Protein-Mediated Inward Translocation of Phospholipids Occurs in both the Apical and Basolateral Plasma Membrane Domains of Epithelial Cells†

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ABSTRACT: The translocation of spin-labeled analogues of phosphatidylcholine (4-doxylpentanoyl-PC, SL-PC), phosphatidylethanolamine (SL-PE), phosphatidylserine (SL-PS), and sphingomyelin (SL-SM) from the outer to the inner leaflet of the plasma membrane bilayer was investigated in dog kidney MDCK II and human colon Caco-2 cells. Disappearance from the outer leaflet was assayed using back-exchange to serum albumin. Experiments with cells in suspension as well as with polarized cells on filters were performed at reduced temperatures (10 and 20 °C) to suppress endocytosis and hydrolysis of spin-labeled lipids. For both epithelial cell lines, a fast ATP-dependent inward movement of the aminophospholipids SL-PS and SL-PE was found, while SL-SM was only slowly internalized without any effect of ATP depletion. The kinetics of redistribution of SL-PC were clearly different between the two cell lines. In MDCK II cells, SL-PC was rapidly internalized in an ATP-dependent and *N*-ethylmaleimide-sensitive manner and at a rate similar to that of the aminophospholipids. In contrast, in Caco-2 cells the inward movement of SL-PC was much slower than that of the aminophospholipids, did not depend on ATP, and was not *N*-ethylmaleimide-sensitive. Inhibitor studies indicated that the outward-translocating multidrug resistance P-glycoprotein present in these cells did not affect the kinetics of inward translocation. Internalization was always similar on the apical and basolateral cell surface, suggesting the presence of the same phospholipid translocator(s) on both surface domains of epithelial cells. We propose that Caco-2 cells contain the well-known aminophospholipid translocase, while MDCK II cells contain either two translocases, namely, the aminophospholipid translocase and a phosphatidylcholine-specific translocase, or one translocase of a new type, translocating aminophospholipids as well as phosphatidylcholine.

Our knowledge of the transbilayer distribution and dynamics of phospholipids in mammalian plasma membranes has grown significantly in the past two decades. The following picture has emerged. The plasma membranes of many cells have a clearly defined transbilayer phospholipid asymmetry (*20*, *23*, *46*, *47*). The aminophospholipids phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) are preferentially located in the cytoplasmic leaflet, while the choline-containing lipids phosphatidylcholine (PC) and sphingomyelin (SM) are found predominantly in the exoplasmic leaflet. Maintenance of membrane phospholipid asymmetry in those cells is most likely controlled by a specific carrier protein, the aminophospholipid translocase, which selectively transports PS and PE from the exoplasmic to the inner leaflet of the plasma membrane at the expense of cytoplasmic ATP. The choline-containing phospholipids (PC and SM) are not recognized by this protein and, under normal conditions, traverse the plasma membrane only slowly by passive diffusion. Under some conditions, however, for example, during activation of blood platelets, PC and SM can rapidly translocate to the inner leaflet presumably via the action of a scramblase (*49*, *50*). In addition, the multidrug resistance gene product mdr2/MDR3 P-glycoprotein can translocate PC from the inner to the outer leaflet of the bile canalicular plasma membrane in hepatocytes (*25*, *31*, *32*, *39*), while the drug transporter MDR1 P-glycoprotein translocates a variety of lipid analogues from the inner to the outer leaflet of the apical membrane (*2*, *39*, *40*). Finally, the multidrug resistance-associated protein MRP1 was found to translocate sphingolipid analogues to the basolateral surface of epithelial pig kidney cells overexpressing the multidrug transporter (R. Raggers, personal communication).

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[1] Abbreviations: BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; EPR, electron paramagnetic resonance; HBSS, Hanks' balanced salt solution without bicarbonate, with 10 mM Hepes (pH 7.4); HBSS-G, HBSS containing 20 mM glucose and 1 mM sodium pyruvate; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PS, phosphatidylserine; SL-, spin-labeled at C2, 4-doxylpentanoyl; SM, sphingomyelin.

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Little is known about the transverse motion of lipids and the involvement of proteins in the apical and basolateral plasma membrane domain of epithelial cells under normal conditions. In these cells, the plasma membrane is divided into two domains, the apical and the basolateral, which differ not only in protein but also in lipid composition. The exoplasmic leaflet of the apical domain facing the external environment has an unusually high sphingolipid content. Up to 90% of the exoplasmic leaflet of the apical membrane is composed of sphingomyelin and glycosphingolipids (*28*, *44*). The basolateral membrane resembles the plasma membrane of nonpolarized cells, in lipid composition (*43*), and presumably in transbilayer lipid distribution as well. The tight junctions, specific zones of cell−cell contact which encircle each cell, act as a barrier to solutes and water between the external environment and the internal milieu of the body (*4*). In addition, they form a barrier to lateral lipid diffusion in the exoplasmic but not in the cytoplasmic leaflet of the plasma membrane (*5*, *6*, *42*). Thus, the differences in lipid composition between the apical and basolateral domain must reside in the exoplasmic leaflet, where mixing is prevented by tight junctions.

Whether proteins are involved in maintaining the transbilayer asymmetry in the plasma membrane of polarized epithelial cells is not known, although it should be noted that the multidrug resistance MDR1 P-glycoprotein is present in the apical plasma membrane domain of most epithelial cells (*35*).

