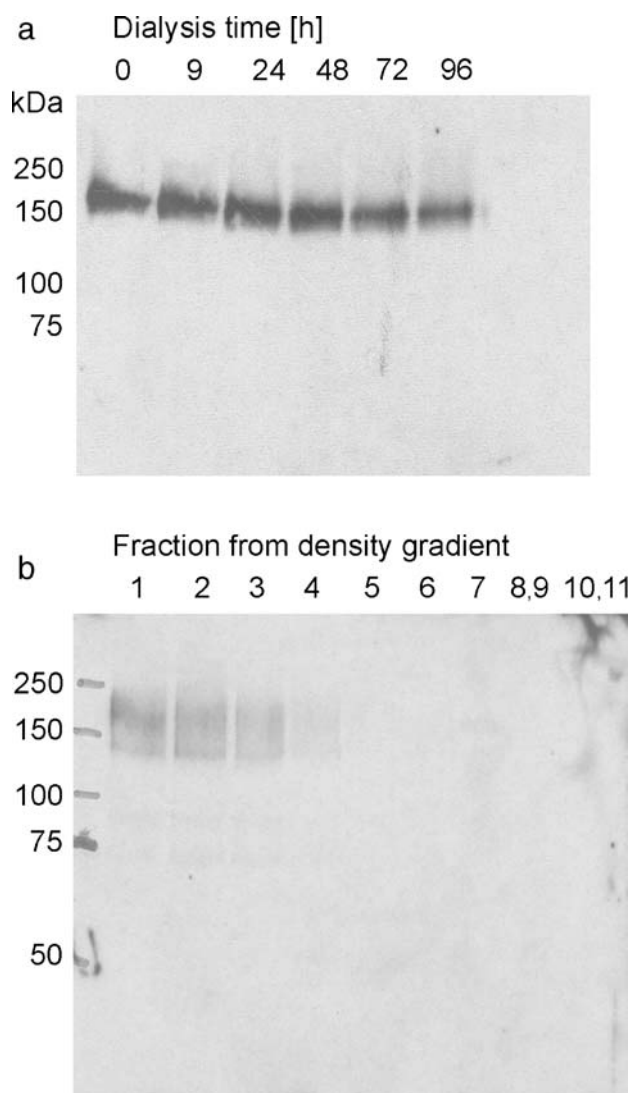
Fig. 1. Confirmation of P-gp reconstitution into proteoliposomes by SDS-PAGE and immunoblotting with the P-gp antibody C219. **a** Samples were taken at the indicated time points during the detergent dialysis. Per slot, 0.08% of the total volume, corresponding to 3.7 μg protein of the final preparation (96 h), was loaded. **b** The final preparation (after 96 h of dialysis) was analyzed by sucrose density centrifugation. Of each fraction (1 to 11, top to bottom) 20 μl were loaded on the gel. Fractions 8 and 9 as well as 10 and 11 were pooled.
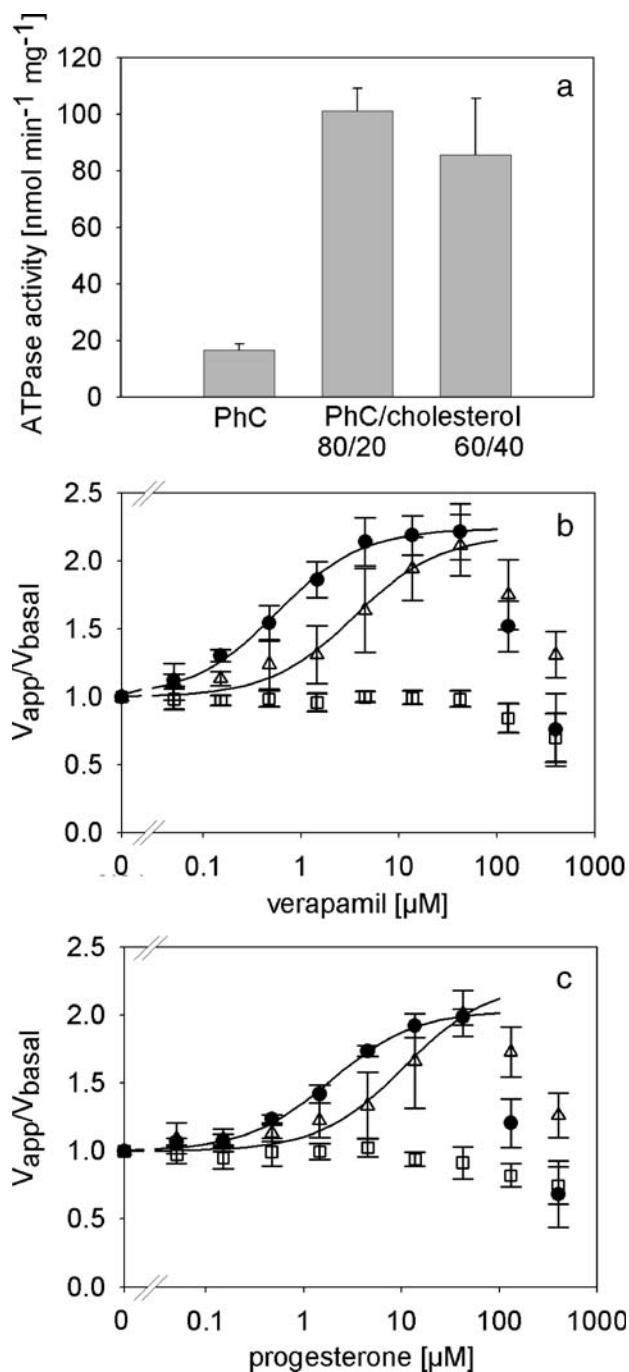
Fig. 2. Influence of cholesterol on the ATPase activity of reconstituted P-gp. The ATPase activities of the P-gp proteoliposomes were determined in the absence (**a**) and the presence of the modulators verapamil (**b**) and progesterone (**c**). (**b**, **c**) PhC proteoliposomes (*squares*); PhC/cholesterol 80/20 (*circles*); PhC/cholesterol 60/40 (*triangles*). The activities shown in **b** and **c** were normalized to the respective basal activities. The concentration-dependent profiles obtained with the PhC/cholesterol proteoliposomes were fitted as described in the text (*solid lines*), the fit parameters are shown in Table II. The symbols are mean values of 6 or 9 activity/ concentration profiles out of 2 or 3 independent experiments. *Error bars* indicate the standard deviations.

