Multidrug Resistance (MDR1) P-glycoprotein Enhances Esterification of Plasma Membrane Cholesterol*

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Class I P-glycoproteins (Pgps) confer multidrug resistance in tumors, but the physiologic function of Pgp in normal tissues remains uncertain. In cells derived from tissues that normally express Pgp, recent data suggest a possible role for Pgp in cholesterol trafficking from the plasma membrane to the endoplasmic reticulum. We investigated the esterification of plasma membrane cholesterol under basal conditions and in response to sphingomyelinase treatment in transfected and drug-selected cell lines expressing differing amounts of functional class I Pgp. Compared with parental NIH 3T3 fibroblasts, cells transfected with human multidrug resistance (MDR1) Pgp esterified more cholesterol both without and with sphingomyelinase. Esterification also was greater in drug-selected Dox 6 myeloma cells than parental 8226 cells, which express low and non-immuno-detectable amounts of Pgp, respectively. However, no differences in total plasma membrane cholesterol were detected. Transfection of fibroblasts with the multidrug resistance-associated protein (MRP) did not alter esterification, showing that cholesterol trafficking was not generally affected by ATP-binding cassette transporters. Steroidal (progesterone, dehydroepiandrosterone) and non-steroidal antagonists (verapamil, PSC 833, LY335979, and GF120918) were evaluated for effects on both cholesterol trafficking and the net content of 99mTc-Sestamibi, a reporter of drug transport activity mediated by Pgp. In Pgp-expressing cells treated with nonselective and selective inhibitors, both the kinetics and efficacy of inhibition of cholesterol esterification differed from the antagonism of drug transport mediated by Pgp. Thus, although the data show that greater expression of class I Pgp within a given cell type is associated with enhanced esterification of plasma membrane cholesterol in support of a physiologic function for Pgp in facilitating cholesterol trafficking, the molecular mechanism is dissociated from the conventional drug transport activity of Pgp.

P-glycoproteins (Pgp)1 are 140–180-kDa plasma membrane glycoproteins that initially were identified because some members of this family confer multidrug resistance (MDR) in tumor cells (1, 2). Mammalian Pgp are encoded by a small gene family, consisting, for example, of two members in humans (MDR1 and MDR2) and three members in mice (mdr1a, mdr1b, and mdr2). Class I Pgp (MDR1, mdr1a, and mdr1b) decrease intracellular concentrations of a wide variety of structurally diverse chemotherapeutic agents, resulting in MDR, whereas the closely related class II Pgp (MDR2, mdr2) are not associated with resistance to drugs (3).

Although class I Pgp are expressed normally in many different tissues (4, 5), the physiologic function(s) and molecular mechanisms of the protein remain under active investigation. Based on expression in epithelia of the intestine, kidney, liver, and endothelial cells of the blood-brain barrier, a role for Pgp in protection from xenobiotics has been proposed. Such a function is supported by studies with mice that have had homozygous disruption of both class I Pgp (6). These mice are phenotypically normal but have increased penetration of drugs into the brain and reduced elimination of these compounds. However, the hypothesis that Pgp functions only to exclude xenobiotics does not readily explain the high levels of the protein in adrenal gland (5) or apical localization of Pgp in epithelial cells of the choroid plexus (7). Other studies suggest that Pgp functions as a flippase for phospholipids. Recently, class I Pgp were shown to translocate a wide variety of short chain analogs of phospholipids from the inner to outer leaflet of the plasma membrane, and Pgp also may function as a flippase for native phosphatidylcholine and sphingomyelin (8–10). Class II Pgp also function as lipid flippases, although these proteins are specific for phosphatidylcholine (11). Mice that are deficient in mdr2 have severe liver disease caused by greatly reduced excretion of phosphatidylcholine and cholesterol into bile (12).

In addition to these possible roles for Pgp in outward translocation of substrates, several recent studies suggest a function for class I Pgp in the trafficking of sterols within cells. Lange and Steck (13) have shown that esterification of plasma membrane cholesterol in rat hepatoma cells is inhibited rapidly by treatment with a wide variety of amphiphilic compounds. Most of the compounds tested in these studies were nonspecific inhibitors of class I Pgp. Likewise, in CaCo-2 cells (14) and in a human hepatoma cell line (15), compounds known to inhibit MDR1 Pgp nonspecifically also inhibited esterification of plasma membrane cholesterol. In CaCo-2 cells, esterification was reduced at concentrations of drugs which did not significantly inhibit acyl-CoA cholesterol acyltransferase (ACAT) in isolated microsomes, suggesting that these nonspecific inhibitors of Pgp affected cholesterol movement from the plasma membrane to the endoplasmic reticulum (ER). Using a series of hydroperiandrosterone; MRP, multidrug resistance-associated protein; MEBSS, modified Earle’s balanced salt solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
steroid hormones, Debry et al. (16) found that inhibition of cholesterol trafficking from the plasma membrane to the ER correlated directly with the hydrophobicity of each steroid and its potency in reversing the effect of Pgp on drug accumulation. These authors also demonstrated that esterification was decreased by non-steroidal inhibitors of class I Pgp. Although these data support a role for Pgp in cholesterol transport from the plasma membrane to the ER, this conclusion is based on results with amphiphiles and inhibitors that also are known to affect targets other than Pgp. Furthermore, cholesterol trafficking has not been analyzed in cells with documented differences in expression and function of class I Pgp. Thus, the correlation of Pgp with esterification of plasma membrane cholesterol remains indirect.