In view of the unique lipid composition of the two plasma membrane domains, it was of interest not only to investigate whether epithelial cells exhibit a protein-mediated and ATP-dependent translocation of lipids from the outer to the inner leaflet of the plasma membrane but also to elucidate whether this activity is domain-specific. Recently, evidence for the existence of an aminophospholipid translocase activity in the apical domain of polarized bovine aortic endothelial cells has been presented (*12*, *13*). In the current study, we focus on two other major epithelial tissues and examine the transbilayer movement of phospholipid analogues in the plasma membrane of kidney-derived MDCK II and colon-derived Caco-2 cells. For this purpose, spin-labeled phospholipids with a short fatty acid chain at the C2 position bearing a paramagnetic nitroxide moiety (*26*) were incorporated into the plasma membrane. EPR measurements allow one to monitor membrane insertion of the analogues, to study the transbilayer movement of the analogues using back-exchange to BSA, and to detect acyl chain hydrolysis at the C2 position (*16*, *26*, *48*). The spin-labeled analogues used in this study have been shown to closely mimic the transbilayer distribution of endogenous phospholipids in erythrocytes (*18*, *22*, *36*). Our results suggest the presence of an aminophospholipid translocase in both plasma membrane domains of Caco-2 cells. In MDCK II cells, the aminophospholipid translocase is accompanied by a PC-specific translocase or a new type of translocase is present with a broad lipid specificity, translocating both aminophospholipids and PC.

MATERIALS AND METHODS

Culture media, antibiotic and antimycotic solutions, and serum were purchased from Biochrom KG (Berlin, Germany) and fatty acid-free bovine serum albumin (BSA), EDTA, trichloroacetic acid, 2-deoxyglucose, and *N*-ethylmaleimide (NEM) from Sigma (Deisenhofen, Germany); diisopropyl fluorophosphate (DFP) was purchased from Aldrich (Steinheim, Germany), and sodium azide and potassium cyanide were purchased from Fluka Chemie AG (Buchs, Switzerland). Hanks' balanced salt solution without bicarbonate (HBSS) was supplemented with 10 mM Hepes. Spin-labeled phospholipid analogues were synthesized as described previously (*8*, *18*). Cyclosporin A was kindly donated by Dr. B. Ryffel (Sandoz AG, Basel, Switzerland); PSC 833 was a kind gift of Sandoz Pharma bv (Uden, The Netherlands), and B8509-035 (dexniguldipine hydrochloride) was kindly provided by V. Gekeler (Byk Gulden, Konstanz, Germany).

Cell Culture. The epithelial kidney cell line MDCK strain II was grown in 175 cm^2 culture flasks (Nunc, Biochrom, Berlin, Germany) at 37 °C in 5% CO_2 using Eagle's minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 mM Hepes. Caco-2 cells (clone PD 7; *3*) were cultured in Dulbecco's Modified Eagle's Medium (high glucose and sodium bicarbonate levels) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 1 mM nonessential amino acids, and 10 mM Hepes. All culture media contained antibiotics and antimycotics: 100 units of penicillin, 100 μg of streptomycin, and 2.5 μg of amphotericin B per milliliter. Polarized cell monolayers were obtained by growing the cells to confluency on tissue culture-treated polycarbonate filter units with a 24 mm diameter and 0.4 μm pore size (Transwells, Costar, Bodenheim, Germany) over a period of 8 days. In the experiments with cells grown on plastic, cells were grown as 8-day-old confluent monolayers in 40 mm diameter Petri dishes.

Suspending of Cells. Cell monolayers grown in plastic flasks (175 cm^2) were rinsed with calcium- and magnesium-free HBSS, and cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA in calcium- and magnesium-free PBS for 10 min at 37 °C, resuspended in culture medium, dispersed by pipetting, and filtered through a nylon mesh (Falcon mesh with a 70 μm pore size, Becton Dickinson, Heidelberg, Germany) to remove cell aggregates. After 30 min on ice, the cell suspension was centrifuged and washed two times with HBSS at 2 °C. Finally, the cell pellet was resuspended in HBSS containing 20 mM glucose and 1 mM sodium pyruvate (HBSS-G). This prevented a decline in the cellular ATP level which was confirmed by the luciferin-luciferase assay (see below). Cells were then counted in a hemocytometer, diluted to a final concentration of 10^8 cells/mL, and immediately used for spin-labeling experiments.

ATP Depletion. Cell suspensions were incubated for 10 min at 37 °C in glucose-free HBSS containing 5 mM sodium azide and 50 mM 2-deoxyglucose. In experiments with filter-grown cells, filters were incubated for 30 min at 37 °C in glucose-free HBSS containing 50 mM 2-deoxyglucose followed by incubation for 15 min at 37 °C in glucose-free HBSS containing 2 mM potassium cyanide, 5 mM sodium azide, and 50 mM 2-deoxyglucose.

ATP Measurement. Samples of the cell suspension or cells on filters were added to a solution of 4% trichloroacetic acid (final concentration) containing 2 mM EDTA and stored at −20 °C. The samples were subsequently measured in a

BioOrbit-Luminometer using a luciferin-luciferase assay (Colora, Lorch, Germany).

Quantification of Cell Phospholipids. Cellular lipids were extracted by the method of Bligh and Dyer (*1*) and destructed with 70% perchloric acid for 30 min at 180 °C. The total phospholipid content was quantified by measuring the amount of phospholipid phosphorus (*24*). Filter-grown MDCK II cells and plastic-grown Caco-2 cells contained 40 ± 14 and 86 ± 4 nmol of phospholipid phosphorus per 10^6 cells (mean ± standard error of the estimate of three determinations), respectively.

Drug Incubations. Cells were preincubated with inhibitors of multidrug resistance MDR1 P-glycoprotein for 10 min at 37 °C. Cyclosporin A (10 μM) and PSC 833 (10 μM) were diluted from ethanolic, B8509-035 (dexniguldipine hydrochloride) (10 μM) from DMSO stocks. The MDR inhibitors were also present during the redistribution kinetics. Control (pre)incubation mixtures contained 0.1% ethanol.

