Biochimica et Biophysica Acta, 1146 (1993) 65–72

BBAMEM 75890

The effects of lipids and detergents on ATPase-active P-glycoprotein

Carl A. Doige [1], Xiaohong Yu and Frances J. Sharom

Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario (Canada)

(Received 13 August 1992)
(Revised manuscript received 16 November 1992)

Key words: P-Glycoprotein; Glycoprotein; Multidrug resistance; ATPase; Detergent; Phospholipid

We previously isolated and characterized a partially purified preparation of ATPase-active P-glycoprotein, the multidrug transporter (Doige, C.A., Yu, X. and Sharom, F.J. (1992) Biochim. Biophys. Acta 1109, 149–160). The effect of various detergents and membrane phospholipids on the ATPase activity of P-glycoprotein has now been investigated. P-Glycoprotein ATPase activity was most stable in CHAPS, with over 50% of the activity retained at a concentration of 8 mM. Octyl glucoside in the low mM range also supported the ATPase, while deoxycholate destroyed all activity at 1 mM. Digitonin and SDS inhibited ATPase activity at very low concentrations. Triton X-100 at 2–10 μM stimulated the ATPase almost 2-fold, while higher levels inhibited activity. Although P-glycoprotein ATPase was sensitive to thermal inactivation, full activity was preserved in the presence of asolectin, but not phosphatidylcholine species. Further studies revealed that asolectin, both saturated and unsaturated phosphatidylethanolamines, and phosphatidylserine, were best able to maintain ATPase activity at 23°C. Saturated phosphatidylethanolamine species activated P-glycoprotein ATPase up to 40% at 23°C, and 80% at 4°C. Following detergent delipidation, various lipids were able to restore P-glycoprotein ATPase activity. Unsaturated phosphatidylcholine and phosphatidylserine were most effective, while saturated species were not able to restore catalytic activity. These results indicate that membrane lipids are necessary for catalytic activity of the ATPase domains of P-glycoprotein. P-Glycoprotein has well-defined lipid preferences, with saturated phosphatidylethanolamines both activating the ATPase and providing protection from thermal inactivation, while fluid lipid mixtures are able to restore activity following delipidation.

Introduction

P-Glycoprotein, the multidrug transporter, is a member of a superfamily of membrane-associated proteins known as 'traffic ATPases' [1]. Members of this class of proteins contain one or two consensus sequences for ATP-binding domains, and are believed to couple ATP hydrolysis to the import or export of various substrates. Examples of traffic ATPases are the bacterial membrane proteins involved in amino acid, sugar and oligopeptide transport [1,2], the yeast STE6 protein [3], the mammalian cystic fibrosis gene product, CFTR [4], and the P-glycoprotein multidrug transporter [5]. While the natural substrates for P-glycoprotein are currently unknown, overexpression of this protein in cell lines grown in vitro confers resistance to several natural product drugs, such as *Vinca* alkaloids and anthracyclines. By analogy to other traffic ATPases, P-glycoprotein is believed to mediate multidrug resistance by acting as an ATP-dependent exporter of drugs to the outside of the cell [6,7].

Although there is substantial evidence for the ATP-dependent transport of drugs mediated by P-glycoprotein [8–12], little is known about the ATPase properties of this transporter. P-glycoprotein ATPase activity was first detected in an immunopurified preparation solubilized in the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [13,14]. The maximum activity was very low (50 nmol $mg^{-1} min^{-1}$), and was stimulated by the addition of verapamil and trifluoperazine (chemosensitizers which

Correspondence to: F.J. Sharom, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

[1] Present address: Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA 94720, USA.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

antagonize multidrug resistance), but not by other drugs in the multidrug resistance spectrum [14].

More recently, we [15] and others [16,17] have detected substantially higher levels of ATPase activity associated with P-glycoprotein. Our approach was to enrich for P-glycoprotein using a rapid CHAPS extraction of multidrug resistant cell plasma membrane, which resulted in a partially purified preparation of P-glycoprotein (30% pure), with a specific activity of around 0.55 μmol mg^{-1} min^{-1} [15]. The ATPase activity of this preparation is due almost entirely to P-glycoprotein, and is substantially free of other membrane ATPases [15]. One of our objectives is to reconstitute ATPase-active P-glycoprotein into defined lipid vesicles, so as to more fully understand its drug transport and ATPase properties. Knowledge of potential interactions of a membrane protein with both detergents and membrane lipids is an essential prerequisite for reconstitution studies. With this in mind, we have investigated the effects of various detergents and phospholipids on the ATPase activity of partially purified P-glycoprotein.

Materials and Methods

CHAPS, sodium deoxycholate, Triton X-100, disodium-ATP, dithioerythritol, dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (PC), and bovine brain phosphatidylserine (PS) were purchased from Sigma (St. Louis, MO). Dipalmitoylphosphatidylethanolamine (DPPE), egg phosphatidylethanolamine (PE) and yeast phosphatidylinositol (PI) were obtained from Serdary Research Laboratories (London, Ontario) and asolectin (30% PC, 31% PI and 30% PE) was obtained from Fluka (Ronkonkoma, NY). Octyl glucoside was purchased from Calbiochem-Behring (La Jolla, CA), digitonin from ICN Biomedicals (St. Laurent, Québec), and sodium dodecyl sulfate (SDS) from Fisher Scientific (Unionville, Ontario, high purity electrophoretic grade). Tissue culture supplies were from Gibco Canada (Burlington, Ontario), and iron-supplemented/defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). Protein was quantitated by a microwell adaptation of the Bradford assay [18] using bovine serum albumin (crystallized and lyophilized, Sigma) as a standard.