To define better the role of Pgp in cholesterol trafficking, we determined the esterification of plasma membrane cholesterol in cells that express differing amounts of functional class I Pgp. Both drug-selected cells expressing increased levels of Pgp and cells transfected with MDR1 Pgp or other ATP-binding cassette transporters (ABC) were examined. In addition to evaluating selected, non-specific steroidal and non-steroidal modulators of Pgp which have been tested previously, we also investigated the effects of several potent antagonists specifically targeted to Pgp on both cholesterol esterification and intracellular accumulation of a known substrate for Pgp. Results showed that within a given cell type, greater expression of Pgp was correlated with increased esterification of plasma membrane cholesterol. Specific modulators all increased the cellular content of a substrate for Pgp but differed with respect to the effects on cholesterol esterification, suggesting dissociation between the functions of Pgp in cholesterol trafficking and MDR. Overall, the data support an additional physiologic function for Pgp in cholesterol trafficking within cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1,2,3-3H]Cholesterol (50 Ci/mmol), [4,5-3H]cholesterol (51.3 mCi/mmol), and [1-3H]cholesterol (55 mCi/mmol) were purchased from Sigma, NEN Life Science Products, and American Radiolabeled Chemicals, respectively. Stock solutions of several steroidal and non-steroidal modulators of Pgp were prepared in dimethyl sulfoxide. Solutions of PSC 833 (gift of DuPont Medical Corp.) as described previously (25) were prepared in dimethyl sulfoxide. Solutions of GF120918 (gift of Glaxo-Wellcome), and LY335979 (gift of Eli Lilly and Co.) were prepared in dimethyl sulfoxide. Solutions of PSC 383 (gift of Mallinckrodt Medical, Inc.) and cholesterol were prepared in ethanol. Sphingomyelinase (Bacillus cereus) and cholesterol oxidase (Brevibacterium spp.) were obtained from Sigma. [3H]-Dehydroepiandrosterone (DHEA) (Sigma), GF199018 (gift of Glaxo-WelCome), and LY335979 (gift of Eli Lilly and Co.) were prepared in dimethyl sulfoxide. Solutions of PSC 833 (gift of Mallinckrodt Medical, Inc.) and cholesterol were prepared in ethanol. Sphingomyelinase (Bacillus cereus) and cholesterol oxidase (Brevibacterium spp.) were obtained from Sigma. [3H]-Dehydroepiandrosterone was prepared with a one-step kit formulation (Cardiolite, gift of DuPont Medical Products Division). Synthesis of the (−)-enantiomer of DHEA was performed as described previously (17), and a stock solution was prepared in dimethyl sulfoxide. All other reagents were from Sigma.

**Cell Culture and Buffers**—NIH 3T3 fibroblasts were obtained from the ATCC and grown in Dulbecco's modified Eagle's medium, 10% heat-inactivated calf serum, and 0.1% penicillin/streptomycin. NIH 3T3 cells stably transfected with human MDR1 (obtained from Michael Gottesman, NIH) or human multidrug-resistance associated protein (MRP) (gift of Gary Kruh, Fox Chase Cancer Center) were maintained in 60 ng/ml colchicine or passaged once/month in 750 μg/ml G418, respectively. Experiments with the MRP transfectants were performed within 1 week of culture in the selection drug. 8226 myeloma cells and the doxorubicin-selected derivative Dox 6 cell line (gift of William Dalton, Moffitt Cancer Center) were grown in suspension culture in RPMI with 5% heat-inactivated fetal bovine serum, 0.1% penicillin/streptomycin, and 1% L-glutamine (18). All cells were cultured in a 5% CO2 incubator at 37 °C. Control buffer for all assays was a modified Earle's balanced salt solution (MEMBS) containing (mM): 145 NaCl, 5.4 K+ (1.2 Ca2+), 8.8 Mg2+, 152 Cl, 0.8 HPO42-, 0.8 SO42-, 5.6 dextrose, 4.0 HEPES, and 0.1% fatty acid-deficient bovine serum albumin (BSA) (w/v), pH 7.4. For cellular accumulation of 3H-Tc-Sestamibi, 1% serum was used instead of BSA (MEMBS-buffer). Assays of ACAT activity in homogenates of cells were performed in 0.1 ml Tris-HCl, 0.25 M sucrose, 1 mg/ml BSA, 1 mM dithiothreitol, pH 7.5 (ACAT buffer) (19). TBS-BSA (150 mM NaCl, 50 mM Tris-Cl, 2 mM BSA, pH 7.4) was used as a wash buffer in cholesterol oxidase assays (20).

**RESULTS**

**Characterization of Pgp in Fibroblast and Myeloma Cell Lines**—To determine relative expression of Pgp, we prepared enriched membrane fractions from the fibroblast and myeloma cell lines and immunoblotted with a monoclonal antibody to Pgp (Fig. 1A). NIH 3T3 cells express a low amount of murine Pgp, whereas immunodetectable Pgp is increased in fibroblasts transfected with human MDR1. In agreement with previous reports (18), parental 8226 cells do not express immunodetectable levels of Pgp, whereas the Dox 6 derivative cell line has a low level of class I Pgp.