Labeling of Cells in Suspension and EPR Measurements. Labeling of cells and measurement of redistribution of spin-labeled phospholipids (SL-PL) were adapted from the methods of Morrot et al. (*18*). Briefly, appropriate amounts of analogues (4.5 nmol of SL-PL for 3×10^7 cells which corresponds to about 0.4 and 0.2 mol % of the total cell phospholipid concentration in MDCK II and Caco-2 cells, respectively) in chloroform/methanol (1:1) were transferred to a glass tube, dried under nitrogen, and vortexed with the desired volume of HBSS-G. For labeling, 2 volumes of cell suspension was mixed at either 10 or 20 °C with 1 volume of label suspension representing time zero for the kinetic measurements on cells in suspension. The known amount of added analogues was set to 100%. Upon addition to a suspension of epithelial cells, spin-labeled phospholipid analogues inserted rapidly into the exoplasmic leaflet of the plasma membrane. The degree of insertion was assessed by measuring the amount of analogues still present in the external medium after centrifugation as well as by following the change of the EPR line shape (data not shown) and was essentially complete within 3 min irrespective of the temperature (10 or 20 °C).

For back-exchange of the analogues from the exoplasmic leaflet, 50 μL samples (3×10^6 cells) were mixed at given time points with 25 μL of ice-cold HBSS containing 10% (w/v) fatty-acid free BSA. After incubation for 1 min on ice, the suspension was centrifuged (13000*g* for 2 min). Fifty microliters of the supernatant was mixed with 5 μL of 100 mM potassium ferricyanide to reoxidize reduced lipid analogues. The amount of probe present in the supernatant which corresponds to lipid analogues originally present in the exoplasmic leaflet was determined from its electron paramagnetic resonance (EPR) spectrum measured using a Bruker ECS 106 instrument (Bruker, Karlsruhe, Germany) and compared to the known amount of added label (see above).

Hydrolysis of the spin-labeled analogues into lyso derivatives and free fatty acids resulted in the appearance of three narrow peaks superimposed on the anisotropic spectrum of BSA-bound probe. This narrow component corresponds to the cleaved spin-labeled fatty acid. Note that, because fatty acids rapidly cross membranes, spin-labeled fatty acids are also depeleted from the intracellular locations during back-exchange. The correct estimation of the amplitude of the anisotropic EPR component as well as the extent of hydrolysis was obtained using computer subtraction as described previously (*8, 18*).

All data presented refer to SL-PL in the BSA-containing supernatant. However, EPR spectra of cell pellets were also recorded after disruption of cells by freezing and addition of potassium ferricyanide to reoxidize reduced lipid analogues.

At the end of the experiments, cell viability was determined by trypan blue exclusion (final concentration of 0.5%). The percentage of nonviable cells did not exceed 7% even in experiments where cells were pretreated with chemical reagents (e.g., NEM).

Labeling of Cells on Filter Supports and EPR Measurements. Cells grown on polycarbonate filters were incubated twice in HBSS for 10 min at 37 °C, and then extensively washed with ice-cold HBSS to suppress all internalization processes. Before labeling, filters were tipped onto a tissue wetted with buffer to remove adhering fluid. Spin-labeled phospholipid analogues were incorporated into the exoplasmic leaflet of either the basolateral or the apical cell membrane at 0 °C by addition of 50 or 200 μL of the ice-cold label suspension (prepared as described above and containing 20 or 5 μM SL-PL) to the basal or apical side of the filter unit, respectively. The total amount of label used was 1 nmol per filter. After labeling for 5 min on ice, nonincorporated analogues were removed by washing (requiring 5 min) the filter supports with ice-cold HBSS. About one-third of the added analogues was incorporated into the exoplasmic membrane leaflet of either the basolateral or apical domain (data not shown). This amount of analogue which was determined by EPR spectroscopy (see below) was set to 100% for kinetic measurements. Following labeling and washing, cells were warmed to 20 °C by replacing the apical and basolateral medium with prewarmed HBSS-G (representing time zero for the kinetic measurements on filter-grown cells). The volume was 200 μL in the apical and 50 μL in the basolateral compartment.

The distribution of the analogues was determined by back-exchange to fatty acid-free BSA. The back-exchange to BSA was performed simultaneously on both domains to check whether the analogues moved from the labeled membrane domain to the outer leaflet of the nonlabeled domain during the time course of the experiment. The filters were removed from the basolateral medium (BM). The BM was collected. Filters were tipped with the basolateral side onto a tissue wetted with buffer to remove adhering fluid. Subsequently, the filters were placed with the basolateral side on a 50 μL droplet of HBSS with BSA (final concentration of 2% w/v) at 0 °C. Then, 50 μL of a 10% BSA solution in HBSS (final concentration of 2% w/v) was added to the apical medium. During the subsequent incubation at 0 °C for 10 min, all analogues from the exoplasmic leaflet of each plasma membrane domain were extracted to BSA. The basolateral BSA-containing medium was pooled with the corresponding BM medium. The apical medium was recovered. Both media were reduced to a volume of 50 μL by speed vac centrifugation. The filters were cut from their rings and incubated in 200 μL of SDS (10%) for 30 min at room temperature to recover analogues inside the cells. The amount of spin-labeled analogue present in the media (apical and basolateral) was determined by recording the respective EPR spectra and