Cell culture and plasma membrane preparation

The drug-sensitive parent Chinese hamster ovary cell line (AuxB1) and the multidrug resistant cell line selected for colchicine resistance (CH^RC5) have been described previously [19]. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in α-minimal essential medium supplemented with 10% heat-inactivated bovine calf serum, penicillin (1000 U/ml), streptomycin (1 mg/ml), and 2 mM L-glutamine.

Plasma membrane vesicles were isolated from CH^RC5 cells by a method involving cell disruption by nitrogen cavitation followed by centrifugation on a 35% (w/w) sucrose cushion as described previously [20]. Isolated plasma membrane vesicles were stored at −70°C for no more than 3 months before use.

Isolation of partially purified P-glycoprotein by selective detergent extraction

P-Glycoprotein was partially purified using a selective CHAPS extraction as described previously [15]. Briefly, plasma membrane vesicles from CH^RC5 were thawed and sedimented at 164000 × *g* for 30 min at 4°C. The pellet was resuspended in 100 μl of solubilization buffer A (25 mM CHAPS, 50 mM Tris-HCl, 0.15 M NH_4Cl, 5 mM $MgCl_2$, 1 mM dithioerythritol, 0.02% NaN_3, pH 7.5 at 23°C) to give a final protein concentration of 10 mg/ml, and then incubated for 30 min at 4°C. Insoluble material was collected by ultracentrifugation at 164000 × *g* for 15 min at 4°C. The pellet was resuspended in 1 ml of solubilization buffer B (8 mM CHAPS, 50 mM Tris-HCl, 0.15 M NH_4Cl, 5 mM $MgCl_2$, 1 mM dithioerythritol, 0.02% NaN_3, pH 7.5 at 23°C) to give a final protein concentration of 0.4–0.5 mg/ml, and incubated for 30 min at 4°C. The sample was then pelleted at 15000 × *g* for 15 min at 4°C. The resulting supernatant, which contained partially purified ATPase-active P-glycoprotein (30% by SDS-PAGE analysis), was divided into aliquots and stored at −70°C.

Measurement of P-glycoprotein ATPase activity

The ATPase activity of P-glycoprotein was determined by measuring the release of inorganic phosphate from ATP, using a colorimetric method adapted from Chifflet et al. [21]. Partially purified P-glycoprotein (1.0–1.5 μg) was diluted with assay buffer (50 mM Tris-HCl, 0.15 M NH_4Cl, 5 mM $MgCl_2$, 0.02% NaN_3, pH 7.5 at 23°C) in a 96-well microtitre plate (total volume 90 μl). To initiate the reaction, 10 μl of ATP solution in assay buffer was added, giving a final concentration of 2 mM ATP, and between 0.4 and 0.8 mM CHAPS. After 20 min at 37°C, the reaction was stopped by adding 100 μl of 6% SDS/3% ascorbate/0.5% ammonium molybdate in 0.5 M HCl. Products were stabilized by the addition of 100 μl of 2% sodium citrate/ 2% sodium arsenite/2% acetic acid. Absorbance of each well at 750 nm was measured using an ELISA plate reader. Background absorbances were determined by performing the assay with heat-inactivated P-glycoprotein. These values did not exceed 10% of the total activity, and were subtracted from the experimental values. Data are presented as the means ± S.E. for triplicate determinations.

Preparation of phospholipids

Stock solutions of phospholipids were prepared in $CHCl_3$-MeOH and stored at −20°C. Lipids were dispensed into glass tubes when required, mixed if necessary, dried under a stream of nitrogen, and then pumped under vacuum for 45 min. The lipids were then suspended in assay buffer and mixed with small diameter glass beads using a vortexer until homogenous. Lipids were freshly prepared prior to each experiment.

Results

Effect of detergents on P-glycoprotein ATPase activity

Reconstitution of solubilized P-glycoprotein into lipid bilayer systems requires exposure of the protein to detergents for extended time periods, and thus some information on the stability of P-glycoprotein in various detergents is needed before such studies can be undertaken. Several commonly used detergents were tested for their effects on P-glycoprotein ATPase activity (Figs. 1A and B). All experimental samples (except those of CHAPS) contained a basal level of 0.4 mM CHAPS in addition to the indicated detergent concentration. The data for CHAPS (Fig. 1A) show the final total concentration of CHAPS in the assay mixture. Octyl glucoside produced a small activation of the ATPase activity at 0.25–0.5 mM, but inhibited the activity at higher concentrations, with 50% inhibition at around 4 mM. Complete inhibition of catalytic activity occurred at 10 mM octyl glucoside (Fig. 1A). Similarly, deoxycholate stimulated the ATPase activity slightly at 0.1–0.25 mM, but produced complete inhibition at 2.5 mM (Fig. 1A). Digitonin and SDS inhibited the ATPase activity at very low concentrations, with complete inhibition occurring at 100 μM (Fig. 1B). In contrast, Triton X-100 had a biphasic effect on ATPase activity, with an almost two-fold stimulation observed at low concentrations (2–10 μM). Higher Triton X-100 concentrations caused progressive loss of activity, with > 80% inhibition at 100 μM (Fig. 1B). P-Glycoprotein ATPase activity was most stable in CHAPS, with 50% inhibition occurring at the relatively high concentration of 8 mM (Fig. 1A). The CHAPS concentrations typically used in ATPase assays (0.4–0.8 mM), showed the maximal ATPase activity achievable in solutions of CHAPS alone (Fig. 1B). A further decrease in the CHAPS concentration to as low as 0.1–0.2 mM did not lead to any additional increase in ATPase activity (data not shown). It should be noted that P-glycoprotein stored in solubilization buffer B (8 mM CHAPS) was stable for several months at −70°C, and lost only 50% of its ATPase activity after 12 days at 4°C. In addition, P-glycoprotein in 8 mM CHAPS required six freeze-thaw cycles to abolish its ATPase activity, while P-glycoprotein that had been dialyzed into 10 μM Triton