ATPase activity of P-gp at concentrations <100 μM but have an inhibitory effect at higher concentrations that can be attributed to unspecific effects on the protein or the membrane (12). The ATPase activities of the cholesterol-free and cholesterol-containing proteoliposomes were determined at verapamil and progesterone concentrations between 50 nM and 400 μM (Fig. 2b, c). For PhC proteoliposomes no activation was detected over the whole concentration range while at concentrations >50 μM the ATPase activity was inhibited by both compounds. In the proteoliposomes containing 20 and 40% cholesterol, respectively, verapamil and progesterone enhanced the ATPase activity at concentrations <100 μM. The maximal activities at 50 μM modulator were about two-fold higher than the respective basal activities. The activity was decreased at concentrations >50 μM. To further analyze the drug induced activity profiles, data were fitted with Eq. 1, excluding the concentrations with the inhibitory effect from the fit. The fits are shown in Fig. 2. The mean ratios of the fitted maximal to basal activities (V'/V_{basal}) and the mean modulator concentrations at half-maximal activation (K) were derived from the fits of the single experiments and are summarized in Table II. The values for K of verapamil and progesterone were dependent on the cholesterol content of the proteoliposomes while V'/V_{basal} was hardly affected. Both drugs were more potent (had lower K values) in inducing the P-gp ATPase activity in proteoliposomes containing 20% cholesterol than with 40% cholesterol. The fitted K of both compounds was about 6 fold lower with 20% than with 40% cholesterol.

Influence of the Detergent CHAPS on the ATPase Activity in Egg PhC Proteoliposomes

Several earlier studies with P-gp reconstituted in PhC (7,22,29) found a drug-induced increase of the ATPase activity in the absence of cholesterol. Our findings are in contrast to these data as the ATPase activity was not affected by the tested modulators in the absence of cholesterol. The differences between our system and published proteoliposomes are the type and removal method of detergents and the content of residual detergents. We therefore tested the influence of CHAPS, a frequently used detergent for P-gp purification and/or reconstitution, on the ATPase activity of egg PhC proteoliposomes prepared by our procedure. The final concentration of 28 μM CHAPS had no significant influence on the basal ATPase activity, however, with verapamil the typical bell-shaped activity/concentration profile was observed (Fig. 3).

