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Decrease of P-Glycoprotein Activity in K562/ADR Cells by MβCD and Filipin and Lack of Effect Induced by Cholesterol Oxidase Indicate That This Transporter Is Not Located in Rafts

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The effect of low-density membrane domains on function of the plasma membrane transporter P-glycoprotéine (P-gp), involved in multidrug resistance (MDR) phenotype, has been investigated in K562/ADR cells. To this end we reversibly altered the cholesterol content of K562/ADR cells by using methyl-β-cyclodextrin as a cholesterol chelator and conversely we repleted them through incubation with cholesterol in culture medium. We also used the cholesterol-binding fluorochrome filipin and cholesterol oxidase. Our data show that either cholesterol depletion or complex formation with filipin resulted in a strong decrease of P-gp activity. However, when cells were incubated with cholesterol oxidase that are known to disrupt rafts, no modification of the P-gp activity was observed. In addition, using a free-detergent methodology to separate by ultracentrifugation, "light," "heavy," and "extra heavy" fractions we show that no P-gp is found in the "light" fraction where rafts are usually detected. Altogether, our data strongly suggest that, in this cell line, P-gp is not localized in rafts.

KEY WORDS: Multidrug resistance; *P*-glycoprotein; transport; cholesterol; raft.

INTRODUCTION

Cancer is a major worldwide health concern, with an estimated 6 millions new cases per year. Until now, the only possible methods of curing systemic cancers, such as leukemia, lymphoma, and unifocal tumors that have spread by metastasis, have involved systemic treatments such as chemotherapy and immunotherapy. Unfortunately, clinical resistance to anticancer drugs has been observed from the onset of chemotherapy. In addition to this intrinsic and primary resistance to first-line chemotherapy, clinicians are often confronted by the progressive resistance of tumors that were initially sensitive to treatment. Therefore, the appearance of cell populations resistant to multidrug-based chemotherapy constitutes the major obstacle to cure (Bordow *et al*., 1994; Baldini *et al*., 1995; Leith *et al*., 1995).

Multidrug resistance (MDR) involves resistance to naturally derived anticancer agents, such as anthracyclines, vinca alkaloïds, epipodophyllotoxins, and taxanes. This form of resistance is due to multifactorial mechanisms, such as decreased cellular topoisomerase II, increased cellular glutathione, and increased ATP-dependent efflux of the cytotoxic agent over the cellular plasma membrane due to the presence of drug efflux pumps (Alvarez *et al*., 1998; Beck *et al*., 1995; Brock *et al*., 1995; Hasegawa *et al*., 1995).

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Key to abbreviations: P-gp, P-glycoprotein; MDR, Multidrug Resistance; MβCD, Methyl-β−cyclodextrin; Amplex Red,10-acetyl-3,7-dihydroxyphenoxazine; HRP, Horseradish peroxidase; COase, cholesterol oxidase; FCS, fetal calf serum; PIRA, pirarubicin; DOX, doxorubicin; DNR, daunorubicin; IDA, idarubicin; CTB, cholera toxin B; TX, Triton X-100.

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Biochemical research has uncovered three types of drug pumps that may play a role in MDR. The most intensively studied is the 170-kDa P-glycoprotein (P-gp) encoded by the MDR1 gene in humans. A variety of studies have shown that P-gp acts as a drug pump, localized in the plasma membrane of tumor cells, which can recognize an astonishing range of cytotoxic molecules and remove them from the cell. The effects of various lipid species on P-gp functions have been reported and overall it appears that optimal function of the protein may be supported by lipids such as cholesterol and the nonbilayer-forming phosphatidyl ethanolamine. MDR cells contain increased numbers of cholesterol- and sphingolipid-rich microdomains containing caveolins known as caveolae. Furthermore, localization of P-gp in these ordered microenvironments within the membrane has been reported (Rothnie *et al*., 2001). Also, Luker *et al*. (2000), have shown that in polarized cells, P-gp is localized in apical membranes, domains that are enriched in sphingolipids and cholesterol compared with basolateral surface. In general, changes in the cholesterol content of biological membranes are known to alter the lipid fluidity and thus membrane integrity (Yeagle, 1989, 1991).

Considering other transport proteins, it has been shown (i) that membrane cholesterol modulates serotonin transport activity (Scanlon *et al*., 2001), (ii) that cholesterol has modulator effects on the function of two structurally related peptide receptors, the oxytocin receptor and the brain cholecystokinin receptor in plasma membranes as well as in intact cells (Gimpl *et al*., 1997).

The effects of low density membrane domains on function of the protein remain almost unexplored in whole cells systems and in this study we have investigated whether membrane cholesterol also modulates the functional properties of the P-gp transporter expressed in K562/ADR cells with respect to substrate transport. To this end, in a first approach we reversibly altered the cholesterol content of K562/ADR cells by using methyl-β-cyclodextrin as a cholesterol chelator and conversely we repleted them through incubation with cholesterol in culture medium. We also used the cholesterol-binding fluorochrome filipin and cholesterol oxidase. Our data show that either cholesterol depletion or complex formation with filipin resulted in a strong decrease of P-gp activity. However, when cells were incubated with cholesterol oxidase that is known to disrupt rafts, no modification of the P-gp activity was observed. In addition, using a free-detergent methodology to separate by ultracentrifugation, "light," "heavy," and "extra heavy" fractions we show that no P-gp is found in the "light" fraction where rafts are usually detected. Altogether, our data strongly suggest that P-gp is not localized in rafts.