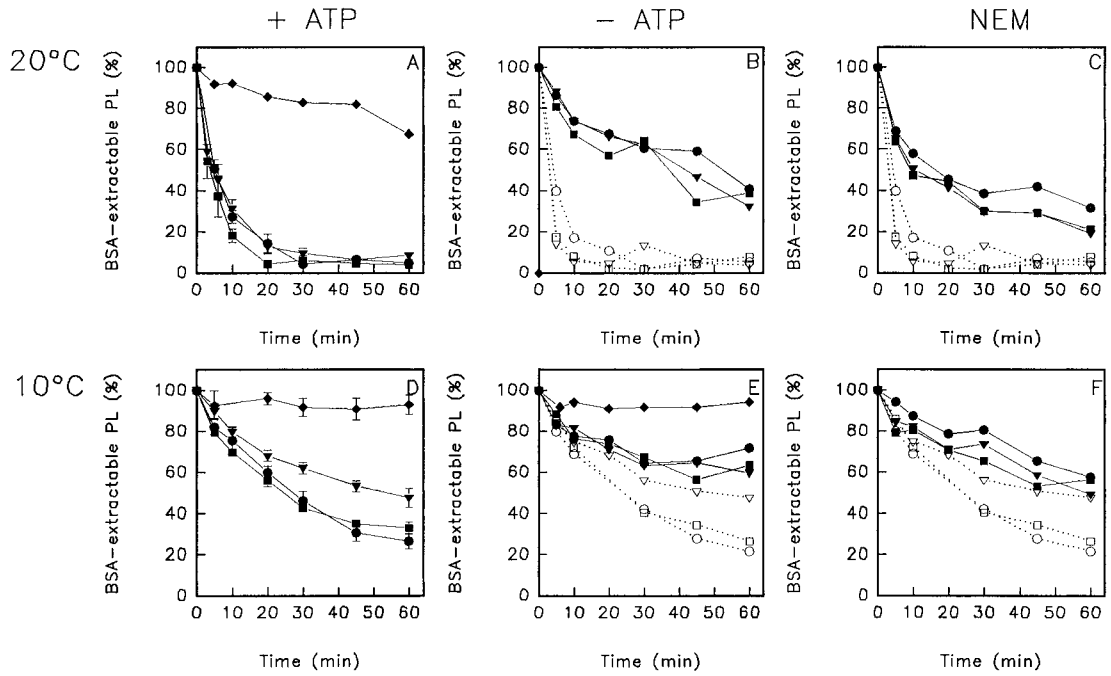

FIGURE 1: Internalization of spin-labeled phospholipid analogues in MDCK II cells. Effect of temperature, ATP depletion, and NEM treatment. At $t = 0$, cells in suspension were labeled with SL-PS (circles), SL-PE (squares), SL-PC (triangles), or SL-SM (diamonds). The amount of analogues present in the exoplasmic leaflet of the plasma membrane was assessed by back-exchange to BSA and expressed as the percentage of total label added at time zero (see Materials and Methods). (A and D) Control cells at 20 and 10 °C. The mean and standard error of three independent experiments are shown; results for SL-SM shown in panel A are from a single experiment representative of two independent experiments. (B and E) Prior to labeling, cells were incubated with 5 mM NaN_3 and 50 mM 2-deoxyglucose in HBSS for 10 min at 37 °C to deplete the level of cellular ATP. (C and F) Cells were pretreated with 2 mM NEM in HBSS-G for 15 min at 37 °C. Before labeling, cells were washed free of NEM. Data at 10 and 20 °C were obtained with independent preparations. For comparison, data for glycerophospholipid analogues obtained for nontreated control cells of the same preparation are included (open symbols and broken lines).

compared to the amount of label initially inserted into the membrane. To obtain the latter value, a cell monolayer was solubilized in SDS directly after labeling and removal of nonincorporated label by washing (see above). The EPR spectrum of the sample was recorded. At each time point of the redistribution kinetics, the amount of spin-labeled analogue present in the SDS-solubilized cell fraction after BSA treatment was determined by recording the respective EPR spectra. All EPR spectra were recorded in the presence of the reoxidizing agent potassium ferricyanide (10 mM).

RESULTS

Epithelial Cells in Suspension

Transverse Redistribution of Spin-Labeled Analogues. In both cell lines, the spin-labeled aminophospholipids PS and PE disappeared rapidly from the exoplasmic leaflet at 20 °C (Figures 1A and 2A). Since BSA extracts all analogues from the exoplasmic monolayer of the plasma membrane, the inaccessible fraction reflects analogues that have been redistributed across the plasma membrane into cells; uptake of analogues by endocytosis is almost negligible at ≤20 °C (*38*). About 72 and 63% of SL-PS and 82 and 44% of SL-PE were internalized from the exoplasmic leaflet within 10 min by MDCK II and Caco-2 cells, respectively. In comparison to the aminophospholipid probes, the choline-containing analogue SL-SM disappeared significantly slower from the exoplasmic leaflet; less than 20% became inaccessible to BSA after 10 min. The kinetics of redistribution of the choline-containing analogue SL-PC were clearly different in MDCK II as compared to those of Caco-2 cells; in MDCK II cells, SL-PC was rapidly internalized at a rate similar to that of the aminophospholipids (Figure 1A), whereas in Caco-2 cells, the inward movement of SL-PC was faster than that of SL-SM but much slower than that of the aminophospholipids (Figure 2A).

During incubation of the cells at 20 °C, a narrow component appeared in the EPR spectrum of the BSA-containing media (not shown). This narrow component corresponds to the spin-labeled fatty acid presumably cleaved off by a phospholipase A_2 activity. The component can be easily separated from the anisotropic spectrum of the phospholipid analogues and quantified (see Materials and Methods). Less than 20% of the analogues of PS, PE, and PC were hydrolyzed in 10 min at 20 °C. Thus, the extent of hydrolysis within the initial phase is much lower than the amount of analogues redistributed from the exoplasmic leaflet. However, for longer incubation times, data on analogue redistribution across the plasma membrane of epithelial cells obtained at 20 °C should be treated with caution. About 50% of SL-PC, SL-PE, and SL-PS initially incorporated into the plasma membrane of MDCK cells were hydrolyzed within 30 min at 20 °C. In Caco-2 cells, 15% of SL-PC, 16% of SL-PE, and 38% of SL-PS were degraded within 30 min at this temperature. In contrast to the glycerophospholipids, the sphingolipid analogue SL-SM was not deacylated.