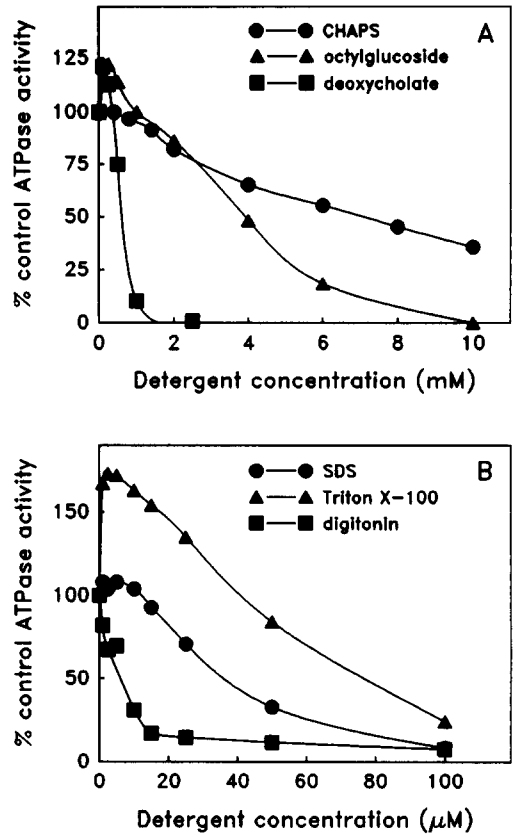

Fig. 1. Effect of various detergents on P-glycoprotein ATPase activity. ATPase activity of partially purified P-glycoprotein (1–2 μg in 100 μl) was determined in the presence of varying concentrations of (A) CHAPS (●), octyl glucoside (▲), and deoxycholate (■), and (B) SDS (●), Triton X-100 (▲), and digitonin (■). The CHAPS concentrations shown represent the total concentration present in the assay mixture. Other samples contained 0.4 mM CHAPS in addition to the indicated detergent concentration. ATP hydrolysis is presented as % of control ATPase activity (mean ± S.E., $n = 3$) measured in the presence of 0.4 mM CHAPS alone, which ranged from 0.305 ± 0.018 to 0.435 ± 0.023 μmol min^{-1} mg^{-1}.

X-100 (with comparable catalytic activity) lost its activity completely after only three freeze-thaw cycles. Thus CHAPS appears to be the detergent of choice for isolation and storage of functional ATPase-active P-glycoprotein. This detergent has the added advantage of being readily removed by dialysis, and is useful for reconstitution purposes.

To test whether the P-glycoprotein ATPase remains active in very low concentrations of CHAPS, we dialyzed the P-glycoprotein preparation against several changes of assay buffer for 24 h at 4°C. This treatment was sufficient to remove greater than 99% of the detergent (as indicated by absorbance at 210 nm), yet it resulted in only a 10–20% loss of activity relative to an undialyzed control. The remaining ATPase activity was relatively stable at 4°C, with only 20% loss of the activity after 6 days.

Ability of phospholipids to protect P-glycoprotein from inactivation

The fact that substantial ATPase activity was retained after removal of most of the CHAPS suggests that P-glycoprotein contains tightly bound annular lipids, which are sufficient to maintain high levels of ATPase activity even in the absence of added detergent. Since we are currently unable to obtain large quantities of purified P-glycoprotein from which bound lipids could be directly extracted and analyzed, we tested the lipid preferences of the P-glycoprotein ATPase by two indirect approaches. One approach stems from the observation that a substantial loss of ATPase activity occurred if the P-glycoprotein preparation was incubated for 1 h at 23°C in the absence of ATP, at 10–15 μg/ml protein and 0.4 mM CHAPS. We therefore tested the ability of several phospholipids for their ability to protect P-glycoprotein from this thermal inactivation.

Fig. 2 shows the rate of ATPase inactivation over 2 h in the absence and presence of 0.5 mg/ml of various phospholipids. While none of the PC lipids was effective in protecting the P-glycoprotein ATPase activity, asolectin maintained 100% of the activity over the entire incubation period. Since asolectin consists of roughly equal proportions of PC, PE, and PI, we tested these and other phospholipids (PS) at different concentrations for their effectiveness in protecting the ATPase activity during an incubation of 1 h at 23°C. Fig. 3A shows that concentrations of asolectin as low as 0.25 mg/ml provided complete protection of the P-glycoprotein ATPase activity, whereas neither DMPC,