Because expression and function of Pgp are not always correlated directly (28), we also characterized the cells functionally based on accumulation of Tc-Sestamibi, a known substrate for class I Pgp (29). The net cell content of Tc-Sestamibi is inversely to expression of functional class I Pgp, and this effect is reversed by GF120918, a specific modulator of the protein (25, 26, 27). Data are reported as mean ± S.E. using the number of replicates for each point as described in figure legends. Pairs were compared by Student's t test. Values of p ≤ 0.05 were considered significant.
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FIG. 1. Detection of class I Pgp. Panel A, expression of Pgp in fibroblast and myeloma cells was detected in 50 µg of enriched membrane fractions using monoclonal C219 as described under “Experimental Procedures.” Lane 1, NIH 3T3 MDR1; lane 2, NIH 3T3; lane 3, 8226; lane 4, Dox 6. The position of 170 kDa is indicated. Functional class I Pgp in NIH 3T3 (panel B) and myeloma cell lines (panel C) was identified by incubating cells with 0.1–0.6 nM 99mTc-Sestamibi in MEBSS-serum containing vehicle alone (■) or 300 nM GF120918 (□) for 30 min at 37 °C. The net cell content of Tc-Sestamibi was quantified as described under “Experimental Procedures.” Columns represent the mean of four determinations; bars represent ± S.E. when large enough to be seen.

26, 30). Cells were incubated with Tc-Sestamibi for 30 min, a time sufficient for the radiotracer to reach a steady-state accumulation and cholesterol esterification. When GF120918 was added. Thus, based on the data for Tc-Sestamibi accumulation, 8226 cells do not express functional Pgp, whereas Dox 6 cells have a modest amount of the protein. Overall, for these cells, the data reflecting transport function of class I Pgp correspond directly with expression levels of the protein determined by Western blotting.

Esterification of Plasma Membrane Cholesterol—Cholesterol is located predominantly in the plasma membranes of cells, separated from ACAT, which resides primarily, if not exclusively, in the ER (31–33). To investigate the function of Pgp in cholesterol trafficking, we used esterification of plasma membrane cholesterol as a marker of cholesterol movement from the cell surface to the ER. Plasma membranes were pulse labeled with [3H]cholesterol at 15 °C for 20 min and then chased at 37 °C for up to 4 h (21). Under basal conditions, the esterification of plasma membrane cholesterol was greater in fibroblasts transfected with MDR1 Pgp than in the parental cells (Fig. 2A). At all time points, the percent cholesteryl oleate in the MDR1 transfectants was increased by approximately 1.3–1.9-fold over the parental cells. To verify that the correlation of Pgp with cholesterol esterification was not unique to fibroblasts and to compare Pgp-negative and -expressing cells directly, we also measured the time course of esterification in 8226 and Dox 6 myeloma cells. Similar to the fibroblasts, more plasma membrane cholesterol was esterified in the Pgp-expressing Dox 6 cells than the 8226 cells during the 4-h time course (Fig. 2B). The percent cholesteryl oleate was approximately 1.2-fold greater in the Dox 6 cells after 30 min and increased to a 3-fold difference by 4 h. The relative differences between cell lines were maintained in all experiments with the fibroblasts and myeloma cells, although the absolute percent cholesteryl oleate varied (see subsequent figures). Similar variations in esterification among experiments have been noted previously in human fibroblasts (34). Pilot data also indicate that the kinetics of cholesterol esterification are enhanced in Pgp-expressing KB-8-5 epidermoid carcinoma cells compared with the Pgp-negative KB-3-1 parental cell line (data not shown). Although Pgp is not essential for cholesterol trafficking from the plasma membrane to the ER under basal conditions in all cells, these data show that cholesterol esterification within a given cell type increases in direct relation to expression of functional Pgp.

Treatment of cells with sphingomyelinase stimulates esterification of plasma membrane cholesterol without expanding total intracellular pools of cholesterol (35). Although the mechanism for this effect is not known, the pathway may involve an energy-independent mechanism of vesicle formation which differs from esterification under basal conditions (36). Nevertheless, modulators of Pgp have been shown to inhibit cholesterol esterification induced by sphingomyelinase, suggesting that this pathway also is affected by Pgp (16). To determine if levels of functional Pgp also correlate with esterification stimulated by sphingomyelinase, we pulse labeled the plasma membrane with [3H]cholesterol and then chased cells in the medium containing sphingomyelinase for various periods of time. Throughout the 4-h chase, more cholesterol was esterified in the MDR1 transfectants than parental NIH 3T3 cells (Fig. 3A). Differences between these two cell lines were greatest at the earlier
time points, with 6-fold more cholesteryl oleate in the MDR1 cells at 1 h. After 4 h of chase, cholesteryl oleate was approximately 1.4-fold greater in the MDR1 transfectants compared with the parental fibroblasts. Relative to basal conditions, stimulation of esterification by sphingomyelinase was greatest at the 30-min time point, with approximately 11- and 6-fold increases in cholesteryl oleate in the MDR1 transfectants and NIH 3T3 cells, respectively. Esterification was approximately 2-fold greater than under basal conditions in both fibroblast cell lines after 4 h of chase.

Similarly, in the myeloma cells, esterification induced by sphingomyelinase correlated with expression of Pgp. Synthesis of cholesteryl oleate was approximately 1.2–1.9-fold greater in Dox 6 cells compared with the Pgp-negative 8226 cells (Fig. 3B). The percent cholesteryl oleate in both cell lines was 2–3-fold greater than comparable time points without sphingomyelinase treatment.

To confirm that these observations were not an artifact of partial labeling of a cholesterol pool by the pulse-chase protocol, cholesterol esterification in response to sphingomyelinase also was determined in cells that were equilibrium labeled with [3H]cholesterol for 72 h. After 1 h of incubation with sphingomyelinase, cholesterol esterification was significantly greater in MDR1-transfected fibroblasts and Dox 6 cells compared with the respective parental cell lines. Cholesteryl oleate increased by 4.8- and 2.8-fold for the MDR1-transfected fibroblasts and parental NIH 3T3 cells (p < 0.02), respectively, whereas the increases were 1.6-fold in Dox 6 and 1.1-fold in 8226 cells (p <
0.01). Thus, using two different methods of labeling with \(^{3}H\)cholesterol, these data show that esterification of plasma membrane cholesterol in response to sphingomyelinase correlates with expression of Pgp.