To determine the stability of the spin-label, we compared the sum of the EPR signal in the supernatant and in the pellet at time zero and 60 min after labeling of cells, more than

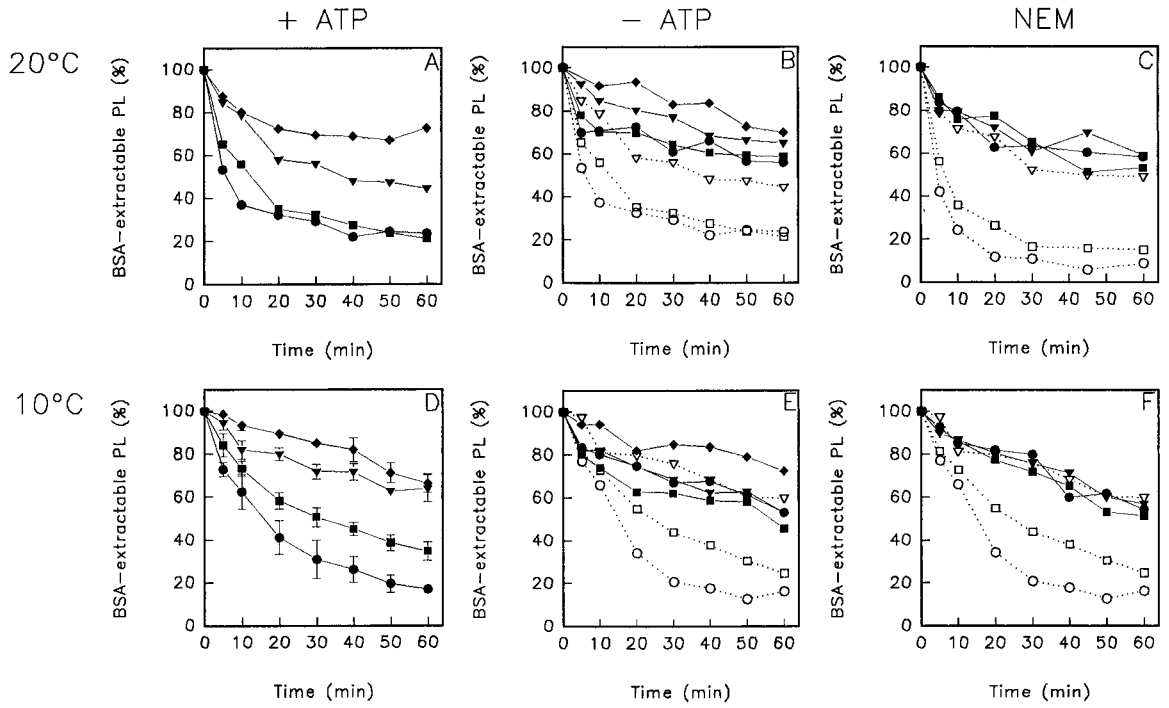

FIGURE 2: Internalization of spin-labeled phospholipid analogues in Caco-2 cells. Effect of temperature, ATP depletion, and NEM treatment. At $t = 0$, cells in suspension were labeled with SL-PS (circles), SL-PE (squares), SL-PC (triangles), or SL-SM (diamonds). The amount of analogues present in the exoplasmic leaflet of the plasma membrane was assessed by back-exchange assay and expressed as the percentage of total label added at time zero (see Materials and Methods). (A and D) Control cells at 20 and 10 °C. In panel A, results shown are for a representative experiment (one of at least two). The results shown in panel D represent the mean and standard error of at least three independent experiments. (B and E) Prior to labeling, cells were incubated with 5 mM NaN_3 and 50 mM 2-deoxyglucose in HBSS for 10 min at 37 °C to deplete the extent of cellular ATP. (C and F) Cells were pretreated with 2 mM NEM in HBSS-G for 15 min at 37 °C. Before labeling, cells were washed free of NEM. Data at 10 and 20 °C were obtained with independent preparations. For comparison, data for glycerophospholipid analogues obtained with nontreated control cells of the same preparation are included (open symbols and broken lines).

90% of the total label could be recovered after 60 min, indicating good stability of the spin-label. Note that the EPR signal in the supernatant results not only from SL-PL but, in the case of hydrolysis, also from spin-labeled fatty acid.

To diminish the extent of hydrolysis of phospholipid analogues, cells were incubated with 5 mM DFP prior to labeling. As shown previously for human erythrocytes (*18*) and found here for epithelial cells, DFP efficiently reduced the extent of hydrolysis of the glycerophospholipid analogues in both cell lines, except for SL-PS in Caco-2 cells at 20 °C (data not shown). Remarkably, DFP treatment was also accompanied by a fast reduction in cellular ATP content in MDCK II and Caco-2 cells to 50 and 75% of control cells, respectively, even after incubation for 5 min. However, at a lower temperature (10 °C), the extent of hydrolysis was significantly reduced for all glycerophospholipids in MDCK II cells and for SL-PE and SL-PC in Caco-2 cells (data not shown) even in the absence of DFP. Except for SL-PS in Caco-2 cells, only about 10% of analogues were hydrolyzed after incubation for 30 min. Therefore, and to avoid the use of DFP, we measured the extent of internalization of analogues in the absence of DFP at 10 °C. In MDCK II cells, the glycerophospholipids rapidly disappeared from the exoplasmic leaflet of the plasma membrane (Figure 1D). The inward motion was slightly faster for PS and PE than for PC. The internalization rate of the sphingolipid analogue was significantly lower when compared to those of the glycerophospholipids. Likewise, in Caco-2 cells a fast internalization of the aminophospholipid analogues was observed, slightly faster for the PS analogue; about 70% of SL-PS and 50% of SL-PE analogues were localized inside the cells after 30 min (Figure 2D). Again the choline-containing derivatives, SL-SM and SL-PC, disappeared at a slower rate from the exoplasmic leaflet of the plasma membrane.

Effect of Intracellular ATP Concentration and of Pretreatment with NEM. To elucidate whether the inward movement of phospholipids requires cytoplasmic ATP and is protein-mediated, the redistribution of the spin-labeled lipid analogues was assessed at 20 and 10 °C for both cell types after depletion of ATP or after treatment with NEM.