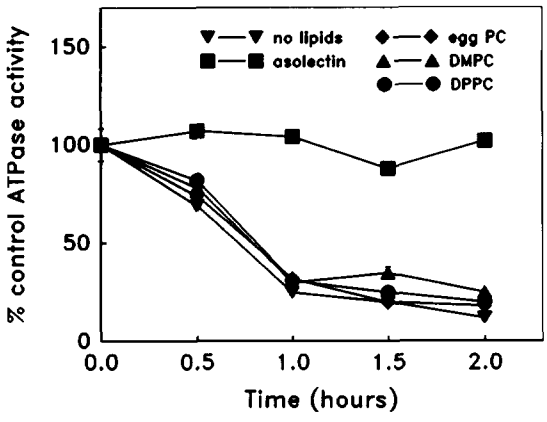

Fig. 2. Effect of various phospholipids on the thermal inactivation of P-glycoprotein ATPase activity. Samples containing partially purified P-glycoprotein (1–2 μg in 100 μl) and 0.4 mM CHAPS in assay buffer, were preincubated for the indicated times at 23°C either alone (▼) or in the presence of one of the following phospholipids at a concentration of 0.5 mg/ml: asolectin (■), egg PC (♦), DMPC (▲), or DPPC (●). The assay was initiated by the addition of ATP as described in Materials and Methods. ATP hydrolysis is presented as % of the control ATPase activity assayed without prior preincubation (means ± S.E., $n = 3$), which ranged from 0.343 ± 0.028 to 0.353 ± 0.009 μmol min^{-1} mg^{-1}.

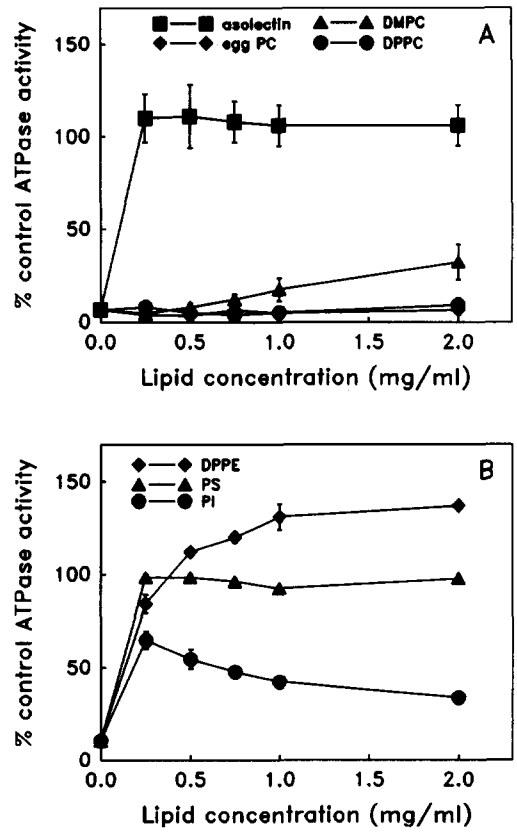

Fig. 3. Concentration dependence of various phospholipids in the prevention of thermal inactivation of P-glycoprotein ATPase activity. Samples containing partially purified P-glycoprotein (1–2 μg in 100 μl) and 0.4 mM CHAPS in assay buffer were preincubated for 1 h at 23°C with various concentrations of the following phospholipids: (A) asolectin (■), egg PC (♦), DMPC (▲), and DPPC (●), and (B) DPPE (♦), PS (▲) and PI (●). The ATPase assay was initiated by the addition of ATP as described in Materials and Methods. ATP hydrolysis is presented as % of the control ATPase activity assayed without prior preincubation (means ± S.E., $n = 3$), which ranged from 0.347 ± 0.032 to 0.453 ± 0.019 μmol min^{-1} mg^{-1}.

DPPC nor egg PC in the concentration range 0.25–2.0 mg/ml supported any substantial catalytic activity. As indicated in Fig. 3B, PI provided only modest protection of the ATPase activity, with the greatest effect occurring at lower concentrations. By contrast, at a concentration of 0.25 mg/ml or greater, PS completely maintained the P-glycoprotein ATPase activity, while DPPE, at a concentration of 1–2 mg/ml, significantly stimulated the ATPase (around 40% over the untreated control, see Fig. 3B). Fig. 4 summarizes the data for the protective effect of various lipids (1 mg/ml) during a 1 h preincubation at 23°C. Notably, in addition to DPPE, two other species of PE, DMPE and egg PE, also supported P-glycoprotein ATPase activity. When the preincubation was conducted at 4°C (a condition where no enzyme inactivation was observed over several hours), DMPE and DPPE at 2 mg/ml substantially activated the ATPase (183% and 175% of control, respectively), while egg PE and PC species had no

effect, and PS and PI caused some inactivation (77% and 37% of control, respectively).

Taken together, these results suggest that membrane phospholipids are essential for stability of the ATPase domains of P-glycoprotein, and that PE may be the most suitable species for maintaining P-glycoprotein ATPase activity during reconstitution. A significant disadvantage, however, to using this lipid for reconstitution studies is that, depending on buffer conditions and the length and the degree of unsaturation of the fatty acyl chains, PE prefers to form the H_{II} hexagonal phase, rather than a lamellar bilayer [22]. H_{II} phase lipids form cylinders that are unsuitable for measurement of vectorial processes such as transport. To counter this problem, one can include bilayer-forming lipids (such as PC) with hexagonal phase-preferring lipids like PE, to stabilize bilayer formation. We therefore tested several combinations of egg PC and DPPE for their ability to stabilize the P-glycoprotein ATPase activity. As shown in Fig. 5, ratios of PC/PE of 2:1 and 1:1 at concentrations of 1–2 mg/ml were adequate to maintain 100% of the P-glycoprotein ATPase activity, whereas a ratio of 1:2 produces a stimulation of a similar magnitude to that found for DPPE alone (compare Fig. 3B and Fig. 5).