To determine if another ABC transporter could increase cholesterol esterification, the effects of MRP on trafficking of plasma membrane cholesterol were investigated. Expression of MRP decreases intracellular accumulation of structurally diverse drugs, some of which overlap with compounds included in the MDR phenotype mediated by class I Pgp (37), including Tc-Sestamibi (38, 39). NIH 3T3 cells stably transfected with human MRP or empty vector were pulse labeled with \(^{3}H\)cholesterol and chased for 1 h in the absence or presence of sphingomyelinase. MDR1-transfected fibroblasts were included in these same experiments to allow direct comparison of both ABC transporters. Under both basal conditions (Fig. 4A) and in response to sphingomyelinase (Fig. 4B), esterification of plasma membrane cholesterol was not increased by expression of MRP compared with vector alone. Relative to the cells expressing MRP, esterification was increased by approximately 3-fold in MDR1-transfected fibroblasts. These data indicate that the function of Pgp in cholesterol trafficking is not a general effect of ABC transporters.

**Effects of Modulators of Pgp**—Previous investigations of the role of Pgp in cholesterol trafficking have used nonspecific modulators such as verapamil and progesterone. Although these compounds are reported to inhibit the effect of Pgp on drug accumulation, they are not specific for this protein. We used concentrations of these compounds which are comparable to concentrations shown previously to inhibit cholesterol esterification in other cell types and tested these drugs for effects on both accumulation of Tc-Sestamibi and cholesterol trafficking (14, 16).

First, in the presence of 100 \(\mu\)M verapamil, the net content of Tc-Sestamibi increased by approximately 18-fold in MDR1-transfected fibroblasts (Fig. 5A) and 35-fold in Dox 6 cells (Fig. 5B), but the amount of Tc-Sestamibi in the 8226 cells was not significantly changed. Accumulation of Tc-Sestamibi in Dox 6 cells increased to the same absolute amount as in 8226 cells, indicating complete inhibition of Pgp in Dox 6 cells. Conversely, progesterone was an ineffective reversal agent of transport of Tc-Sestamibi mediated by Pgp. In MDR1-transfected fibroblasts, accumulation of Tc-Sestamibi was not increased significantly by 20 \(\mu\)M progesterone (Fig. 5A), whereas in Dox 6 cells, the radiotracer content was only 2-fold greater (Fig. 5C). The Tc-Sestamibi content in 8226 cells was not affected by progesterone.

We then tested these modulators for effects on cholesterol esterification, using pulse labeling of plasma membrane cholesterol followed by a 1-h chase period. Verapamil inhibited esterification in MDR1-transfected fibroblasts, reducing cholesteryl ester to 31% of control when the modulator was added only to the chase buffer (Fig. 5A). Adding verapamil only to the chase medium also had a significant inhibitory effect on esterification in the Pgp-expressing Dox 6 cells, reducing esterification to 53% of cells treated with vehicle alone; the cholesteryl oleate was decreased only to 75% of vehicle control in 8226 cells (Fig. 5B). Incubating Dox 6 cells with verapamil for 24 h did not inhibit esterification to a greater percentage than only including the drug in the chase medium. However, treatment with verapamil for 48 h further decreased esterification to 20% of control values for these cells, but effects caused by cell toxicity could not be excluded over this length of time. Cell growth was diminished markedly under this condition, and cells were not viable when incubated with verapamil for 72 h. Compared with including verapamil only in the chase medium, prolonged incubation of 8226 cells with verapamil produced a minimal additional reduction in synthesis of cholesteryl oleate.

Progesterone was an effective inhibitor of cholesterol esterification in MDR1-transfected fibroblasts, unlike the effects of this steroid on the accumulation of Tc-Sestamibi. Esterification decreased to 24% of control when these cells were treated with 20 \(\mu\)M progesterone (Fig. 5A). Similarly, progesterone inhibited cholesterol esterification in both myeloma cell lines, with a greater effect on Pgp-expressing Dox 6 cells. Using progesterone in the chase buffer, the cholesteryl oleate was 77 and 64% of control in 8226 and Dox 6 cells, respectively (Fig. 5C).

Because these first-generation modulators are known to interact with cellular targets other than Pgp, it was of interest to study compounds developed specifically to inhibit Pgp in MDR.
We used three such compounds (PSC 833, GF120918, and LY335979) at concentrations that maximally antagonize the effects of class I Pgp on drug accumulation (25, 30, 40, 41) to investigate further the relationships of Pgp to the intracellular content of drugs and cholesterol trafficking. Using 2 mM PSC 833, 300 nM GF120918, or 1 mM LY335979, the net cell content of Tc-Sestamibi increased by approximately 40-fold in both MDR1-transfected fibroblasts and Dox 6 cells (see Figs. 6–8). At the concentrations tested, none of the compounds altered the accumulation of Tc-Sestamibi in Pgp-negative 8226 cells. In Dox 6 cells, the accumulation of Tc-Sestamibi increased to the amount present in the parental myeloma cells. The antagonism was seen without preincubation of cells in the modulator prior to adding the radiotracer substrate, showing that these com-