MATERIALS AND METHODS

Cell Lines and Culture

K562 leukemia cells and P-glycoprotein expressing K562/ADR cells (Lozzio and Lozzio, 1975) were cultured in RPMI 1640 (Sigma, St Louis, MO) medium supplemented with 10% fetal calf serum (Biomedia, Boussens, France) at 37°C in a humidified incubator with 5% CO_2. Every month, resistant K562/ADR cells were cultured for three days with 400 nM doxorubicin. The cell line was then used, 1 week later, during 3 weeks. Before each experiment the P-gp expression stability was checked by measuring the P-gp functionality, i.e., the rate of the P-gp-mediated efflux of pirarubicin (see later). Under these conditions, P-gp activity did not change. Cell cultures used for experiments were split 1:2 one day before use in order to assure logarithmic growth. Cells (10^6/mL; 2 mL/cuvette) were energy-depleted via preincubation for 30 min in Hepes buffer with sodium azide but without glucose.

Drugs and Chemicals

Purified doxorubicin (DOX), daunorubicin (DNR), idarubicin (IDA), and pirarubicin (PIRA) were kindly provided by Laboratoire Pharmacia-Upjohn. Concentrations were determined by diluting stock solutions to approximately 10^{-5} M with $\varepsilon 480 = 11500\ M^{-1}\ cm^{-1}$. Stock solutions were prepared just before use. Methyl-β-cyclodextrin (MβCD) (mean degree substitution: 10.5–14.7), horseradish peroxidase (HRP), cholesterol oxidase (COase), filipin, and Triton X-100 (TX) were from Sigma and were dissolved in water. 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was from Molecular Probes (Eugene, OR). C219 anti-P-gp antibody was from DAKO (Carpinteria, CA). Other chemicals were from the highest grade available Before the experiments, the cells were counted, centrifuged and resuspended in Hepes buffer solutions containing 20 mM Hepes plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ at pH 7.3, with or without 5 mM glucose. All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments.

Determination of the Cholesterol Content of K562 cells

Nonesterified cholesterol assay was adapted from a spectrofluorometric method used in the kit assay from Molecular Probe (Amundson and Zhou, 1999). Briefly,

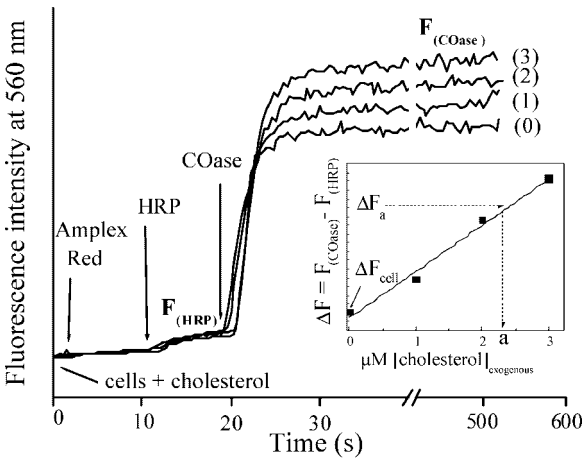

Fig. 1. Spectrofluorometric method for the quantification of the cellular nonesterified cholesterol. The fluorescence intensity at 585 nm ($\lambda_{ex} = 560$ nm) was recorded when the following compounds were added to cells (10^6/mL) suspended in Hepes buffer: (1) a micromole cholesterol with $a = 0, 1, 2$, or 3; (2) 50 μM Amplex Red; (3) 0.5 U/mL HRP; (4) 0.1 U/mL COase. $\Delta F = F_{(COase)}$-$F_{(HRP)}$ represents the increase of fluorescence intensity due after the addition of COase. Inset, $\Delta F = F_{(COase)}$-$F_{(HRP)}$ has been plotted as a function of the exogenous cholesterol concentration (a) added to the cells, the slope is $\rho = (\Delta F_a - \Delta F_{cell})/a$ where ΔF_{cell} and ΔF_a are the variation of fluorescence in the absence and in the presence of a micromole exogenous cholesterol. The endogenous molar concentration of cholesterol in the assay is then $C = a \times 10^{-6} \times \Delta F_{cell}/(\Delta F_a - \Delta F_{cell})$ and the number of mole of cholesterol per cell is 4×10^9. C (see Material and Method section).

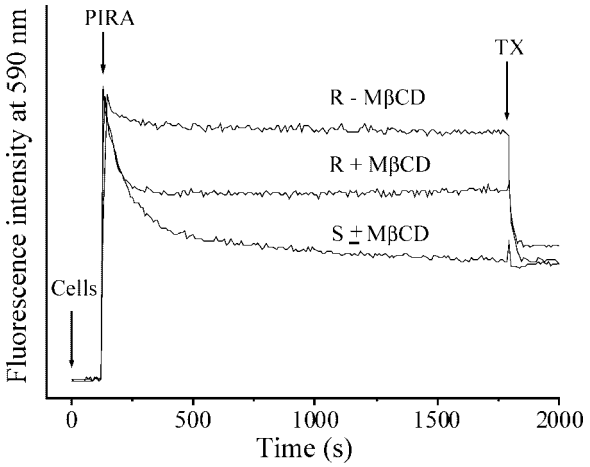

Fig. 2. Time course of uptake of pirarubicin (PIRA) by K562 cells after incubation with MβCD. Cells either sensitive (S) or resistant (R), 10^6/mL, were incubated with 0 or 10 mM MβCD for 10 min. Cells were then centrifuged as was explained in the Materials and Methods section and incubated with 1 μM PIRA. The fluorescence intensity at 590 nm ($\lambda_{ex} = 480$ nm) was recorded as a function of the time of incubation. Arrow indicates the addition of 0.05% (w/v) triton X-100.

cells, 2.5×10^6/mL, were washed once with PBS buffer and suspended in 1 mL of reaction buffer. The reaction buffer at pH 7.4 contained 0.1 M PBS, 0.05 M NaCl, 0.1% TX. Samples were incubated at 37°C for 15 min, followed by three times 30-s sonication on ice and then one additional hour incubation at 37°C under continuous stirring. Unless otherwise indicated, 160 μL of this sample were added to the reaction buffer (total volume 1.6 mL) and the fluorescence signal at 560 nm ($\lambda_{ex} = 585$ nm) was monitored continuously when the following reactant were added: 50 μM Amplex Red, 0.5 U/mL HRP, 0.1 U/mL COase. The concentration of cholesterol in the solution was proportional to the difference of the fluorescence signal $\Delta F = F_{COase}$-F_{HRP} (Fig. 1). Because the interaction of the cell suspension with HRP yielded a small change in the fluorescence signal (not shown), standard was systematically carried out in the presence of 160 μL of sonicated cells suspension to which 0 to 10 μM of cholesterol solution was added. The curve $\Delta F = F_{COase}$-F_{HRP} against the exogenous cholesterol concentration was linear within the range 0–5 μM. Cholesterol titration was not affected by the presence of MβCD at concentrations used in this study.