Restoration of P-glycoprotein ATPase activity by phospholipids

The lipid specificity of a number of membrane bound enzymes, including the Ca^{2+}-ATPase [23,24], the Na^+,K^+-ATPase [25], the membrane ATPases from oat roots and yeast [26], and cytochrome-*c* oxidase [27], has been determined using detergent-induced delipidation with cholate, deoxycholate, or Triton X-100. Detergent delipidation results in loss of enzymatic activity, which

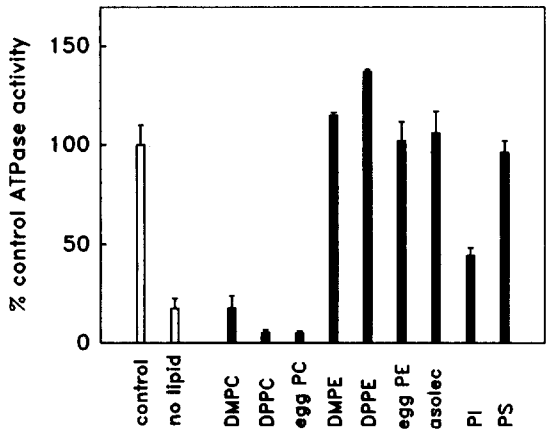

Fig. 4. Relative efficiency of various phospholipids in preventing thermal inactivation of P-glycoprotein ATPase activity. Samples containing partially purified P-glycoprotein (1–2 μg in 100 μl) and 0.4 mM CHAPS in assay buffer were preincubated for 1 h at 23°C either alone, or in the presence of the indicated lipids at a concentration of 1 mg/ml. ATP hydrolysis is presented as % of the control ATPase activity assayed without prior preincubation (means ± S.E., $n = 3$), which ranged from 0.314 ± 0.032 to 0.473 ± 0.019 μmol min^{-1} mg^{-1}.

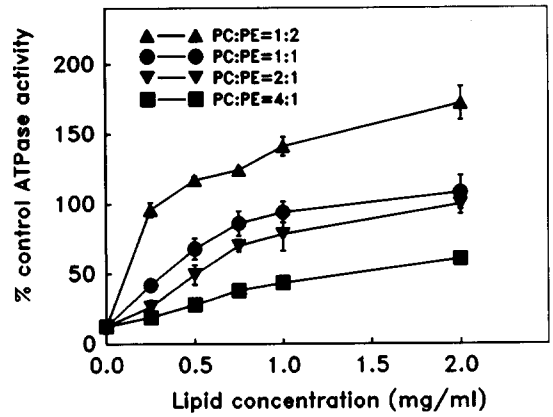

Fig. 5. Ability of various mixtures of DPPE and egg PC to prevent the thermal inactivation of P-glycoprotein ATPase activity. Samples containing partially purified P-glycoprotein (1–2 μg in 100 μl) and 0.4 mM CHAPS in assay buffer were preincubated for 1 h at 23°C with different mixtures of egg PC and DPPE. The lipid concentration represents the sum of the concentrations of the individual lipids. ATP hydrolysis is presented as % of the control ATPase activity assayed without prior preincubation (means ± S.E., $n = 3$), which was equivalent to 0.473 ± 0.005 μmol min^{-1} mg^{-1}.

can be restored by the re-addition of specific phospholipids. As was previously shown in Figs. 1A and B, both deoxycholate and Triton X-100 are potent inactivators of the P-glycoprotein ATPase activity, most likely through their ability to remove tightly-associated boundary lipids. Therefore, our second approach to studying the lipid specificity of the P-glycoprotein ATPase was to test the ability of various phospholipids to restore catalytic activity after delipidation by either Triton X-100 or deoxycholate. This approach is similar to one used previously to determine the lipid specificity of *Neurospora crassa* plasma membrane ATPase [28].

Figs. 6A and B summarize the results from experiments of this kind. Although, in general, phospholipids were more effective at restoring the ATPase activity of P-glycoprotein that had been delipidated by Triton X-100, the lipid specificity was similar regardless of the detergent used. Highly fluid natural lipid mixtures containing PC (egg PC, asolectin) were the most effective, followed by natural mixtures of PE, PS, and PI (Figs. 6A and B). Egg PE, PS, and PI provided significant restoration of activity (around 50%) following Triton X-100-induced inactivation (Fig. 6B), while these same lipids and DPPC produced only modest activity after deoxycholate inactivation (Fig. 6A). Within the PC series, the ability to restore activity decreased with decreasing fluidity (egg PC > DMPC > DPPC). Saturated species of PE were ineffective at restoring ATPase activity.