**FIG. 5.** Effects of nonspecific modulators on plasma membrane cholesterol esterification and Tc-Sestamibi accumulation. The plasma membranes of NIH 3T3 MDR1 (panel A), or 8226 (■) and Dox 6 (□) cells (panels B and C) were labeled with [3H]cholesterol as described in Fig. 2 and then incubated for 1 h at 37 °C in MEBSS containing 10 μM cholesterol and vehicle (veh), 100 μM verapamil (verap), or 20 μM progesterone (prog). In panel B, myeloma cells were pretreated with vehicle or verapamil for 24 or 48 h before labeling with [3H]cholesterol. Cells that were pretreated with verapamil also were incubated with the modulator during the 1-h chase period. [3H]Cholesterol and [3H]cholesteryl oleate in cells were determined as described under “Experimental Procedures.” Each column is the mean of three (fibroblasts) or four (myeloma cells) determinations; bars represent ± S.E. Data for the fibroblasts are representative of two independent experiments; data for the myeloma cells are from single experiments for each modulator. The inset to all panels shows cell accumulation of Tc-Sestamibi during a 30-min assay in MEBSS-serum containing vehicle or the indicated modulator. Each column is the mean of four determinations for all cell lines; bars represent ± S.E. when larger than the column.
pounds rapidly inhibit Pgp-mediated effects on drug accumulation during short term assays.

We then determined the effects of these same concentrations of modulators on cholesterol trafficking in both fibroblasts and myeloma cells, using pulse labeling of the plasma membrane with \(^{3}H\)cholesterol followed by a 1-h chase. In MDR1-transfected fibroblasts, cholesterol esterification was not affected by PSC 833 when the drug was added only during the chase. Because prolonged incubation with PSC 833 has been reported to alter transmembrane distribution of lipids (10), cells were incubated with drug for up to 72 h before the assay. Prolonged pretreatment with PSC 833 also did not affect cholesterol esterification in MDR1-transfected fibroblasts (Fig. 6A). Conversely, including PSC 833 in the chase buffer inhibited esterification in both myeloma cell lines, reducing the cholesteryl oleate to 51 and 28% of control in 8226 and Dox 6 cells, respectively (Fig. 6B). The effect of PSC 833 was immediate in these cells, reducing the percent cholesteryl oleate to approximately equivalent levels with minimal further reductions from culturing cells in PSC 833 prior to the experiment. Inhibition of cholesterol esterification in Pgp-negative cells by PSC 833 may be the result of effects on another unidentified transporter. For example, PSC 833 has been reported to reduce bile excretion of digoxin, another substrate for Pgp, in mice lacking both mdr1a and mdr1b (42).

In MDR1-transfected fibroblasts, LY335979 decreased esterification to 60% of control when added only during the chase period (Fig. 7A). LY335979 had a greater effect when MDR1-transfected fibroblasts were cultured with modulator for up to 72 h before the assay. Under these conditions, esterification was inhibited progressively, decreasing to only 28% of control after 72 h of pretreatment with this modulator. In the myeloma cells, LY335979 had a similar effect on cholesterol trafficking. Esterification decreased to 86% of control values in Dox 6 cells when the drug was included only in the chase medium (Fig. 7B). However, inhibition was progressively greater when this modulator was included in the culture medium of Dox 6 cells for 48–72 h prior to the experiment, with cholesteryl oleate reduced to approximately 72% of control. LY335979 did not inhibit esterification in 8226 cells under any of the conditions tested.

Unlike the other two modulators, GF120918 had minimal effects on the esterification of plasma membrane cholesterol, regardless of expression of Pgp (Fig. 8). In MDR1-transfected fibroblasts, GF120918 had no effect when added during the 1-h chase, and incubation with the drug for 72 h only reduced esterification to 85% of control (Fig. 8A). The percent cholesteryl oleate was unaffected in 8226 and Dox 6 cells, even after prolonged incubations with the modulator (Fig. 8B). The same discordant effects of LY335979 and GF120918 were also seen when esterification of plasma membrane cholesterol was stimulated by sphingomyelinase in the MDR1-transfected fibroblasts (data not shown).

To determine if the effects of these modulators on esterification were the result of direct inhibition of ACAT, we measured the activity of this enzyme in homogenates of fibroblasts. Homogenates were incubated with vehicle or appropriate drug and an excess of cholesterol before adding 25 \(\mu M\) [\(^{14}C\)]cholesterol coenzyme A to begin esterification. Synthesis of cholesteryl oleate was reduced by only 18% after treatment of homogenized cells with 100 \(\mu M\) verapamil and was not affected significantly by any of the specific modulators of Pgp (Table I). By comparison, cholesterol esterification in the presence of 20 \(\mu M\) progesterone was only 28% of control, which is approximately equal to inhibition of esterification in whole fibroblasts and exceeds the effect of this steroid in intact myeloma cells. Thus, unlike the results with all other modulators tested, decreases in cholesterol esterification mediated by progesterone may be caused by inhibition of ACAT rather than cholesterol transport from the plasma membrane to the ER.