Influence of Cholesterol on the Binding of Verapamil and Progesterone to Reconstituted P-gp

To investigate whether cholesterol has an effect on the binding of the modulators to P-gp, we studied the affinity of verapamil and progesterone to cholesterol-free and cholesterol-containing P-gp proteoliposomes by means of equilibrium dialysis at drug concentrations between 10^{-10} and 10^{-4} M as described under "Materials and Methods" section. Figure 4 shows the Scatchard plots of both drugs in proteoliposomes containing 20% cholesterol. The plots revealed two binding classes, one with a high affinity but a low number of interaction sites and a second one with a lower affinity but a higher number of interaction sites. The results were similar for proteoliposomes with 40% cholesterol. The high affinity was assigned to the binding to P-gp and the low affinity interaction to the partitioning into the lipid bilayer. The binding data were fitted with Eq. 2. The respective fit functions are shown in Fig. 4. The good agreement between the experimental data and the fit function corroborates the underlying model, i.e., the co-existence of a relatively strong specific binding with a low number of binding sites and an unspecific affinity with high capacity. The first is interpreted as the binding to P-glycoprotein, the latter as the membrane affinity. The differences between the fitted parameters of the proteoliposomes with 20 and 40% cholesterol were within the experimental error of the single experiments. As the purity of the enriched P-gp protein fractions is not known, no absolute binding constants were calculated.

In order to elucidate the reason for the lack of P-gp activation in the cholesterol-free proteoliposomes, we also studied the binding affinity of verapamil and progesterone to P-gp reconstituted in PhC proteoliposomes. The Scatchard plots shown in Fig. 5 revealed only unspecific binding for both compounds within the tested concentration range (see above). In contrast to the cholesterol-containing proteoliposomes, no increase in binding affinity was detected at the low concentrations. The lack of a distinct slope in the Scatchard plot results from a high number of association sites. The observed affinity was therefore assigned to the partitioning of the drugs into the lipid bilayer. The calculated value of r/c_u was in accordance with the partition coefficients revealed with liposomes (see below). In conclusion, no binding of the drugs to P-gp was detectable in the absence of cholesterol.

Influence of Cholesterol on the Lipid Bilayer Affinity of Verapamil and Progesterone

P-gp is proposed to extract its substrates directly from the inner leaflet of the membrane (2). As the composition of the lipid bilayer has a major influence on the drug-membrane affinity and on the local drug concentration and arrangement inside the membrane, it could affect the drug–P-gp interactions. The influence of cholesterol on the lipid bilayer affinity of verapamil and progesterone was determined with PhC liposomes containing 0, 20 and 40% cholesterol, respectively. The membrane affinity of the compounds was measured by means of equilibrium dialysis and verapamil data were fitted using Equation 4. Figure 6 shows the influence of cholesterol on the lipid bilayer partitioning (D) of verapamil (between pH 5 and 12) and progesterone (between pH 2 and 12), respectively, and on the fitted partition coefficients of the neutral compounds (P^N) and of protonated verapamil (P^I). The P^N of progesterone was decreased 1.8 fold upon incorporation of 20% cholesterol in PhC liposomes. Increasing the cholesterol content to 40% led to a further decrease of P^N by a factor of 1.6. In the case of verapamil, the presence of 20% cholesterol decreased the affinity of the neutral species (P^N) 1.2 fold, increasing the cholesterol to 40 % led to a further decrease by a factor of 2.4. The affinity of the protonated verapamil (P^I) was decreased 1.8 fold by incorporation of 20% cholesterol and 1.4 fold by increasing the amount of cholesterol from 20 to 40%.