Discussion

CHAPS was previously reported to be the best detergent for maintenance of the native ATPase activity

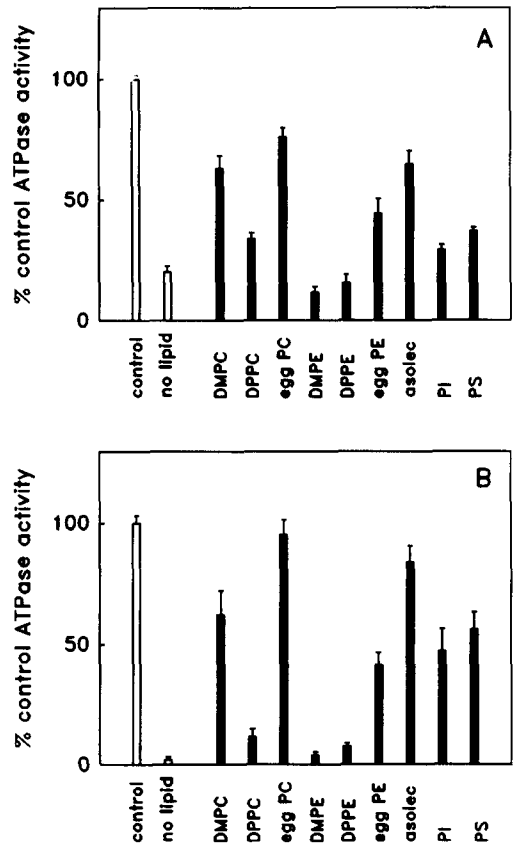

Fig. 6. Ability of various phospholipids to restore P-glycoprotein ATPase activity after inactivation by delipidation with (A) deoxycholate or (B) Triton X-100. Samples containing partially purified P-glycoprotein (1–2 μg in 100 μl) and 0.4 mM CHAPS in assay buffer were preincubated for 10 min at 4°C with either 200 μM Triton X-100 or 1 mM deoxycholate. Phospholipids were then added, where indicated, to a final concentration of 1 mg/ml. The assay was then initiated by the addition of ATP as described in Materials and Methods. ATP hydrolysis is presented as % of the ATPase control assayed without detergent or lipid treatment (mean ± S.E., $n = 3$), which ranged from 0.484 ± 0.008 to 0.504 ± 0.016 μmol min^{-1} mg^{-1}.

of P-glycoprotein [13–15]. We therefore tested the effect of various concentrations of CHAPS on partially purified P-glycoprotein ATPase activity, to determine the concentration range compatible with high levels of activity. Since concentrations of CHAPS in the range 0.4–0.8 mM gave maximal ATPase activity, these were routinely used in subsequent assays. These concentrations are lower than those reported by Hamada and Tsuruo [12], who found optimal activity for an immunoprecipitated P-glycoprotein-antibody complex at 1.7–3.4 mM CHAPS. These differences may result from restricted accessibility of the Sepharose-immobilized protein to molecules in the surrounding environment.

Recently, Sarkadi et al. detected high-level drug-dependent ATPase activity in the plasma membranes of Sf9 insect cells transfected with *mdr1* cDNA [16]. By taking into account that P-glycoprotein constitutes 3% of the insect cell membrane protein, these researchers estimated that the maximum P-glycoprotein ATPase activity in the native membrane is about 3–5 μmol min^{-1} mg^{-1}. In addition, they also reported that treatment of their membranes with 1.6 mM CHAPS completely obliterated the drug-dependent P-glycoprotein ATPase activity. The maximum specific activity of our partially purified preparation of P-glycoprotein (about 30% pure by SDS-PAGE) was previously determined to be around 0.55 μmol min^{-1} mg^{-1} [15]. Assuming no losses in activity, complete purification would result in a maximum specific activity of approximately 1.7 μmol min^{-1} mg^{-1}. Since this represents about 50% of the maximum activity estimated by Sarkadi et al. [16], we conclude that CHAPS treatment during partial purification of P-glycoprotein (25 mM in the first step and 8 mM in the second) does not result in a substantial overall loss of catalytic activity. It does, however, result in partial uncoupling of the ATPase activity from drug binding [15].

There are several possible reasons for the lower sensitivity of our P-glycoprotein preparation to CHAPS inactivation, compared to that of Sarkadi et al. First, the recombinant protein, as expressed in these cells, is severely under-glycosylated, with a molecular mass of 130 kDa [16], compared to 170–180 kDa for the fully glycosylated form. Glycosylation of proteins is known to play a role in their conformation, stability, and susceptibility to proteolysis and inactivation by adverse conditions [29,30]. It seems likely that the absence of the large amount of carbohydrate normally found on P-glycoprotein could alter the protein conformation, and thus its sensitivity to inactivation by detergents. Second, the recombinant protein is a human *mdr1* gene product, while the P-glycoprotein in the present study results from expression of both the hamster *pgp1* and *pgp2* gene products [31]. Lastly, the recombinant P-glycoprotein is expressed in a non-mammalian membrane, which could potentially have a very different lipid composition from that of the Chinese hamster ovary cell membrane from which our P-glycoprotein was isolated. The results of the present study suggest that the sensitivity of P-glycoprotein to detergents will depend on the lipids present in its immediate environment.

Another detergent that might prove suitable for solubilizing ATPase-active P-glycoprotein is octyl glucoside. All other detergents tested in this study completely inactivated the catalytic activity at concentrations in the sub-millimolar range. Since ATPase activity could be fully restored by the addition of specific phospholipids (Fig. 6), it seems likely that inactivation results from detergent-induced delipidation, rather than a direct inhibitory interaction of the detergents with P-glycoprotein. One interesting feature of the interaction of Triton X-100 with P-glycoprotein is the almost 2-fold stimulation of catalytic activity observed in the 2–8 μM concentration range. Other membrane-

bound enzymes have also been reported to be activated by amphiphiles [32–34]. Multidrug resistance spectrum drugs (e.g., verapamil) also show similar biphasic stimulation and inhibition of ATPase activity [15]. The multidrug resistant cells used in this work displayed collateral sensitivity to Triton X-100. We have recently shown that Triton X-100 blocks azidopine photoaffinity labelling of P-glycoprotein in the 2–8 μM concentration range (Loe, D.W. and Sharom, F.J., submitted), and it is possible that this particular nonionic detergent interacts with P-glycoprotein in a specific fashion.