Effects of Enantiomers of DHEA on Esterification—The hydrophobicity of steroid hormones has been correlated with inhibition of both the function of Pgp and esterification of cholesterol derived from the plasma membrane (16). DHEA has been reported previously to modulate reduced drug accumulation mediated by Pgp, and it is one of the most effective steroids at blocking esterification of plasma membrane cholesterol stimulated by sphingomyelinase (16, 43). To test directly the effects of hydrophobicity on the accumulation of Tc-Sestamibi and cholesterol esterification, we synthesized the (−)-enantiomer of DHEA for comparison with the naturally occurring (+)-enantiomer. Because these compounds have identical hydrophobicity (as well as other physical properties), differences in the effects on accumulation of Tc-Sestamibi or esterification of cholesterol reflect enantio-specific interactions. MDR1-transfected fibroblasts and both myeloma cell lines were incubated with Tc-Sestamibi and either vehicle alone or an enantiomer of this steroid. Low intracellular accumulation of Tc-Sestamibi (1.1 ± 0.1 and 5.7 ± 0.3 fmol of Tc-Sestamibi (mg of protein)\(^{-1}\) (nmol)\(^{-1}\)) in MDR1-transfected fibroblasts and Dox 6 cells, respectively) was not increased significantly by either enantiomer of DHEA at concentrations up to 40 \(\mu M\). DHEA also did not increase the net cell content of Tc-Sestamibi (141.2 ± 3.6 fmol of Tc-Sestamibi (mg of protein)\(^{-1}\) (nmol)\(^{-1}\)) in Pgp-negative 8226 cells. These data indicate that DHEA is not a modulator of Pgp transport function in MDR1-transfected fibroblasts or Dox 6 myeloma cells as probed by Tc-Sestamibi.

To determine the effects of DHEA on cholesterol trafficking, the plasma membranes of MDR1-transfected NIH 3T3 cells were pulse labeled with \(^{3}H\)cholesterol and then incubated with sphingomyelinase in the absence or presence of differing concentrations of each enantiomer of DHEA. Cholesterol esterification was inhibited in a dose-dependent manner by each steroid (Fig. 9A). At concentrations between 1 and 20 \(\mu M\), the (−)-enantiomer of DHEA inhibited esterification in the MDR1-transfected fibroblasts less than the (+)-enantiomer. These effects on esterification were not the result of direct inhibition of ACAT because the activity of the enzyme in cell homogenates was not affected by a 20 \(\mu M\) concentration of either enantiomer (Table I). Thus, these data show evidence of enantio-specific inhibition of cholesterol trafficking in fibroblasts treated with (+) or (−)-DHEA.

To evaluate further the effects of DHEA on esterification of plasma membrane cholesterol, we tested the enantiomers of this steroid in both myeloma cell lines (Fig. 9, B and C). In these cells, cholesterol esterification was inhibited by both enantiomers with no consistent stereo-specific differences over the range of concentrations tested. The effects of DHEA also were independent of the expression of Pgp. At 40 \(\mu M\) (+)-DHEA, esterification was reduced to 43 and 36% of control in 8226 and Dox 6 cells, respectively. Decreased esterification of plasma membrane cholesterol was not simply the result of ACAT inhibition because esterification in cell homogenates treated with 40 \(\mu M\) steroid was reduced only to 72–80% of control and did not show enantio-specific effects (Table I). The mechanisms underlying the differing results seen with enantiomers of DHEA between fibroblasts and myeloma cells are unknown. Nevertheless, enantio-specific inhibition of esterification in MDR1-transfected fibroblasts demonstrates that hydrophobicity alone is not the only determinant of the effects of DHEA on intracellular cholesterol transport in all cell types.

Sensitivity to Cholesterol Oxidase—A potential explanation
for the effect of Pgp on cholesterol esterification is changes in either the overall cholesterol content in the plasma membrane or the sterol distribution in the membrane domains. To investigate total cholesterol in the plasma membrane, we probed cells with cholesterol oxidase. After labeling for 72 h with $[3H]$cholesterol, cells were then treated with cholesterol oxidase according to the method of Porn and Slotte (23). Only cholesterol in the plasma membranes is oxidized using this method (44). Approximately 80% of total cell cholesterol was oxidized to cholestenone in both the fibroblast and myeloma cell lines (Table II). The base-line percent of oxidizable cholesterol did not differ among the cell lines and was not related to

**FIG. 6. Effects of PSC 833 on plasma membrane cholesterol esterification and Tc-Sestamibi accumulation.** NIH 3T3 MDR1 (panel A) or 8226 (panel B) cells were pretreated with vehicle (veh) or 2 $\mu M$ PSC 833 (PSC) in growth medium for 24, 48, or 72 h before labeling plasma membranes with $[3H]$cholesterol as described in Fig. 2. After labeling, cells were incubated for 1 h in MEBSS with or without 2 $\mu M$ PSC 833 as described in Fig. 5. One set of cells received vehicle alone during both the pretreatment and chase periods, and another group was incubated with PSC 833 only during the chase. All cells that were pretreated with PSC 833 also were incubated with the modulator during the 1-h chase. The percent $[3H]$cholesterol and $[3H]$cholesteryl oleate under each condition was determined as described under “Experimental Procedures.” Data are expressed as the mean $\pm$ S.E. for three determinations and are representative of two independent assays. The inset in panels A and B shows the steady-state content of Tc-Sestamibi in each cell line after a 30-min incubation in MEBSS-serum containing vehicle or PSC 833. Data for Tc-Sestamibi accumulation are the mean $\pm$ S.E. for four determinations under each condition.
the levels of Pgp.