Table II. Influence of Cholesterol on the Basal and Drug-induced P-gp ATPase Activity

	V_{basal}[a]	V'[b]		V'/V_{basal}[b]		K[b]	
		Verap	Progest	Verap	Progest	Verap	Progest
PhC	16.6 ± 2.1	–	–	–	–	–	–
PhC:Chol 80:20	101.0 ± 8.1	227.0 ± 14.8	196.7 ± 15.9	2.24 ± 0.02	2.04 ± 0.02	0.56 ± 0.05	1.88 ± 0.12
PhC:Chol 60:40	85.6 ± 20.0	182.1 ± 42.7	175.8 ± 44.1	2.19 ± 0.05	2.24 ± 0.08	3.56 ± 0.49	10.58 ± 1.81

V_{basal} and V' [nmol $\times$ min^{-1} $\times$ mg^{-1}]; K [µM]

Verap, verapamil; Progest, progesterone

[a] Mean values $\pm$ standard deviations ($n \geq 36$); [b] means of fitted values $\pm$ standard deviations from six or nine activity/concentration profiles out of two or three independent experiments each.
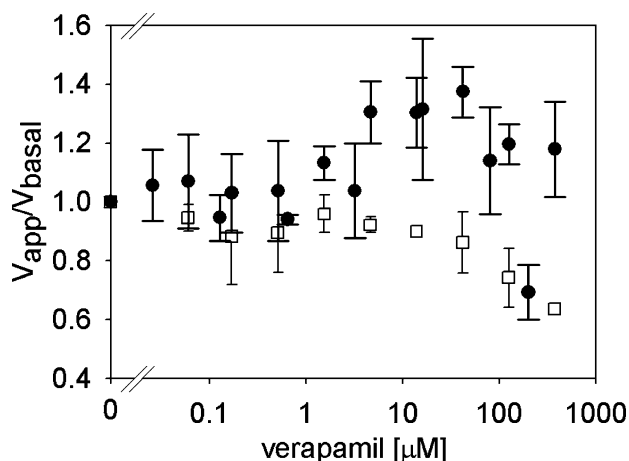
DISCUSSION

The present study was conducted with P-gp proteoliposomes which contained negligible amounts of residual detergents. As P-gp was solubilized and purified with SDS according to Dong *et al.* (33) it can also be expected that no residual lipids from the P-gp source were present. The absence of significant amounts of detergents and source lipids makes this system unique and allows to investigate the influence of the lipid environment and of drug compounds on P-gp functions under well defined conditions.
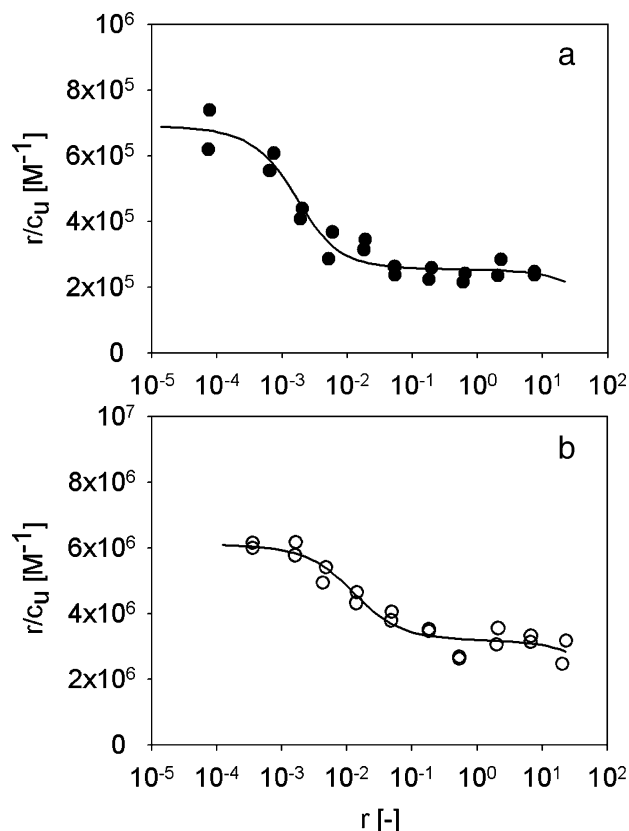
Residual detergents in P-gp proteoliposomes may significantly influence the ATPase activity of the transporter (31). The detergent CHAPS is frequently used for the purification and reconstitution of P-gp. Romsicki and Sharom (42) studied the influence of additional CHAPS on the ATPase activity of P-gp in di-myristoyl PhC (DMPC) proteoliposomes prepared from P-gp/lipid/CHAPS micelles. The residual detergent concentration was <0.02% of the starting concentration, corresponding to a concentration of <40 µM. The addition of more detergent ($\geq$ ~30 µM) led to a significant increase in the ATPase activity. The authors assigned this effect to the permeabilization of the vesicles. However, these data could also disclose the influence of the