Cellular Anthracycline Accumulation

The rationale and validation of our experimental setup for measuring the kinetics of the transport of anthracyclines in tumor cells has been extensively described and discussed before (Frézard and Garnier-Suillerot, 1991a,b; Borrel *et al.*, 1994; Mankhetkorn *et al.*, 1996; Marbeuf-Gueye *et al.*, 1999). It is based on the continuous spectrofluorometric monitoring (Perkin Elmer LS50B spectrofluorometer) of the decrease in the fluorescence signal of anthracycline at 590 nm ($\lambda_{ex} = 480$ nm) after incubation with the cells in a 1-cm quartz cuvette (Fig. 2). The decrease in fluorescence occurring during incubation with cells is due to the quenching of fluorescence after intercalation of anthracycline between the base pairs of DNA. We have previously shown that this methodology allows the accurate measurement of the free cytosolic concentration of anthracyclines under steady-state conditions, their initial rates of uptake, and kinetics of active efflux (Frézard and Garnier-Suillerot, 1991a,b; Borrel *et al.*, 1994; Mankhetkorn *et al.*, 1996; Marbeuf-Gueye *et al.*, 1999). This methodology was used to check the effect of the treatment of cells with MβCD, filipin and COase on membrane permeability. For this purpose doxorubicin was used because the rate of uptake of doxorubicin by cells being very low (Kimmich *et al.*, 1975), the impact of membrane permeabilization by any compound can be easily detected. Cells were thus treated with these different compounds, as explained later, and the decrease

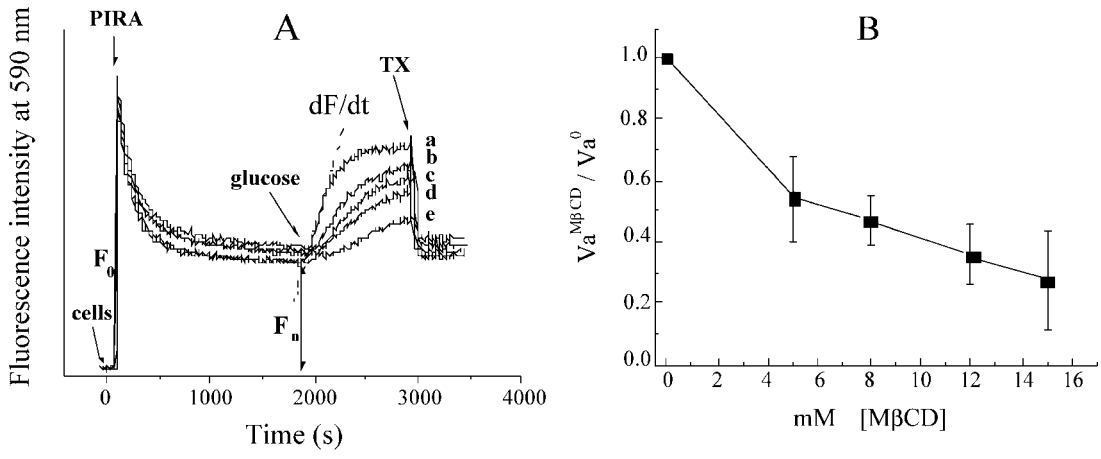

Fig. 3. Incorporation of pirarubicin in energy-depleted cells K562/ADR cells and determination of the active efflux rate (V_a). Cells, 10^6/mL, were incubated for 10 min with MβCD at concentrations equal to 0 mM (a), 5 mM (b), 8 mM (c), 12 mM (d), and15 mM (e). Cells were then centrifuged as explained under Materials and Methods and incubated with 1 μM PIRA. (A) The fluorescence intensity at 590 nm ($\lambda_{ex} = 480$ nm) was recorded as a function of the time of incubation of cells with PIRA. The active efflux rate (V_a) was determined from dF/dt after the addition of glucose. (B) The ratio $V_a^{\beta MCD}/V_a^0$, has been plotted as a function of the MβCD concentrations. $V_a^{\beta MCD}$ and V_a^0 are the rate of P-gp-mediated efflux of daunorubicin in the presence and absence of MβCD respectively. The points are the mean $\pm$ SD of three independent experiments.

of the fluorescent signal was recorded as a function of time.

Determination of the P-gp-Mediated Efflux of Anthracyclines

Cells (1×10^6/mL; 2 mL/cuvette) were preincubated for 30 min in HEPES buffer with 10 mM sodium azide, but without glucose (energy-deprived cells). Depletion of ATP in these cells was 90%, as checked with the luciferin-luciferase test (Marbeuf-Gueye *et al.*, 1998). The cells remained viable throughout the experiment, as checked with Trypan blue and calcein vital stain (not shown). After addition of anthracycline, the decrease in the signal was monitored until steady state was reached. Since the pH of the buffer was chosen to equal the intracellular pH, at steady state the extracellular free drug concentration (C_e) was equal to the cytosolic free drug concentration (C_i). At this point, $C_i = C_e$ was calculated from the nonquenched fluorescence: $C_e = C_T(F_0 - F_n)/F_0$, where F_0 is the fluorescence of a C_T micromolar anthracycline solution and F_n, the fluorescence at steady state. Then glucose was added, which led to the restoration of control ATP levels within 2 min and to an increase in the fluorescence signal due to the efflux of anthracycline. Since under these conditions, at the moment of addition of glucose $C_i = C_e$, the passive influx and efflux were equal, the net initial efflux represents the P-gp-mediated active efflux only (Mankhetkorn *et al.*, 1996; Marbeuf-Gueye *et al.*, 1998), which was calculated $V_a = C_T/ F_0$ (dF/dt), where dF/dt is the slope of the tangent to the curve (see Fig. 3).