The cholesterol oxidase technique described by Porn and Slotte uses fixation with glutaraldehyde prior to treatment with cholesterol oxidase. Because glutaraldehyde has been reported to mask the existence of different pools of cholesterol in some cell types (45), we also determined the oxidizable pool of

FIG. 7. Effects of LY335979 on plasma membrane cholesterol esterification and Tc-Sestamibi accumulation. NIH 3T3 MDR1 (panel A) or 8226 (■) and Dox 6 (■) (panel B) cells were pretreated with vehicle or 1 μM LY335979 in growth medium for 24, 48, or 72 h before labeling plasma membranes with [3H]cholesterol as described in Fig. 2. After labeling, cells were incubated for 1 h in MEBSS with or without 1 μM LY335979 as described in Fig. 6. The percent [3H]cholesterol and [3H]cholesteryl olate under each condition was determined as described under “Experimental Procedures.” Data are expressed as the mean ± S.E. for three determinations and are representative of three independent assays. The inset in panels A and B shows the steady-state content of Tc-Sestamibi in each cell line after a 30-min incubation in MEBSS-serum containing vehicle or LY335979. Data for Tc-Sestamibi accumulation are the mean ± S.E. for four determinations under each condition.
plasma membrane cholesterol in the absence of fixation, using the protocol of Smart et al. (24). By this method, the cholesterol sensitive to oxidation localizes to caveolae, which are membrane specializations enriched in cholesterol and sphingomyelin (46). Caveolae are reported to mediate cholesterol efflux from cells (47) and are also the sites at which newly synthesized cholesterol enters the plasma membrane (48). Although neither 8226 nor Dox 6 cells are expected to express caveolin,
cholesterol that is oxidized potentially localizes to detergent-resistant membrane fractions enriched in cholesterol and glycosphingolipids that can exist without caveolin (49–51). In all cell lines, approximately 3–5% of cholesterol was oxidized to cholestenone in both the parental and Pgp-expressing cells (Table II). These data are comparable to the 5–7% of oxidizable cholesterol reported previously for human fibroblasts and Chinese hamster ovary cells (20, 24). Overall, the data show that expression of Pgp does not confer any change in cholesterol content of the plasma membranes as examined with cholesterol oxidase.

DISCUSSION

Cholesterol is distributed asymmetrically among cell membranes, with up to 90% of free cholesterol located in the plasma membrane (31). However, the enzymes and transcription factors that control cholesterol metabolism are located primarily in the ER, an organelle that is relatively poor in cholesterol (32, 33, 52). Thus, for the bulk of free cholesterol in cells to communicate with the regulatory pool of cholesterol in the ER, cholesterol from the plasma membrane and other intracellular organelles must move to the ER. However, the mechanisms and regulation of intracellular cholesterol trafficking remain poorly characterized.

In this report, we show that class I Pgp functions to increase esterification of cholesterol derived from the plasma membrane under basal conditions and in response to sphingomyelinase, demonstrating that the protein affects a step that is common to both pathways. Because regulation of ACAT by sterols in cultured mammalian cells is dependent on the supply of cholesterol and not the amount of enzyme or rate of catalysis (53), the data indicate that Pgp increases esterification by facilitating cholesterol movement from the plasma membrane to the ER. The effects of Pgp on transport were not simply the result of the general expression of an ABC transporter because esterification was not increased in fibroblasts transfected with MRP.

Furthermore, these effects on cholesterol trafficking occurred without differences in plasma membrane cholesterol as measured using cholesterol oxidase with or without glutaraldehyde fixation.

As seen with both steroidal and non-steroidal antagonists, inhibition of cholesterol trafficking was not always related to reversal of Pgp-mediated effects on drug accumulation. For example, verapamil increased the net content of Tc-Sestamibi in Dox 6 cells without affecting the Pgp-negative 8226 cell line, whereas this drug inhibited cholesterol esterification in both cell lines, albeit with a greater effect on Dox 6 cells. Conversely, progesterone and DHEA, which have been reported to reverse the Pgp-mediated effects on the intracellular content of some substrates (43, 54), were not effective antagonists of the reduced accumulation of Tc-Sestamibi mediated by Pgp in these fibroblast and myeloma cells at the concentrations tested. The reasons for a lack of effect in the current study are unknown but may relate to the use of differing cell lines and drug transport substrates for Pgp. Cell type-specific differences in interactions of Pgp with both modulators and substrates have been reported previously (for review, see Ref. 55). Although these steroids decreased cholesterol esterification, the effect of progesterone is likely the result of direct inhibition of ACAT, whereas the data indicate that DHEA affected cholesterol trafficking from the plasma membrane to the ER in the presence or absence of Pgp. Steroid hydrophobicity has been reported to correlate with the inhibition of cholesterol esterification (16). At least for DHEA, hydrophobicity alone cannot account for the effects on cholesterol trafficking in all cell types, as evidenced by the greater effectiveness of the naturally occurring (+)-enantiomer than the (−)-enantiomer in fibroblasts. The lack of an enanti-specific effect of DHEA in myeloma cells likely reflects differences between these cells and fibroblasts in the mechanisms regulating cholesterol transport.

Specific modulators of Pgp all immediately increased the content of Tc-Sestamibi in cells expressing class I Pgp and did not significantly affect accumulation of the radiotracer in Pgp-negative cells. However, these drugs differed markedly in their effects on esterification of plasma membrane cholesterol. GF120918 had minimal effects on esterification in the fibroblast and myeloma cell lines, independent of Pgp. PSC 833 did not inhibit esterification in MDRI-transfected fibroblasts, whereas synthesis of cholesteryl oleate in the presence of this drug was reduced in both myeloma cell lines. Essentially all of the effect of PSC 833 on cholesterol trafficking in myeloma cells was observed when the drug was added during the chase period; prolonged exposure of cells to this antagonist before the assay did not result in further inhibition of esterification. Interestingly, LY335979 inhibited esterification selectively in cells with functional class I Pgp, showing an even greater effect when used to pretreat cells for a prolonged period prior to the esterification assay. We cannot exclude that the effects of LY335979 on cholesterol trafficking may be mediated by a metabolite of this antagonist because the drug is metabolized rapidly when incubated with liver microsomes (56). Nevertheless, these data with LY335979 suggest that part of the effect of class I Pgp on cholesterol trafficking depends on changes in cells which are not immediately reversed. Potentially, the differing effects of the more potent and selective MDR modulators may be used to probe mechanistic differences between cholesterol trafficking and drug transport functions mediated by Pgp. A corollary of this observation is that although all of these drugs are well characterized as potent reversal agents of MDR, some possess cross-reactive molecular targets.