detergent on the ATPase activity of the protein. CHAPS restores P-gp ATPase activity even in the absence of lipids (31) and verapamil was able to increase the ATPase activity of P-gp in 0.4 mM of the detergent (32).
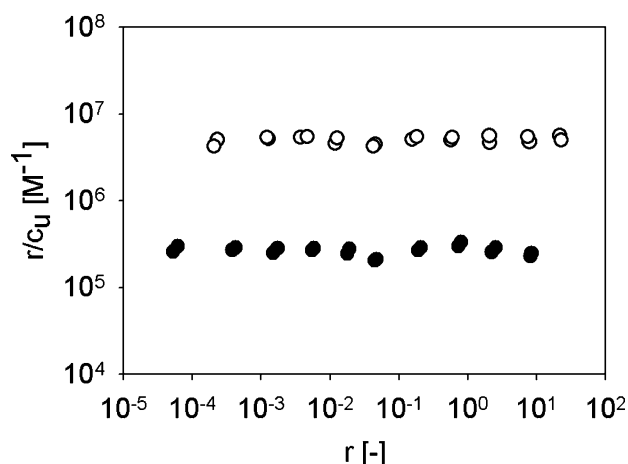
In our egg PhC proteoliposomes the P-gp ATPase activity was not inducible by verapamil and progesterone in the absence of cholesterol. This is in contrast to the findings of other studies with PhC proteoliposomes (7,22,29). However, the incubation with CHAPS restored the verapamil-

Fig. 4. Scatchard plots of verapamil and progesterone binding to P-gp PhC/cholesterol 80/20 proteoliposomes. Verapamil (**a**) and progesterone (**b**) were incubated at various concentrations between 10^{-10} and 10^{-4} M with proteoliposomes (protein concentration between 0.1 and 0.5 mg/ml). Binding was determined by equilibrium dialysis at 37°C. *Solid lines* show the fit functions for two binding classes as described in the text. Results are from one representative experiment each. Proteoliposomes with 40% cholesterol revealed similar Scatchard plots.

Fig. 3. Influence of CHAPS on the ATPase activity of P-gp in egg PhC proteoliposomes at different verapamil concentrations. Circles, after incubation of the proteoliposomes with CHAPS, the final detergent concentration in the assay was 28 µM. *Squares*, influence of verapamil in the absence of detergent. Shown are mean values $\pm$ standard deviations of 4 independent experiments each. The basal activity after CHAPS incubation did not significantly differ from the activity in the absence of CHAPS.

Fig. 5. Scatchard plots for the binding of verapamil and progesterone to P-gp proteoliposomes in the absence of cholesterol. Verapamil (*closed circles*) and progesterone (*open circles*) binding to P-gp PhC proteoliposomes was determined as described in Fig. 4 and in the text. Only unspecific binding, i.e., partitioning into the lipid bilayer, was detected as seen from the concentration-independent r/c_u values. Data are from one representative experiment each.

inducible ATPase activity in the absence of cholesterol in our assay. This indicates that P-gp is potentially functional in egg PhC proteoliposomes. It confirms that the non-inducibility by verapamil and progesterone, respectively, is indeed due to the absence of cholesterol or any other membrane constituent which induces functionality of the ATPase and not due to irreversible inactivation during the purification and reconstitution procedures. It further emphasizes the influence of residual detergent on the ATPase activity of the transporter. The overlapping effect of cholesterol and CHAPS on the function of P-gp, as found in our study, is not surprising, considering the similarity in their molecular structure.

We can not exclude that P-gp is partially denatured in our system due to the contact with the relatively strong detergents SDS and cholate. However, the increase in ATPase activity during dialysis and the typical behavior of the PhC/cholesterol proteoliposomes in terms of ATPase activity and activation by the modulators, indicates that at least a significant part of the transporter is correctly folded. Investigations by Dong et al. (33) and by Naito and Tsuruo (57) have shown before that P-gp which was solubilized with SDS or cholate regains its function after the removal of the detergents.