For ATP depletion, MDCK II and Caco-2 cells were preincubated for 10 min at 37 °C in glucose-free HBSS containing a glycolytic (50 mM 2-deoxyglucose) and a mitochondrial (5 mM sodium azide) inhibitor. This treatment reduced the intracellular ATP level to 2% (MDCK II) and 4% (Caco-2) of that in control cells. Under these conditions, the rapid inward movement of aminophospholipid analogues was inhibited in both cell lines (Figures 1B,E and 2B,E). In the case of ATP-depleted Caco-2 cells, the rate of uptake of SL-PC was only slightly reduced at 20 °C and not affected at 10 °C. For energy-depleted MDCK II cells, a strong reduction of SL-PC internalization similar to that of aminophospholipid analogues was observed at 20 °C, while at 10 °C, the influence of ATP depletion was essentially absent. The internalization of SL-SM was not reduced in these cells, but still slower than that of glycerophospholipid analogues.

When MDCK II and Caco-2 cells were incubated for 15 min at 37 °C with NEM (2 mM) prior to labeling, all glycerophospholipid analogues were internalized at equivalent rates (Figures 1C,F and 2C,F) much lower than those

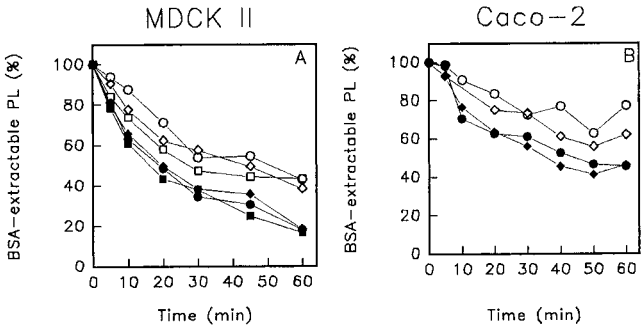

FIGURE 3: Internalization of spin-labeled phospholipid analogues into MDCK II cells (A) and Caco-2 cells (B) at 10 °C in the presence of inhibitors of the multidrug resistance P-glycoprotein. Cells in suspension were incubated for 10 min at 37 °C in HBSS-G in the absence (control cells, circles) or presence of 10 μM PSC 833 (squares in panel A) or 10 μM B8509-035 (dexniguldipine hydrochloride) (diamonds). Then, cells were cooled to 10 °C and labeled with SL-PE (black symbols) or SL-PC (white symbols) at $t = 0$. The amount of analogues present in the exoplasmic leaflet of the plasma membrane was assessed by the back-exchange assay and expressed as the percentage of total label added at time zero (see Materials and Methods). Results shown are from a single experiment representative of two independent experiments.

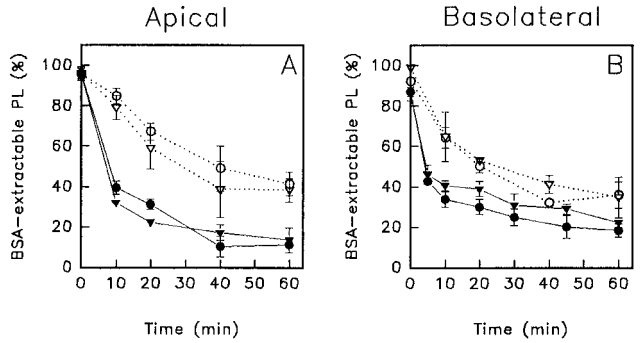

FIGURE 4: Internalization of spin-labeled phospholipid analogues in filter-grown MDCK II cells at 20 °C and effect of ATP depletion. Confluent cell monolayers on filters were labeled either at the apical side (A) or at the basolateral side (B) with SL-PS (circles) or SL-PC (triangles) (filled symbols, control cells; open symbols, ATP-depleted cells). ATP depletion and determination of the amount of analogues present in the exoplasmic leaflet of the plasma membrane were performed as described in Materials and Methods. For both analogues, hydrolysis did not exceed 35% of the total label. The results shown represent the mean ± the standard error of estimate of three independent experiments.

of aminophospholipid analogues in control cells. Again, under these conditions, a decrease in the rate of internalization of SL-PC was detected in MDCK II cells but not in Caco-2 cells. Unfortunately, pretreatment of MDCK II and Caco-2 cells with NEM resulted in a reduction of the cellular ATP level to 2 and 6% of that in control cells, respectively. Therefore, in these experiments, we cannot separate a direct effect of NEM on (amino)phospholipid translocation from an indirect effect on lipid translocation as a result of ATP depletion.

Influence of Inhibitors of the Multidrug Resistance P-Glycoprotein on the Internalization of Spin-Labeled Phospholipid Analogues. MDCK II and Caco-2 cells express the multidrug resistance MDR1 P-glycoprotein at their apical surface (*9*−*11*). A recent study has demonstrated that the MDR1 P-glycoprotein can actively translocate a wide variety of lipid analogues from the cytosolic side to the exoplasmic leaflet of the plasma membrane via an ATP-dependent process (*39*, *40*).

To determine whether any lipid transport activity of the multidrug resistance P-glycoprotein could affect the kinetics of analogue redistribution to the cytoplasmic leaflet, we have measured the degrees of internalization of SL-PE and SL-PC from the plasma membrane of MDCK II and Caco-2 cells at 10 °C in the presence of multidrug resistance inhibitors cyclosporin A and its more efficient analogue PSC 833 as well as B8509-035. As shown in panels A and B of Figure 3, the presence of PSC 833 or B8509-035 did not affect the internalization of the spin-labeled analogues; differences are within the experimental error. The same results were obtained with the inhibitor cyclosporin A (data not shown).