Treatment of Cells With MβCD

The methylated derivatives of β-cylodextrin are known to trap preferentially membrane cholesterol compared to other cyclodextrins, which have also affinity to phospholipids and proteins (Ohvo and Slotte, 1996). We then used a 10.5–14.7 times methylated β-cylodextrin (MβCD) to study the effect of cholesterol depletion on K562/ADR and K562 cells. Cells were grown as described. The standard culture medium was replaced with Hepes buffer to which 2–20 mM MβCD had been added. Unless otherwise stated, cells were then incubated for 30 min at 37°C. MβCD interacted with anthracycline and modified the fluorescence signal, therefore cells were washed with Hepes buffer and then transport activity was measured as described above.

Treatment of Cells With Filipin

To study the effect of filipin on P-gp functionality, transport activity was measured as described above with the difference that 1–10 μM filipin were added 10 min prior to the addition of glucose. The effect of filipin on the passive diffusion of doxorubicin was also determined.

Incorporation of Cholesterol into Cholesterol-Depleted Cells

Cells were treated with MβCD as described above to remove endogenous cholesterol. Cells were then incubated with 100 μM cholesterol in standard culture medium with gentle agitation for 30 min to 2 h at 37°C. The cells were then washed and resuspended in Hepes buffer and transport activity was measured as described above.

Treatment of Cells with Cholesterol Oxidase

K562/ADR cells overexpressing P-gp were pretreated at 37°C for 30 min with 0.5 U/mL COase in Hepes buffer.

Isolation of "Light" and "Heavy" Membrane Fractions

Purification of membrane domains was performed using a detergent free procedure. "Light" and "heavy" fractions were isolated according to the method of Monneron and d'Alayer (1978a,b) with the following modifications. K562/ADR cells, $10 - 15 \times 10^7$ cells, were washed two times with PBS buffer and suspended in this buffer, 2×10^6 cells/mL. They were then incubated for 30 min in the absence or presence of 15 mM MβCD at 37° under stirring. Cells were then again washed two times with PBS buffer. The pellet was suspended in 500 μL of sodium carbonate buffer at pH 9–10, and sonicated in a cold bath three times 30s (50 HZ, 117 v, and 80 W) (Vibra cell, Sonics & Materials, Inc., Danbury, CT). The lysate was mixed with 80% sucrose to yield 2 mL of 40% sucrose solution. This mixture was transferred to the bottom of the ultracentifugation tube (Beckman Instruments, Palo Alto, CA) and was overlaid with 3 mL 35% sucrose, 3 mL 22% sucrose, and 3 mL 10% sucrose. The weight of the ultracentrifugation tube was equilibrated with 10% sucrose and the gradients were inserted into a SW41 rotor. The ultracentrifugation was performed at 160,000 g for 14 h at 4°C. Light-scattering bands confined at the 10–22% sucrose and 22–35% sucrose interfaces respectively were observed. Eleven 1-mL fractions were collected by suction with a syringe from the top to the bottom. The velocity of aspiration was kept low in order to avoid disturbance of the sucrose layers. The first fraction was called F1 and the last fraction was called F11. The total protein concentration was measured in each fraction using the Bradford reagent (BC assay kit, Interchim France).

Western Blotting Measurement of P-Glycoprotein Expression

Equal volume (12 μL) of sucrose density gradient fractions were mixed with concentrated SDS reducing buffer (final concentrations are 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, bromophenol blue). Protein samples were separated on 7.5% SDS-PAGE then transferred onto PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The membrane was blocked with 5% nonfat dry milk in 0.1% Tween-PBS and treated with 0.5 μg/mL C219 anti-P-glycoprotein antibody (DAKO). Detection was carried out by enhanced chemiluminescence (ECL plus^R kit with mouse IgG, HRP linked whole antibody from Amersham Pharmacia Biotech). The P-gp expression has been evaluated after densitometric scanning of the films and analysis with Image J 1.30 software.

Localization of Ganglioside GM1

GM1 is commonly found in high concentrations in rafts (Harder *et al*., 1998), and it can be labeled using fluorescent FITC-conjugated cholera toxin B. Cholera toxin B (CTB) (Dietrich *et al*., 2001) binds to the glycosphingolipid GM1 and we used it as marker for raft localization in "light" and "heavy" plasma membrane fractions. We employed a CTB-horseradish peroxidase (CTB-HRP) conjugate for fast simple detection of ganglioside GM1. Briefly, after activation of a PVDF membrane with methanol and washes with water and 0.1% Tween-PBS (PBS-T), 4 μL of each gradients were dotted onto the wet membrane. The air-dried membrane was reactivated and blocked with 5% nonfat dry milk in PBS-T. After washes with PBS-buffer, the membrane was incubated with HRP conjugated cholera (dilution 1:5000, Sigma) in PBS-T for 90 min, rinsed several times with PBS-buffer and then detected by enhance chemiluminescence (Amersham Pharmacia Biotech) and analyzed with Image J 1.30 software.

RESULTS

Nonesterified Membrane Cholesterol in Sensitive and Resistant cell Lines

Cholesterol content in K562 and P-gp overexpressing cell lines K562/ADR was similar and equal to $2.7 \pm 0.4 \times 10^{-14}$ mol/cell. However, the rate of cholesterol depletion by MβCD was slightly different. This can be seen in Table I in which the number of moles of cholesterol per

Table I. Percentage of Cellular Cholesterol Content After Treatment With Different MβCD Concentrations for Various Times

MβCD mM	% mol chol/cell after Δt = 10 min K562	% mol chol/cell after Δt = 10 min K562/ADR	% mol chol/cell after Δt = 15 min K562	% mol chol/cell after Δt = 15 min K562/ADR
0	100[a]	100	100	100
5	52	65	37	42
10	31	48	12	30
15	25	36	10	30

[a]Corresponds to $2.7 \pm 0.3 \times 10^{-14}$ mol/cell.

cell has been indicated after various times of incubation with different concentrations of MβCD.