The tremendous increase in the basal ATPase activity caused by cholesterol is in line with all discussed possible functions of the sterol: (a) It could be transported by P-gp and therefore increase its ATPase activity. (b) Cholesterol could affect the conformation and/or conformational flexibility of P-gp, i.e., freedom to change conformation upon drug or ATP binding, by a direct interaction and therefore enable its functionality as an ATPase without being transported. (c) Cholesterol could indirectly affect the conformation and conformational flexibility of P-gp *via* its influence on the membrane properties, and therefore induce its activity as an ATPase.

The binding to P-gp and the enhancement of the P-gp ATPase activity by the two modulators verapamil and progesterone was only observed in the presence of choles-

terol in the bilayer. This indicates that cholesterol has a direct or indirect effect on the protein conformation, enabling the interaction with the modulators. This effect would be independent of the recognition of cholesterol as a transport substrate by P-gp but would correspond to the above discussed possible functions (b) and (c), respectively, of cholesterol.

Saeki et al. (29) found a similar influence of cholesterol on azidopine binding to a reconstituted P-gp fusion protein. Evidence that the membrane environment affects the efficiency of allosteric conformational changes of the protein was demonstrated by Modok et al. (22). Their data suggest that a raft-like lipid composition enhances the drug transporter activity of P-gp.

Direct binding of cholesterol to P-gp could lead to the above discussed conformational change in the protein (b). A direct interaction of cholesterol with the P-gp site of daunorubicin binding was suggested by Wang et al. (19) based on ATPase assays with membrane vesicles from P-gp overexpressing cells. The authors found a mixed type (noncompetitive) inhibition of the daunorubicin-induced ATPase activity by cholesterol.
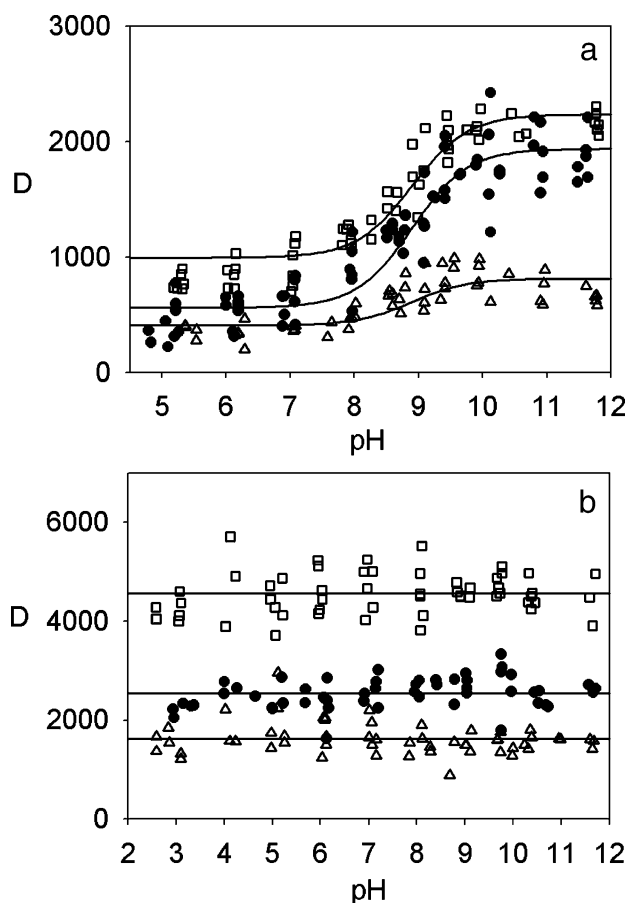
Fig. 6. Influence of cholesterol on the lipid bilayer affinities of verapamil and progesterone. The pH-dependent partitioning of verapamil (**a**) and progesterone (**b**) was determined with PhC (*squares*), PhC/cholesterol 80/20 (*circles*) and PhC/cholesterol 60/40 (*triangles*) liposomes. The *solid lines* show the fit functions as described in the text for (**a**) and mean values for (**b**).

Further evidence of a direct interaction between cholesterol and P-gp was described by Kimura et al. (14). Cholesterol bound to P-gp in the absence of lipids. This could, however, be different within the lipid bilayer, considering the high affinity of the sterol to phospholipids and the interaction between P-gp and phospholipids. Both equilibria are in competition with the binding between cholesterol and P-gp. It is still open how cholesterol distributes between P-gp and the lipid environment.