Polarized Epithelial Cells

Transverse Redistribution of Spin-Labeled Analogues in the Apical and Basolateral Plasma Membrane Domain. The experiments with cells in suspension do not allow a discrimination between the two domains of the plasma membrane, the apical and the basolateral domain. To determine whether the aminophospholipid translocase activity is present in both domains of the epithelial plasma membrane, we measured the kinetics of internalization of spin-labeled analogues using polarized monolayers of MDCK II and Caco-2 cells grown on permeable polycarbonate filter supports. This approach also enables one to verify whether short trypsinization of the cell monolayers affects the internalization behavior of the various analogues used. After insertion of spin-labeled analogues into either the apical or basolateral plasma membrane at 0 °C and removal of noninserted analogues, cells were incubated at 20 °C ($t = 0$ min for the reorientation kinetics). Note that at time zero of kinetics, i.e., after labeling for 5 min and washing for 5 min on ice, part of the analogues could not be removed even after repeated washing with HBSS-containing BSA, indicating their movement to the inner leaflet. Consistent with that is the appearance of an EPR signal in the pellet. During the time course of the experiment, analogues were never found in the BSA medium added to the nonlabeled domain, indicating the presence of intact tight junctions. We found a reduced amount of analogue hydrolysis in filter-grown cells, compared to cells in suspension; only about 35% of SL-PS and 10% of SL-PC were degraded within 60 min at 20 °C in both cell lines. No difference in hydrolysis was observed between analogues inserted in the apical and basolateral domain.

Panels A and B of Figure 4 show the redistribution kinetics of SL-PS and SL-PC in the apical and basolateral plasma membrane domain of MDCK II cells, respectively. Similar to that for the cells in suspension, a rapid disappearance of the PS and PC analogue from the exoplasmic leaflet was observed, slightly faster on the apical domain. The depletion of the ATP level to 5% of that of control cells slowed internalization to the same extent for both phospholipid analogues. However, the effect of ATP depletion on analogue internalization was less pronounced for the basolateral domain.[2]

In Caco-2 cells, a fast internalization of the aminophospholipid analogues was observed in both plasma membrane domains, while the transbilayer movement of SL-PC and SL-

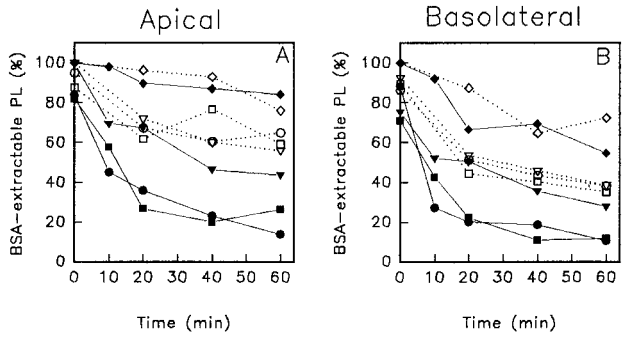

FIGURE 5: Internalization of spin-labeled phospholipid analogues in filter-grown Caco-2 cells at 20 °C and effect of ATP depletion. Confluent cell monolayers on filters were labeled either at the apical side (A) or at the basolateral side (B) with SL-PS (circles), SL-PE (squares), SL-PC (triangles), or SL-SM (diamonds) (filled symbols, control cells; open symbols, ATP-depleted cells). ATP depletion and determination of the amount of analogues present in the exoplasmic leaflet of the plasma membrane were performed as described in Materials and Methods. Hydrolysis of SL-PS, SL-PE, and SL-PC did not exceed 35% of the total label. For SL-SM, hydrolysis was not observed. Results shown are from a single experiment representative of two independent experiments.

SM was relatively slow (Figure 5A,B). The rapid inward movement of aminophospholipids at 20 °C was inhibited in ATP-depleted cells, becoming comparable to that of SL-PC. The internalization of SL-PC and SL-SM was only slightly affected by ATP depletion.

These results are in agreement with the results obtained on cells in suspension and suggest the presence of an ATP-dependent phospholipid translocating activity in both the apical and basolateral plasma membrane domain of epithelial cells.

DISCUSSION

The aim of this study was to characterize the transbilayer movement of phospholipids in the apical and basolateral membrane domains of epithelial cells. Toward this end, we studied the transbilayer movement of various spin-labeled phospholipid analogues across the plasma membrane of dog kidney MDCK II and human colon Caco-2 cells in suspension as well as grown on filters. Epithelial cells grown on permeable filter supports form a well-characterized polarized cell layer that mimics the epithelial organization observed in vivo, and this model system is often used to examine mechanisms involved in generating cell surface polarity of proteins (*17*, *27*). Both cell lines showed an ATP- and NEM-sensitive rapid redistribution of phospholipid analogues strongly suggesting protein-mediated transbilayer movement. This protein-mediated transbilayer movement was dependent on the phospholipid headgroup, and interestingly, we found significant differences between MDCK II and Caco-2 cells in lipid specificity of translocation. A rapid inward movement for the aminophospholipid analogues SL-PS and SL-PE was observed from the apical and basolateral domain in both cell lines. Surprisingly, a transbilayer movement with the same characteristics was detected for SL-PC in MDCK II, but not in Caco-2 cells. In Caco-2 cells, SL-PC redistribution across the plasma membranes was much slower and almost insensitive to ATP depletion and NEM treatment.