Effect of MβCD Treatment on P-gp-Mediated Anthracycline Transport

The incubation of cells, either sensitive or resistant, with MβCD yielded an increase of the cell membrane permeability to the drugs. In other words, the rate of passive uptake of the drug in presence of MβCD, $V_+^{M\beta CD}$, is higher than the rate, V_+, in its absence. The slow diffusing anthracycline, DOX, was used to clearly check this point: the number of mole of DOX accumulated per sensitive cell after 1 h was $1.1 \pm 0.1 \times 10^{-15}$ mol/cell in the absence of MβCD and $3.9 \pm 0.3 \times 10^{-15}$ mol/cell after incubation for 1 h with 15 mM MβCD.

The incorporation of PIRA, IDA or DNR in resistant and sensitive cells was then measured in the presence of various MβCD concentrations, after different times of incubation. Figure 2 shows the data obtained when cells were incubated with 1 μM PIRA after incubation, for 10 min, with 0–10 mM MβCD. As can be seen, the amount of PIRA accumulated inside the resistant cells, at steady state, increased in a dose-dependent manner. No modification was observed for sensitive cells. As, in resistant cell, this parameter depends on both the rate of passive diffusion of the drug and on the rate of its P-gp-mediated efflux, it was difficult to determine an eventual impact of MβCD on the rate of P-gp-mediated efflux of DNR. For this reason, experiments were performed with energy-depleted resistant cells, which were incubated with MβCD as previously described. At steady state the incorporation of PIRA was the same as in sensitive cells (Frézard and Garnier-Suillerot, 1991a,b; Borrel *et al.*, 1994; Mankhetkorn *et al.*, 1996; Marbeuf-Gueye *et al.*, 1999). At this stage, the addition of 5 mM glucose yielded an increase of the fluorescent signal due to the P-gp-mediated efflux of the drug only, and as there was no gradient of concentration across the plasma membrane the effect of MβCD on passive PIRA diffusion had not to be taken into account. Figure 3A shows the records of such experiments performed after cells incubation with different MβCD concentrations. One observed a dose-dependent effect of MβCD on the rate of P-gp-mediated efflux of PIRA (Fig. 3B). Similar experiments were performed with other anthracyclines, IDA and DNR, and analogous data were obtained. These experiments clearly show that the pump functionality is highly sensitive to cholesterol removal.

To determine whether V_M and/or K_m were modified by cholesterol depletion, we have measured the rate of P-gp-mediated efflux of pirarubicin, V_a, as a function of intracellular pirarubicin concentration, (i) in intact cells, (ii) in cells that had been incubated with 15 mM MβCD for 10 min and which, according to the above data, contained $50 \pm 5\%$ of cholesterol versus untreated cells. The variation of V_a as a function of C_i is shown in Fig. 4. The

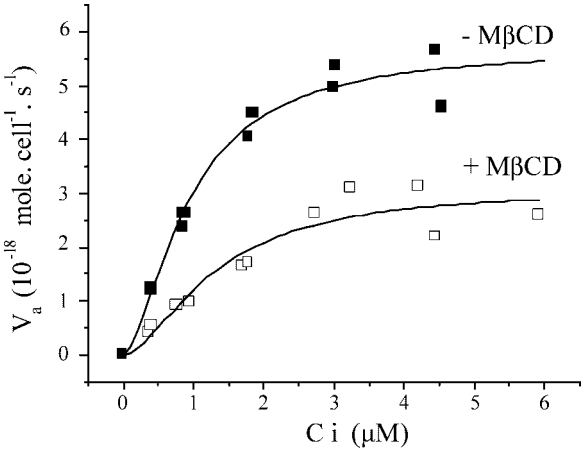

Fig. 4. Rate of the P-gp-mediated efflux of pirarubicin plotted as a function of the intracellular free pirarubicin concentration. The cells were incubated without (full square) or with (empty square) 15 mM MβCD for 10 min. Data points are from 3–5 independent experiments performed on different days. V_a and Ci were determined as described under materials and methods. The data, from a typical experiment, were fitted using equation (1).

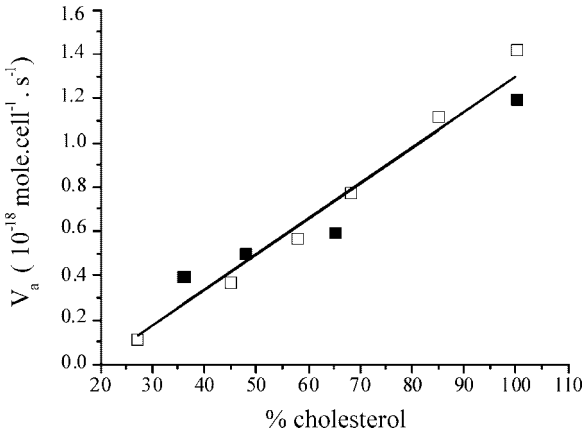

Fig. 5. Rate (V_a) of the P-gp-mediated efflux of PIRA as a function of the percentage of cholesterol in the cells. Cholesterol depletion was obtained through incubation of cells for 10 min with various MβCD ranging from 5 to 10 mM (full square). Cholesterol repletion was performed through incubation of the cells in culture medium in the presence of exogenous cholesterol (open square).