An indirect effect of cholesterol on the conformation of P-gp (c) could arise from its influence on the bilayer rigidity and structure (21) or on the membrane thickness. The addition of cholesterol to egg PhC bilayers could lead to a favorable hydrophobic matching of the bilayer and the protein, enabling its optimal function as an ATPase. Inclusion of cholesterol to egg PhC bilayers increases the hydrophobic thickness of the membrane from 26 to 33 Å (43,44) which better matches the hydrophobic core of P-gp that has been estimated to be 34 Å (45). A hydrophobic mismatch could lead to a change in the transmembrane orientation of the α-helices, distribution of conformational states or conformational mobility of the transmembrane domains (46). This could affect P-gp activity as the protein undergoes large conformational rearrangements during its transport cycle (47).

Further insight into the effect of cholesterol on P-gp function is given by the comparison of the kinetics in the presence of 20 and 40% cholesterol in the bilayers. In our study, the modulator concentrations at half maximal induction of the ATPase activity (K) are dependent on the membrane content of cholesterol. They are higher with 40% cholesterol than with 20%. The membrane affinities of both modulators are higher with 20% cholesterol than with 40%. Assuming Michaelis-Menten conditions, K is related to the binding affinity of the modulators to P-gp. In this case, cholesterol would influence the binding of the modulators to P-gp. Alternatively, the differences in K could reflect differences in processes such as the release of the modulator after ATP hydrolysis or the interaction of the protein with ATP. In addition, K could be affected by the membrane affinity, if one (or both) of the partitioning rate constants is rate-determining for the binding to the active protein or the release from the binding site. However, increasing the cholesterol content from 20 to 40% had a higher impact on the concentrations K than on the membrane affinities D of the two modulators. This makes it unlikely that the partitioning kinetics are rate-limiting for the P-gp binding kinetics. We would also like to note here, that the membrane affinity must not necessarily be positively related with the P-gp binding affinity. An increase in membrane affinity caused by the lipid composition is at the cost of both equilibria the drug partitioning between the water phase and the membrane as well as the drug partitioning between the protein and the membrane. At equilibrium, the protein binding constant (determined from the protein bound concentration and the aqueous concentration) may be independent of the membrane affinity.

The maximal ATPase activities (V') induced by verapamil and progesterone in the presence of 20 and 40% cholesterol, respectively, were all similar, indicating that the P-gp function and therefore conformation is unchanged comparing the two membrane environments. The fact that the cholesterol content (≥20%) has an influence on K but has no influence on the functionality of P-gp, indicates that cholesterol—in addition to enhancing the basal ATPase activity and rendering the protein sensitive towards the modulators—may indeed interact with the binding sites of the modulators in a competitive manner.

Kimura et al. (14) observed a similar influence of cholesterol on K and V' of verapamil with reconstituted P-gp. Whether K was affected by cholesterol was dependent on the molecular weight of the tested compounds in their study. While cholesterol affected the K of verapamil (mw 455) it had no effect on the K of paclitaxel (mw 854). Their model suggests that cholesterol may fill up the binding site when small compounds, such as verapamil bind to P-gp, but is excluded from the binding site when larger compounds, such as paclitaxel bind. In their system P-gp was activated by the tested compounds in the absence of cholesterol while P-gp was not activated under these conditions in our study. It will be interesting to see whether paclitaxel and other larger compounds can activate P-gp under our conditions in the absence of cholesterol. This would support the model of Kimura et al., i.e., that cholesterol is only involved in the binding and activation by small compounds, such as verapamil.

Comparing the concentrations of cholesterol and modulator within the bilayer at maximal ATPase activation in our study, the model of competitive binding appears realistic. The local modulator concentrations within the membrane at maximal ATPase activation are about 50 mM as estimated from the liposome partition coefficients D ($\sim10^3$) and the aqueous modulator concentrations (50 μM). The local cholesterol concentration within the bilayer is in the range of 260–500 mM (the total lipid concentration within the bilayer is ~1.3 M). Depending on the protein binding affinity of the modulator and of cholesterol, respectively, and on their respective affinities to the membrane lipids—which may compete with the protein binding—a significant competition between cholesterol and the modulator for a P-gp binding site is possible. This model of competitive binding is in agreement with the increased K at the higher cholesterol/lipid ratio.