To measure the transbilayer lipid movement of the analogues, two prerequisites have to be fulfilled: (i) internalization of analogues by endocytosis should be negligible and (ii) the analogues must be chemically (metabolically) stable. Experimental conditions were chosen to meet the first precondition. Lipid endocytosis of cells is inhibited at 10 °C (*30*, *37*, *38*) and almost negligible at 20 °C (*38*). Even the slow inward movement of SL-PC and SL-SM at 10 °C is probably not due to endocytosis, since the degree of internalization was not reduced by ATP depletion which is known to inhibit endocytosis (*29*, *30*). Since we realized the same conclusion for analogue internalization from experiments at 10 and 20 °C, we can infer that endocytosis does not contribute significantly to the internalization of analogues at 20 °C, if it all. As shown, the second prerequisite was not fulfilled in particular for suspended MDCK II cells at 20 °C. We observed a strong hydrolysis of analogues into lyso derivatives and free fatty acids. Incubation of epithelial cells with DFP blocked hydrolysis of spin-labeled analogues, but, for reasons unknown, at the same time strongly reduced the level of cellular ATP. Thus, an ATP-dependent redistribution of (amino)phospholipids cannot be measured accurately in the presence of DFP. However, in the initial phase of the experiments, the extent of hydrolysis was lower in comparison to the amount of analogues that disappeared from the exoplasmic leaflet even in the absence of DFP. Thus, the ATP dependence and NEM sensitivity of the time-dependent increase of the sum of the amount of probe in the inner leaflet and amount of probe hydrolyzed are evidence of ATP-dependent protein-mediated transport. To support this conclusion, experiments were performed at 10 °C in the absence of DFP. Although under these experimental conditions not only hydrolysis of the analogues but also the inward movement was reduced, we were able to differentiate between ATP-dependent and ATP-independent translocation.

Transbilayer Movement in Caco-2 Cells. The transbilayer movement of analogues in a Caco-2 cell suspension is very similar to that in other eukaryotic cells, such as human erythrocytes (*26*), fibroblasts (*15*, *21*), hepatocytes (*19*), and yeast cells (*14*). The aminophospholipids SL-PS and SL-PE were rapidly internalized, and internalization was significantly suppressed after depletion of cytosolic ATP or pretreatment of cells with NEM. In comparison, the choline-containing derivatives SL-SM and SL-PC were only slowly internalized with almost no effect of ATP depletion or NEM pretreatment of cells. As has been found for human erythrocytes (*18*, *45*), the inward movement of SL-PE was slower than that of SL-PS but still faster than that of choline-containing phospholipids such as SL-PC and SL-SM. Taken together, these results strongly suggest the existence of an aminophospholipid translocase activity in the plasma membrane of Caco-2 cells and an asymmetric transbilayer lipid distribution with aminophospholipids preferentially located in the cytoplasmic leaflet and SM in the exoplasmic leaflet. This conclusion is supported by earlier calculations on transbilayer phospholipid distribution suggesting that 65−

[2] In some experiments with ATP-depleted cells, analogues could be extracted from the nonlabeled domain by BSA. This was never observed for control cells. Two explanations may account for this observation, namely, (i) bypassing of the intracellular lumen of analogues by opening of the tight junctions and/or (ii) redistribution of analogues between both domains across the cytoplasm. However, data from these experiments were omitted.

90% of the PE and 10−25% of the PC are localized in the cytoplasmic leaflet of epithelial cells (*41*, *42*) which is very similar to the situation found in human erythrocytes (*20*, *47*).

In the case of polarized Caco-2 cells grown as a confluent monolayer on filters, for both domains the same differences in the internalization rates between the aminophospholipids and choline-containing phospholipids were found like those observed in Caco-2 cell suspensions, suggesting that short trypsinization of epithelial cell monolayers does not affect the aminophospholipid translocase activity. Depletion of cytosolic ATP reduced the rate of the fast inward motion of PS and PE, indicating an energy-dependent aminophospholipid translocation. The choline-containing derivatives SL-SM and SL-PC were only slowly internalized without any effect of ATP depletion. These findings demonstrate that an aminophospholipid translocase activity is present in both the apical and basolateral domains of the epithelial plasma membrane. This is in agreement with recent results of Julien et al. (*12*, *13*) showing an energy-dependent and protein-mediated aminophospholipid translocation in the apical plasma membrane of polarized bovine aortic endothelial cells.

Transbilayer Movement in MDCK II Cells. We observed the same characteristics of transbilayer movement for SL-PS, SL-PE, and SL-SM in MDCK II cells in suspension or grown on filters as we observed for Caco-2 cells. Interestingly, the kinetics of redistribution of the choline-containing analogue SL-PC were clearly different in MDCK II as compared to Caco-2 cells. SL-PC was rapidly internalized at a rate similar to that of the aminophospholipids in suspended as well as in filter-grown MDCK II cells. This rapid internalization of SL-PS, SL-PE, and SL-PC was observed in both the apical and basolateral domain. Upon depletion of ATP or treatment with NEM, we found a similar reduction of the extent of internalization for SL-PC and aminophospholipid analogues. At the present stage of investigation, we cannot be sure that the PS, PE, and PC analogues are transported by one protein (complex) with a broad lipid specificity. Although in the opposite direction, such a flippase activity has been recently reported for gastric versicles (*33*). Alternatively, the PC analogue is moved across the membrane by a PC-specific translocator acting alongside a separate aminophospholipid translocase. The only proteins known so far to have an energy-dependent PC translocating activity are the mdr2/MDR3 P-glycoprotein (*7*, *25*, *31*, *32*, *39*) in the bile canalicular membrane of the hepatocyte and the MDR1 P-glycoprotein (*2*, *39*) which is present in the apical membrane of many epithelial cells. However, these proteins mediate lipid transport toward the *outer* leaflet of the plasma membrane and cannot be responsible for the rapid *internalization* of SL-PC in MDCK II cells. As expected, the internalization of the spin-labeled analogues was not affected by the multidrug resistance inhibitors.

In conclusion, it remains to be established whether the fast internalization of PC in the plasma membrane of MDCK II cells is mediated by a protein that controls specifically the movement of this phospholipid. This question can possibly be answered as soon as antibodies against proteins become available that mediate transbilayer movement of the different lipid classes, e.g., the ATPase II of the chromaffin granule membrane (*34*). In this respect, it would be extremely interesting to determine whether the active transport of the aminophospholipids in epithelial cells is mediated by two different proteins, one apical and one basolateral.

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