Like other membrane-bound ATPases (e.g., Ca^{2+}- and Na^+,K^+-ATPases), P-glycoprotein appears to require phospholipids for ATPase activity. Our results are consistent with the findings of Shimabuku et al. [17], who showed that a P-glycoprotein β-galactosidase fusion protein required the presence of phospholipids for optimal ATPase activity. The maximum ATPase activity of their immunopurified fusion protein in the presence of lipids was 180 nmol min^{-1} mg^{-1}, approximately 10-fold lower than the activity of the P-glycoprotein preparation in the present study. The requirement for lipids suggests that stabilization of the hydrophobic transmembrane domains of P-glycoprotein is necessary for ATP hydrolysis at the nucleotide binding domains, which are usually thought of as being located in the cytoplasmic compartment. However, an alternative possibility is that the ATP binding domains in fact interact with the membrane, so that lipids are essential for their integrity and activity. In this regard, Ames has proposed that the ATP-binding subunits (hisP) of the histidine permease complex (a prokaryotic traffic ATPase) span the cytoplasmic membrane [35,36]. The results presented in this work indicate that the P-glycoprotein ATP-binding domains might interact more closely with the lipid bilayer of the membrane than has been previously envisaged.

Using a P-glycoprotein preparation at 10–15 μg/ml, most lipids showed maximum stimulation at 0.5 mg/ml, which represents a 33–50-fold excess of lipid. The β-galactosidase fusion protein also showed maximal stimulation at a similar lipid concentration (0.83 mg/ml), although the overall lipid/protein ratio was around 1600:1 [17]. Since the lipids were added as multilamellar liposomes in both cases, it is not possible to draw any conclusions about the stoichiometry of the P-glycoprotein–lipid interaction.

Many membrane proteins have preferences for specific membrane lipids. Specificity is often directed towards the polar headgroup, although preference for particular acyl chain lengths and unsaturation has been observed for the *Schizosaccharomyces pombe* ATPase [37]. Different lipid specificities may be obtained, depending on whether a non-vectorial or a vectorial process is being measured. For example, ATP hydrolysis by the Ca^{2+}-ATPase shows a preference for PC [23, 24], while Ca^{2+} transport has a strict requirement for PE [23,37]. In this study, lipids were able to both prevent thermal inactivation of P-glycoprotein, and restore catalytic activity following detergent delipidation. However, the profile of preferred lipids was distinctly different in each case. PE species, especially those with saturated acyl chains, were best able to protect P-glycoprotein ATPase activity from inactivation, and in the case of DPPE and DMPE, resulted in substantial activation. PS was also effective, and PI less so, while PC species were completely unable to protect the ATPase activity. PE:PC mixtures were also excellent at maintaining activity, and may be useful for reconstitution purposes. It is interesting to note that PE is often located exclusively in the inner leaflet of the plasma membrane of mammalian cells [39], where it would be well-positioned to interact with the ATP binding domains of P-glycoprotein. It should also be noted that the headgroups of PE, PC, and PS are closely related structurally; choline is *N,N,N*-trimethylethanolamine, and serine is 2-carboxy-ethanolamine. Despite these structural similarities, the different phospholipid headgroups had strikingly different effects on the ATPase function of P-glycoprotein.

A different group of lipids was able to restore catalytic activity following detergent delipidation. This profile is likely representative of the tightly bound annular lipids preferred by P-glycoprotein. In this case, there appeared to be a strong correlation between lipid fluidity and the ability to restore activity; those lipids with low T_m values were highly effective, those with high T_m values were ineffective. Thus the preferred headgroups for restoration of activity are PC > PE > PS > PI, but P-glycoprotein shows a strong preference for short or unsaturated fluid lipids over longer, saturated species. The lipid preferences are known for various membrane ATPases. Some enzymes (e.g., the Na^+,K^+-ATPase [25], and the H^+-ATPases from *Neurospora crassa* [40] and *Saccharomyces cerevisiae* [26]) are most active in the presence of acidic phospholipids, while others (e.g., the Ca^{2+}-ATPase [23,24] and the ATPase from oat roots [26]), prefer PC, and to a lesser extent, PE. The P-glycoprotein ATPase resembles the latter two proteins.

The differences in the lipids required by P-glycoprotein for thermal stability and restoration of activity following detergent delipidation may arise for several reasons. First, tightly bound endogenous lipids are present in the P-glycoprotein used for thermal denaturation studies, while the bulk of these lipids are likely stripped off by detergent treatment. Second, different mixtures and concentrations of detergents are present in each case. Lastly, other membrane proteins are present in the preparation, and may modify the behaviour of P-glycoprotein. The information arising from the present investigation gives some insight into the

interaction of P-glycoprotein with membrane lipids, and should prove useful in reconstitution studies, which are ongoing in our laboratory.