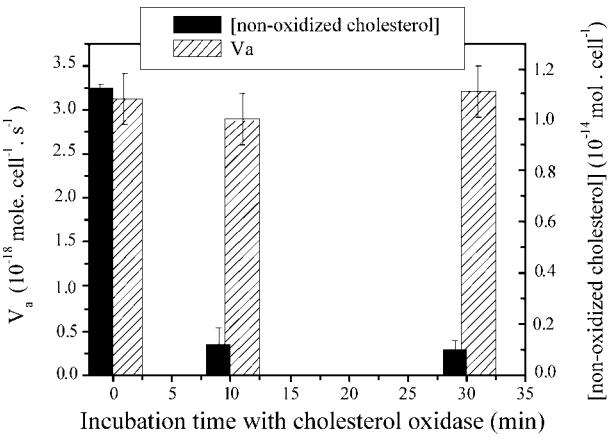

Fig. 6. Effect of cholesterol oxidase on the rate of P-gp-mediated efflux of pirarubicin and on the cellular cholesterol content. Cells, 10^6/mL, were incubated for 0, 10, and 30 min with 0.1 U/mL cholesterol oxidase.

relation between V_a and C_i is

$$V_a = V_M \cdot C_i^h / \left(K_m^h + C_i^h\right) \quad (1)$$

A curve fitting of the data of Fig. 4 with Eq. (1) yielded $V_M = (5.7 \pm 0.4) \times 10^{-18}$ mol/cell/s, $K_m = 0.93 \pm 0.13$ μM, $h = 1.6 \pm 0.4$ in untreated cells and $V_M = (3.1 \pm 0.5) \times 10^{-18}$ mol/cell/s, $K_m = 1.3 \pm 0.4$ μM, $h = 1.7 \pm 0.4$ in MβCD treated cells. The two V_M values are significantly different whereas the two K_m values are similar within the limit of incertitude.

To determine if this decrease of the P-gp functionality was reversible, we studied the effect of cholesterol incubation after treatment of cells with MβCD. We observed that when cells, cholesterol-depleted through incubation with MβCD, were incubated with cholesterol in culture medium the P-gp-mediated efflux of PIRA was almost fully restored. The cholesterol content of the cells was also determined after such treatment. Figure 5 shows the plot of the rate of P-gp-mediated efflux of PIRA as a function of the cholesterol content of the cells (i) during cholesterol depletion of the cells through MβCD action and (ii) during cholesterol repletion. A very interesting data is that the rate of the P-gp-mediated efflux of pirarubicin is closely correlated to the cholesterol content of the cells.

Effect of Cholesterol Oxidation on P-gp Activity

In a second approach, membrane cholesterol was chemically modified using cholesterol oxidase. The rate of P-gp-mediated efflux of PIRA was measured on cells without any treatment ($t = 0$) and after incubation with 0.5 U/mL COase during 10 and 30 min respectively. The amount of nonoxidized cellular cholesterol was also measured under the same experimental conditions. Figure 6 shows that the incubation of cells with COase gave rise to the oxidation of about 90% of cholesterol without yielding modification of V_a. Therefore, we can infer that the P-gp activity does not depend on the oxidation state of cholesterol.

Effect of Filipin on P-gp Functionality

In another approach, filipin, a cholesterol binding fluorochrome, was used to assess the dependence of P-gp functionality on cholesterol. Within the membrane, filipin forms complex with cholesterol therefore altering the cholesterol repartition. The effect of a 10 min-filipin treatment on both the passive diffusion of DOX and the P-gp-mediated efflux of PIRA was measured. Figure 7 shows that the efflux rate of PIRA was totally inhibited at 2 μM without increase of the membrane permeability to DOX. At higher concentrations, filipin drastically increased the permeability of the cells likely because of a detergent or pore forming effect.

Assignment of the Source of Membrane Fractions

Digitalization of the images allowed the determination of GM1 and P-gp present in the different fractions (Fig. 8). To determine the effect of MβCD on the integrity of rafts, K562/ADR cells were treated for 30 min in the absence or in the presence of 15 mM MβCD. Low density membrane fractions were then isolated, and the distribution of GM1 and P-gp was assessed by Western

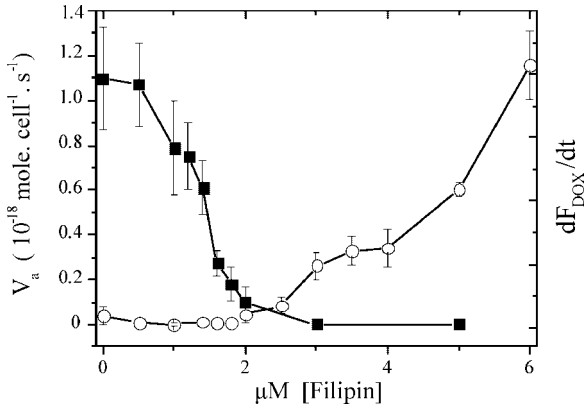

Fig. 7. Effect of filipin on the rate of P-gp-mediated efflux of pirarubicin and on the membrane permeability to doxorubicin. Energy-deprived resistant cells, 10^6/mL, were incubated with 1 μM pirarubicin. After 20 min, filipin, at concentration ranging from 0 to 6 μM, was added, i.e., 10 min before the addition of glucose, and the rate of P-gp-mediated efflux of PIRA was measured (full square). Resistant cells, 10^6/mL, were incubated with 1 μM doxorubicin. After 20 min filipin, at concentration ranging from 0 to 5 μM was added, and after 10 min the slope *dF/dt* of the decrease of the DOX fluorescence was measured (open circle).