Romsicki and Sharom (7) found a good correlation between P-gp binding affinities and membrane affinities with vinblastine, verapamil and daunorubicin in egg PhC, DMPC and di-palmitoyl PhC (DPPC) membranes (note that these proteoliposomes do not contain cholesterol but may contain residual CHAPS). In the same work the membrane affinity and P-gp binding affinity, respectively, of verapamil did not appear to affect K in the respective proteoliposomes. In turn, the ratio between the maximal inducible ATPase activity and the basal activity (V'/V_{basal}) was lipid-dependent. These findings could point to an effect of the lipid environment on the protein which is not directly related to the drug binding site, i.e., an effect of incompetitive character. In their study, the lipid environment could affect the P-gp function in dependence of the lipid chain length and order, which determine the thickness and rigidity of the bilayer.

In general, further effects of cholesterol may occur and be relevant for P-gp function: cholesterol may be needed as a substrate for counter transport when drug substrates or modulators are moved from the cytoplasmic to the exoplas-

mic membrane leaflet by P-gp. Cholesterol and the lipid composition in general, could bring P-gp substrates or modulators in a position within the bilayer which is favorable for their recognition by P-gp. An inhomogeneous distribution of the lipids within the bilayer, e.g., an increase of cholesterol in the vicinity of P-gp, could affect the local distribution of drug compound within the membrane. Finally, P-gp is discussed as a phopholipid transporter (23,48) and could therefore have an indirect effect on the distribution of cholesterol between the two membrane leaflets.

Based on our results and on studies cited in this work, one could speculate that cholesterol is transported by P-gp and regulates the P-gp transport (and ATPase) function *via* its content within the bilayer. In the absence of cholesterol, P-gp displays low activity and only in the presence of cholesterol, P-gp gets fully functional and regulates the distribution of the sterol between the cytosolic and exosolic membrane leaflets. In this conformation, P-gp is further activated by the modulators verapamil and progesterone, which compete with cholesterol for the binding to the transporter. Further investigations have to show whether other membrane constituents than cholesterol can activate P-gp and whether other compounds than those we tested can interact with P-gp in the absence of the sterol.

Considering the substantial influence of the membrane environment on P-gp, findings with reconstituted P-gp have to be interpreted in the light of all system components. Detergents and additional adjuvants from the purification and reconstitution procedures can affect the behavior of the transporter, as we have shown for CHAPS. The most frequently used detergents for the purification of P-gp beside CHAPS (26,28,29,32,39,42) are n-octyl β-D-glucopyranoside (octyl glucoside) (27,30,49–53) and n-dodecyl or n-decyl β-D-maltoside (14,22,25,33,51,54–56). Octyl glucoside had activating as well as inhibiting effects on the ATPase activity of CHAPS-solubilized P-gp, depending on the glucoside concentration (39). Alternatively, other detergents such as cholates (57), lyso PhC (58) and some non-ionic detergents (59) were investigated. The influence of most of these detergents on membrane-reconstituted P-gp remains to be investigated. Several systems contain protease inhibitors in addition and preparations of histidine-tagged P-gp generally contain considerable amounts of imidazole. These additional compounds are potential substrates, modulators or inhibitors of P-gp. Their assay concentrations are generally not known and their influence on the P-gp behavior is rarely examined.

The lipid composition of the reconstituted system is crucial for the behavior of the transporter. Depending on the purification technique the protein is delipidated to different degrees. Lipid compositions for reconstitution range from single phospholipids of synthetic or natural origin to lipid mixtures of phospholipids with or without cholesterol.

Beside reconstituted P-gp, membrane vesicles of P-gp-expressing cells are used to investigate P-gp characteristics. Depending on the cell type, the lipid composition can vary strikingly. Membrane vesicles were prepared from mammalian cells (60–64) and from P-gp-transfected insect cells (65,66). In the light of our findings it is interesting to note that plasma membranes from insect cells (Sf9) contain about ten times less cholesterol than plasma membranes from higher eukaryotic cells (67).

In conclusion, cholesterol exerts three effects on P-gp: (a) it increases its basal ATPase activity, (b) it enables P-gp binding and activation by the modulators verapamil and progesterone and (c) it may compete with verapamil and progesterone for the same binding site(s).

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