Acknowledgements

The authors would like to thank Dr. Victor Ling, of the Ontario Cancer Institute, for providing the multidrug-resistant cell lines used in this study. This research was supported by a grant from the National Cancer Institute of Canada. C.A.D. was supported by a graduate student scholarship from the Natural Sciences and Engineering Research Council of Canada.

References

1 Ames, G.F.-L., Mimura, C. and Shyamala, V. (1990) FEMS Microbiol. Rev. 75, 429–446.
2 Higgins, C.F., Hyde, S.C., Mimmack, M.M., Gileadi, U., Gill, D.R. and Gallagher, M.P. (1990) J. Bioenerg. Biomembr. 22, 571–592.
3 Kuchler, K. and Thorner, J. (1990) Curr. Opin. Cell Biol. 2, 617–624.
4 Riordan, J.R., Rommens, J.M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S. and Tsui, L.-C. (1989) Science 245, 1066–1073.
5 Juranka, P.F., Zastawny, R.L. and Ling, V. (1989) FASEB J. 3, 2583–2592.
6 Kane, S.E., Pastan, I. and Gottesman, M.M. (1990) J. Bioenerg. Biomembr. 22, 593–618.
7 Pastan, I. and Gottesman, M.M. (1991) Annu. Rev. Med. 42, 277–286.
8 Doige, C.A. and Sharom, F.J. (1992) Biochim. Biophys. Acta 1109, 161–171.
9 Horio, M., Lovelace, E., Pastan, I. and Gottesman, M.M. (1991) Biochim. Biophys. Acta 1061, 106–110.
10 Tamai, I. and Safa, A.R. (1990) J. Biol. Chem. 265, 16509–16513.
11 Kamimoto, Y., Gatmaitan, Z., Hsu, J. and Arias, I.M. (1989) J. Biol. Chem. 264, 11693–11698.
12 Horio, M., Gottesman, M.M. and Pastan, I. (1988) Proc. Natl. Acad. Sci. USA 85, 3580–3584.
13 Hamada, H. and Tsuruo, T. (1988) J. Biol. Chem. 263, 1454–1458.
14 Hamada, H. and Tsuruo, T. (1988) Cancer Res. 48, 4926–4932.
15 Doige, C.A., Yu, X. and Sharom, F.J. (1992) Biochim. Biophys. Acta 1109, 149–160.
16 Sarkadi, B., Price, E.M., Boucher, R.C., Germann, U.A. and Scarborough, G.A. (1992) J. Biol. Chem. 267, 4854–4858.
17 Shimabuku, A.M., Nishimoto, T., Ueda, K. and Komano, T. (1992) J. Biol. Chem. 267, 4308–4311.
18 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
19 Kartner, N., Evernden-Porelle, D., Bradley, G. and Ling, V. (1985) Nature 316, 820–823.
20 Doige, C.A. and Sharom, F.J. (1991) Protein Express. Purif. 2, 256–265.
21 Chifflet, S., Torriglia, A., Chiesa, R. and Tolosa, S. (1988) Anal. Biochem. 168, 1–4.
22 Cornelius, F. (1991) Biochim. Biophys. Acta 1071, 19–66.
23 Knowles, A.F., Eytan, E. and Racker E. (1976) J. Biol. Chem. 251, 5161–5165.
24 Bennet, J.P., Smith, G.A., Houslay, M.D., Hesketh, T.R., Metcalfe, J.C. and Warren, G.B. (1978) Biochim. Biophys. Acta 513, 310–320.
25 Kimelberg, H.K. and Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 282, 277–292.
26 Serrano, R., Montesinos, C. and Sanchez, J. (1988) Plant Sci. 56, 117–122.
27 Robinson, N.C., Strey, F. and Talbert, L. (1980) Biochemistry 19, 3656–3661.
28 Scarborough, G.A. (1977) Arch. Biochem. Biophys. 180, 384–393.
29 Elbein, A.D. (1991) Trends in Biotech. 9, 346–352.
30 West, C.M. (1986) Mol. Cell. Biochem. 72, 3–20.
31 Endicott, J.A., Juranka, P.F., Sarangi, F., Gerlach, J.H., Deuchars, K.L. and Ling, V. (1987) Mol. Cell. Biol. 7, 4075–4081.
32 Bruni, A., Van Dijck, P.W.M. and De Gier, J. (1975) Biochim. Biophys. Acta 406, 315–328.
33 Grover, A.K., Slotboom, A.J., De Haas, G.H. and Hammes, G.G. (1975) J. Biol. Chem. 250, 31–38.
34 Gazzotti, P., Bock, H.-G. and Fleischer, S. (1975) J. Biol. Chem. 250, 5782–5790.
35 Kerppola, R.E., Shymala, V.K., Klebba, P. and Ames, G.F.-L. (1991) J. Biol. Chem. 266, 9857–9865.
36 Ames, G.F.-L., Mimura, C.S., Holbrook, S.R. and Shymala, V. (1992) Adv. Enzymol. 65, 1–47.
37 Dufour, J.-P. and Goffeau, A. (1980) J. Biol. Chem. 255, 10591–10598.
38 Gould, G.W., McWhirter, J.M., East, J.M. and Lee, A.G. (1987) Biochim. Biophys. Acta 904, 36–44.
39 Benga, G. and Holmes, R.P. (1984) Prog. Biophys. Mol. Biol. 43, 195–257.
40 Sandermann, H. (1978) Biochim. Biophys. Acta 515, 209–237.