The mechanism through which Pgp affects cholesterol trafficking from the plasma membrane to the ER remains unknown. One possibility is that Pgp removes an endogenous inhibitor of cholesterol transport. Among the substrates for Pgp are a wide variety of hydrophobic peptides (57). Cholesterol esterification in response to lipoproteins or exogenous cholesterol was significantly decreased in macrophages and Chinese hamster ovary cells treated with an inhibitor of cysteine proteases, suggesting that cholesterol trafficking is regulated by an intracellular peptide (58). Pgp may decrease the intracellular...
The increased concentration of such a peptide, thus accounting for increased cholesterol esterification.

Another possibility is that Pgp may increase cholesterol esterification through direct effects on vesicular trafficking. Pgp has been shown to undergo constitutive recycling through an endosomal compartment associated with Rab 5 (59), and changes in this endocytic pathway alter the function of Pgp in MDR. By analogy, the fusion of endosomes in vivo is stimulated by cystic fibrosis transmembrane conductance regulator, another ABC transporter related to Pgp (60, 61). Thus, Pgp may promote cholesterol transport from the plasma membrane to the ER by increasing endocytic trafficking.

A third possible mechanism is that Pgp alters the organization of the plasma membrane, perhaps by functioning as a flippase for lipids. Sphingomyelin, a lipid that interacts strongly with cholesterol (62, 63), is among the lipids that may be translocated by Pgp. PSC 833 inhibited translocation of short chain analogs of sphingomyelin from the inner to the outer leaflet of the plasma membrane during a 3-h assay in cells transfected with class I Pgp (8). The distribution of native sphingomyelin in cells expressing endogenous Pgp also is reported to be altered by PSC 833, although 72 h of treatment with antagonist was required to decrease the pool of native sphingomyelin located in the outer leaflet of the plasma membrane (10). In addition, PSC 833 is reported to inhibit translocation of both short chain analogs and full-length phosphatidylcholine (8, 9). By altering the transbilayer distribution of sphingomyelin or other phospholipids, Pgp could affect the size or composition of lipid domains within the plasma membrane. Such domains could promote cholesterol trafficking from the plasma membrane to intracellular organelles such as the ER without changing the total amount of cholesterol in the cell membrane.

In conclusion, we have shown that increased expression and function of class I Pgp in a given cell type correlate with increased esterification of plasma membrane cholesterol, both under basal conditions and in response to sphingomyelinase.

Fig. 9. Effects of enantiomers of DHEA on plasma membrane cholesterol esterification in response to sphingomyelinase. NIH

| Cell line          | % choleskenone | % cholestenone |
|--------------------|----------------|----------------|
| NIH 3T3            | 83.7 ± 0.9     | 5.4 ± 0.9      |
| NIH 3T3 MDR1       | 81.7 ± 0.9     | 4.1 ± 0.3      |
| 8226               | 81.8 ± 0.8     | 3.6 ± 0.3      |
| Dox 6              | 80.7 ± 0.5     | 3.5 ± 0.2      |

3T3 MDR1 (panel A), 8226 (panel B), and Dox 6 (panel C) cells were incubated with [3H]cholesterol to label plasma membranes as described in Fig. 2. Cells were then incubated for 1 h in buffer containing 50 milliunits/ml sphingomyelinase and vehicle or differing concentrations of (+)-DHEA (●) or (−)-DHEA (○). Cells were harvested and extracted for quantification of percent [3H]cholesterol and [3H]cholesteryl oleate as described under “Experimental Procedures.” Each data point is the mean of three determinations; bars represent ± S.E. when larger than the symbol. Data for each cell line are representative of three independent experiments.

Table II

Susceptibility of plasma membrane cholesterol to cholesterol oxidase in fibroblast and myeloma cell lines

NIH (3T3 and 3T3 MDR1) cell lines were seeded at 3–4 × 10⁵ cells/well in six-well plates, and myeloma cells (8226 and Dox 6) were incubated at an initial density of 1 × 10⁶ cells/10 ml. Cells were cultured with [3H]cholesterol in growth medium for 72 h and then treated with cholesterol oxidase according to either the method of Porn and Slotte or Smart et al., as described under “Experimental Procedures.” Radiolabeled sterols were extracted and separated by TLC and quantified by liquid scintillation. Data are the mean ± S.E. of six samples from two independent experiments for all conditions except 8226 and Dox 6 cells using the method of Smart et al., for which n = 3 from one experiment.
However, although it is a facilitator, functional class I Pgp is not essential for cholesterol trafficking from the plasma membrane to the ER in all cell lines. The effect of Pgp on cholesterol transport is not through a mechanism identical to its function in MDR, as evidenced by discordant inhibition of cholesterol esterification and modulation of drug transport by many of the MDR antagonists that were tested. We also demonstrated cell type-specific differences of inhibitors on cholesterol esterification, suggesting that mechanisms of sterol trafficking differ between fibroblast and myeloma cell lines. These data expand on previous investigations and document a physiologic function between fibroblast and myeloma cell lines. These data expand on previous investigations and document a physiologic function between fibroblast and myeloma cell lines. These data expand on previous investigations and document a physiologic function between fibroblast and myeloma cell lines.

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