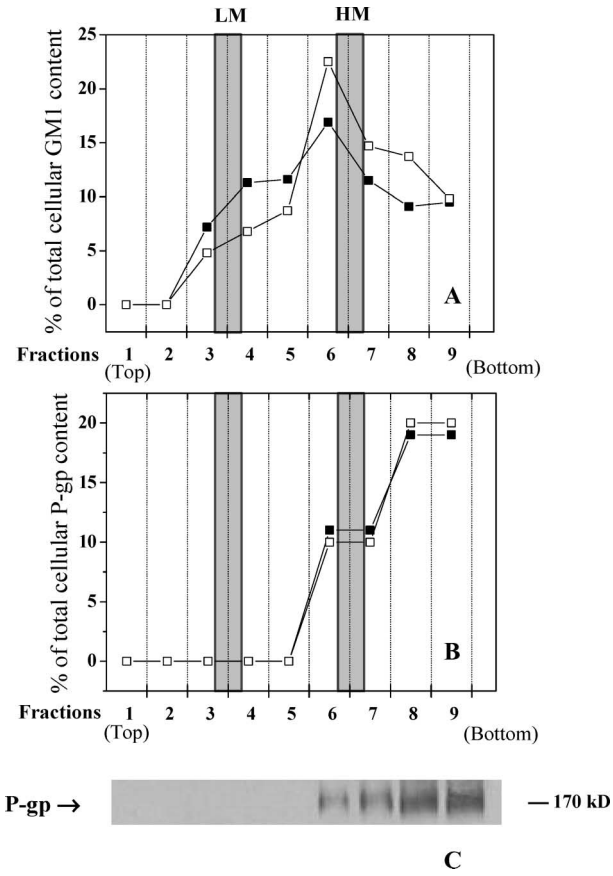

Fig. 8. Detection of GM1 and P-gp in K562/ADR cell lysates. K562/ADR cells were lysed by sonication, before (full square) or after (empty square) treatment with MβCD. The lysate was separated by density centrifugation and collected from the top in 1 mL fractions. Fraction 1 is from the top of the gradient. (A) GM1 content and (B) P-gp expression have been analysed with Image J v1.30 software. (C) Immunodetection of P-gp in the absence of treatment with MβCD. Fractions 3–4 (LM, dashed) and fractions 6–7 (HM, dashed) contain the 10/22% and 22/35% sucrose interface respectively.

and dot blotting of the analytical density gradients. Fraction 1 represents the top of the gradient, and fraction 11 is the bottom of the gradient. Fractions 3–4 and fractions 6–7 contain the 10/22% and 22/35% sucrose interface respectively. Fractions 3–4 were found to have low concentrations of proteins, about nine times less than in fractions 6–7. In untreated cells, about 25% of the GM1was found in fractions 3–4, 34% in fractions 6–7 and 41% in fraction 8–11 corresponding to the cytoplasm and pellet. After mild treatment with MβCD (15 mM, 30 min) the amount of GM1 present in fractions 3–4 decreased to ~16% whereas in fractions 6–7 it increased up to ~43%. Following more drastic treatment, (30 mM, 1 h) GM1 was lost from the low density region of the gradient and recovered instead in the high density region of the gradient. We therefore identified fraction 3–4 as the raft fractions. No significant amount of P-gp was detectable in fraction 3–4, 20% was recovered in fractions 6–7 and the rest in fractions corresponding to cytoplasm and pellet. Treatment with MβCD did not yield modification of this distribution.

DISCUSSION

Domains rich in cholesterol and sphingomyelin have been subject of great interest recently in cell biology because some important integral membrane proteins may be preferentially located within them. Such domains that form around the protein caveolin, referred to as caveolae, have been unambiguously shown to exist (Anderson, 1998). When caveolin is not present, the domains are known as rafts. Raft association of proteins can be assayed by manipulating the lipid composition of rafts. If cholesterol or sphingomyelin are depleted from membrane, lipid rafts are dissociated, and previously associated proteins are no longer in rafts (Simons and Toomre, 2000).

In terms of their cholesterol metabolism and transport, most oncogenically transformed and cancer cells have been classified as type A cells, i.e., cells having many cell surface lipoprotein receptors but few, if any, caveolae and in which regulation of cholesterol homeostasis occurs at the level of the influx (Liscovitch and Lavie, 2000). However, recent results have shown that MDR cancer cells express very high caveolin levels and exhibit a high surface density of caveolae (Lavie *et al.*, 1998). One

immediate implication of the up-regulation of caveolin and caveolae in MDR cells is that during acquisition of the MDR phenotype the cells revert from being cholesterol homeostasis type A cells to cholesterol homeostasis type B cells (i.e., cells having few cell surface lipoprotein receptors but many caveolae and in which regulation of cholesterol homeostasis occurs at the level of the efflux). Being type B cells, MDR cancer cells must have an active cholesterol active pathway and therefore one question is: does P-gp play a role in cholesterol transport and/or metabolism? Actually, a recent paper has proposed a coupling between the basal ATPase activity of P-gp and its intramembrane cholesterol-redistribution function consistent with the possibility that P-gp may actively translocate cholesterol in the membrane (Garrigues *et al*., 2002). This P-gp-mediated cholesterol redistribution in the cell membrane makes it likely that P-gp contributes in stabilizing the cholesterol-rich microdomains, rafts and caveolae, and that it is involved in the regulation of cholesterol trafficking in cells.

Given that the functioning of P-gp is intimately connected with the membrane, we might expect the transport properties of P-gp to be strongly influenced by the physical properties (composition) of the bilayer. The K562/ADR cells used in this work do not express caveolin and therefore no caveolae are expected (Mannechez *et al*., in press). However the possibility that P-gp could be localized within rafts still remains and in the present work, our goal was to check this possibility and to explore the effect of lipid environment on the rate of ATP-driven drug transport by P-gp in intact cells. For this purpose we have used different approaches to affect the putative microdomains at the cholesterol level. In a first set of experiments, in order to determine whether cholesterol affects P-gp function, we used MβCD to extract cholesterol from the lipid phase of intact living cells. MβCD is a highly hydrophilic cyclic oligosaccharide that specifically binds sterol, rather than other membrane lipids, to form water-soluble complexes (Ohvo and Slotte, 1996), without causing further membrane perturbation by insertion (Gimpl *et al*., 1997). This treatment gave rise to a strong decrease of the P-gp-mediated efflux of anthracycline. This was reversible and repletion of cells with cholesterol restored the P-gp functionality. Interestingly, the P-gp functionality, which was quantified by the V_a measurement, was quasi linearly linked with the amount of nonesterified cholesterol present in the cells down to about 30% (see Fig. 5). It must be remind that about 90% of nonesterified cholesterol is localized in the plasma membrane (Lange *et al*., 1993). On the other hand, when cells cholesterol content was decreased to about 50% of the initial value, the decrease of the rate of P-gp-mediated efflux of pirarubicin was due to a decrease of the V_M value whereas the K_m value does not change significantly. This suggest that the site of binding of pirarubicin to P-gp is not strongly affected by 50% cholesterol removal.

In a second set of experiments, filipin which is known to complex cholesterol within the membrane and therefore to destroy the rafts was added to the cells. Here also the P-gp-mediated efflux of anthracycline was strongly affected, the rate being close to 0 at 2 μM filipin. However, in this case, the inhibition of the P-gp-mediated efflux of pirarubicin due to a specific filipin-transporter interaction cannot be excluded.

Treating cells with MβCD to deplete them with cholesterol is the common method to eliminate biological rafts, but it is a harsh treatment. The use of COase appears to be a gentler procedure (Samsonov *et al*., 2001): COase converts cholesterol to cholestenone and because cholestenone does not possess a 3β-hydroxyl group, it does not interact with sphingomyelin. Recently, it has been shown that application of COase eliminated rafts in bilayer membrane (Samsonov *et al*., 2001). In addition, the whole cholesterol present in the plasma membrane is rapidly oxidized by the action of extracellular COase, the transbilayer diffusion (flip-flop) of cholesterol proceeding with a half time of <1 s at 37°C (Steck *et al*., 2002). It must also be emphasized that the oxidation of cholesterol to cholestenone does not modify the membrane fluidity (Gimpl *et al*., 1997). Therefore, in a third set of experiments cholesterol was oxidized by action of COase. No modification of the efflux was observed.

These last sets of experiments strongly suggest that P-gp is not localized in rafts and it is likely that the decrease of the P-gp-functionality observed with cholesterol depletion is due to an increase in membrane fluidity. The promotion of ordered lipid phases within bilayers is a characteristic effect of cholesterol incorporation and such domains may modulate the function of P-gp. Actually, many membrane transporters show very low, or negligible, rates of transport when the host lipid bilayer is in the rigid gel phase, i.e., below *T*m. In contrast, it has been shown by Lu *et al*. (2001) that the rate of tetramethylrosamine, a rhodamine derivative, transport by P-gp in PamMyrGroPCho bilayers is actually at its highest in the gel phase, below 28°C. These authors have also reported that P-gp catalyzed ATP hydrolysis quite well in the gel phase, and the energy activation value is substantially lower than that measured in liquid crystalline bilayers (Romsicki and Sharom, 1998). Overall, P-gp-mediated transport of tetramethylrosamine displayed highly unusual temperature-dependence characteristics. Thus, the uptake of [^{3}H]colchicine into reconstituted proteoliposomes of Myr2Gro*P*Cho is more than twofold higher at the *T*m of

24°C than in the fluid liquid crystalline phase at 32°C (Sharom, 1997). On the other hand, it has been shown that the addition of chemical fluidizing agents led to a decrease in P-gp-mediated transport of vinblastine and DNR in canalicular membrane vesicles (Sinicrope *et al*., 1992) of calcein-AM and pirarubicin in MDR cells (Regev *et al*., 1999; Borrel *et al*., 1995).

Concerning cholesterol and P-gp functionality other authors have reported data that are at variance with ours. Luker *et al*. (2000) have studied the effects of cholesterol on P-gp localization and function in low-density membrane domains in different cell lines. They have observed that depletion of cholesterol with MβCD shows cell-type- and substrate-dependent effects on the transport activity of P-gp. Thus, depletion of cholesterol affects both function and inhibition of class I P-gp differently in CHO cells than in any of the cells expressing human MDR1 P-gp. It seems that such observation is not a general rule since, in the present work, we have used a human cell line and observed that depletion of cholesterol strongly affects P-gp functionality. On the other hand, Garrigues *et al*. (2002) have observed that in cholesterol-depleted MDR vesicles, P-gp exhibits an ATPase activity in the presence of various P-gp substrates, such as verapamil or progesterone, which stimulate P-gp ATPase activity in native membranes. They have concluded that therefore, P-gp is fully functional in cholesterol-depleted MDR vesicles and is able to interact with its substrates.

To support our data, we have also performed experiments to localize the transporter in relation to rafts. It is widely believed that rafts can be isolated from biological membranes by detergents such as TX that solubilizes the lipids and proteins of the fluid regions and leaves rafts as large, detergent-resistant membrane fragments (Yu *et al*., 1973; Brown and Rose, 1992; London and Brown, 2000). However, Heerklotz *et al*. (2003) have recently performed a thermodynamic description of the interactions governing the formation or disintegration of domains and shown that unfavorable interactions between TX and sphingomyelin in a mixed membrane drive the separation of these molecules into separate domains above a critical TX content. This result is in excellent agreement with the phenomenon of TX-induced raft formation reported by Heerklotz (2002). On the other hand, Luria *et al*. (2002) have shown that light membrane fractions isolated from *Xenopus* egg plasma membrane in a detergent-free environment have properties similar to those of detergent-resistant membranes. For these reasons we chose to isolate membrane fractions without detergent extraction. It should be noticed that we have found comparable amount of GM1 in "light" and "heavy" membranes. However, only the one present in light fraction was modified by MβCD treatment. On the other hand, we have found a high percentage of P-gp in the cytoplasm fraction of the cells. This was not unexpected as similar observation has been made by Labroille *et al*. (1998).

In conclusion, from our experiments we can infer that in K562/ADR cells P-gp is not localized in raft. However, cholesterol depletion strongly affects the rate of P-gp-mediated efflux of anthracycline that can be assigned to an increase in membrane fluidity.

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