Aminophospholipids Have No Access to the Luminal Side of the Biliary Canaliculus

IMPLICATIONS FOR THE SPECIFIC LIPID COMPOSITION OF THE BILE FLUID*

Received for publication, February 28, 2003, and in revised form, July 23, 2003
Published, JBC Papers in Press, August 6, 2003, DOI 10.1074/jbc.M302131200

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About 95% of the bile phospholipids are phosphatidylcholine. Although the fractions of phosphatidylcholine and of both aminophospholipids phosphatidylserine and phosphatidylethanolamine in the canalicular membrane are in the same order of about 35% of total lipids, both aminophospholipids are almost absent from the bile. To rationalize this observation, we studied the intracellular uptake of various fluorescent phospholipid analogues and their subsequent enrichment in the bile canaliculus (BC) of HepG2 cells. Diacylaminophospholipid analogues but not phosphatidylcholine analogues became rapidly internalized by an aminophospholipid translocase (APLT) activity in the plasma membrane of HepG2 cells. We observed only low labeling of BC by diacylaminophospholipids but extensive staining by phosphatidylcholine analogues. In the presence of suramin, known to inhibit APLT, a strong labeling of BC by diacylaminophospholipid analogues was found that declined to a level observed for control cells after removal of suramin. Unlike diacylphosphatidylserine, diether phosphatidylserine analogue, which is not an appropriate substrate of APLT, accumulated in the BC. The correlation between low labeling of BC and an APLT-mediated transbilayer movement suggests the presence of an APLT activity in the canalicular membrane that prevents exposure of aminophospholipids to the bile.

An important function of the liver is the synthesis and secretion of bile fluid by the hepatocytes. The plasma membrane of hepatocytes is organized into distinct domains, the basolateral and the apical (or canalicular) membrane, separated by tight junctions. The basolateral membrane faces the blood vessel in vivo. The apical domains of hepatocytes form small tubuli, the bile canaliculi (BC), into which the bile constituents are secreted.

A major component of bile fluid are phospholipids. Their uptake from the canalicular membrane into the BC requires the solubilizing activity of bile salts (1, 2). Phosphatidylcholine (PC) accounts for about 95% of the bile phospholipids (3), whereas it constitutes only 35% of the canalicular membrane phospholipids (4). In agreement with the composition of mammalian plasma membranes, the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) represent about 24 and 11%, respectively (4), of the canalicular membrane lipids. However, PS is virtually absent from the bile, and PE represents only 4.5% of biliary phospholipids. Because previous work has shown that the interaction of bile salts with phospholipids is independent of the phospholipid head group (5), other mechanisms that prevent specific phospholipids located in the apical membrane from being solubilized into BC must ensure the characteristic phospholipid composition of the bile fluid.

We hypothesized that the absence of the aminophospholipids PS and PE from bile is a consequence of their almost exclusive localization on the cytoplasmic leaflet of the apical membrane (6, 7). This asymmetric localization could be the result of the activity of aminophospholipid translocase (APLT), a protein shown to be ubiquitous in the plasma membrane of mammalian cells. APLT rapidly transports the aminophospholipids PS and PE from the exoplasmic to the cytoplasmic leaflet at the expense of ATP (8–10), a process first shown for the erythrocyte membrane (8, 11, 12). In contrast, PC and sphingomyelin are not recognized by APLT. Employing spin-labeled lipid analogues, the half-time of APLT-mediated inward transport in red blood cells was found to be in the order of 2–5 min and 30 min for PS and PE, respectively (13). Although the molecular identity of APLT is awaiting, it is very likely to be a member of the P-type ATPase family (14, 15).

To address the presence of an APLT activity in the canalicular membrane we studied the enrichment of fluorescent phospholipid analogues in the BC of HepG2 cells. This human hepatoma cell line is able to polarize and form a biliary vacuole that resembles the tubular BC of the liver (16, 17). Although we suggested recently the presence of an APLT activity in the plasma membrane of nonpolarized HepG2 cells by employing spin-labeled phospholipid analogues (18, 19), this approach did not allow us to address whether the canalicular membrane itself harbors an APLT activity. Here, using a panel of fluorescent phospholipid analogues with differing affinities toward APLT, we found that analogues that are efficiently transported by APLT are essentially excluded from the BC, whereas those that are not transported by APLT were enriched in the BC. These observations provide strong evidence for an APLT activ-
Lipid Transport in HepG2 Cells

EXPERIMENTAL PROCEDURES

Chemicals—NBD-Labeled phosphatidycholine 1-palmitoyl-2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-sn-glycero-3-phosphatidylethanolamine (diacyl-NBD-PE) were obtained from Avanti Polar Lipids (Birmingham, AL). Synthesis of 1-0-tetradecanoyl-sn-glycero-3-phosphatidylethanolamine (diacyl-NBD-PS), and phosphatidylethanolamine (1-palmitoyl-2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino[caproyl]-sn-glycero-3-phosphatidylserine (diacyl-NBD-PS), and phosphatidylethanolamine (1-palmitoyl-2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino[hexanoxyl]-sn-glycero-3-phosphatidylserine (diether NBD-PS) and of 1-0-tetradecanoyl-sn-glycero-3-phosphatidylethanolamine (diacyl-NBD-PE) were obtained from Seromed (Biochrom, Berlin, Germany). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Hyclone Laboratories (Logan, UT). Poly-l-lysine (21) was from Polysciences (Warrington, PA). Polyethylene glycol (PEG) was from Sigma (St. Louis, MO). Dithionite (25) was from Fluka (Buchs, Switzerland). Chloroform and methanol were from Merck (Darmstadt, Germany). Other chemicals were obtained from Sigma.

Cell Culture—HepG2 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose supplemented with 10% heat-inactivated fetal bovine serum. Cells were routinely passaged in 25-cm² plastic culture flasks coated with collagen and 0.02% EDTA in PBS (supplemented with 20 mM glucose, 1 mM sodium pyruvate, and 10 mM HEPES to prevent ATP depletion and pH shift) for 10–14 days. For microscopic observations, cells were grown on microcover glasses (18 × 18 mm, BRAND, Germany) coated with poly-l-lysine (21). After 3 days, when the highest degree of polarization was obtained (16, 22), cells were used for experiments.

Cell Labeling—Polarized cells on cover glasses were incubated with the label (final concentration 4 μmol/liter HBSS) for 20 min on ice, washed with HBSS, and incubated for 30 min at 37 or 14 °C (7). Taking into account the less efficient incorporation of diacyl-NBD-PE into membranes compared with the diacyl analogues of PS and PC (20, 23), labeling with the analogues was performed by incubating the cells for 30 min with the analogue at 37 °C followed by washing with HBSS* and further incubation for 30 min at 37 °C.

For measurements in suspensions, cells (4 × 10⁶ cells) were incubated with 600 μmol/liter of PS containing the respective lipid analogue. Labeling was performed for 5 min with 12 nmol of diacyl analogues or 15 min with 24 nmol of diether analogues on ice to achieve comparable extent of labeling. To prevent hydrolysis of the diacyl lipid analogues 5 mM DFP was added in parallel and was present in all following steps. Subsequently washed with PBS for 5 min. For confocal laser scanning microscopy (CLSM) of cells labeled with diaeryl-NBD-PS a Leica Confocal laser scanning microscope (Leica Lasertechnik GmbH, Wetzlar, Germany) equipped with NPL Fluorat 40×/1.3 oil objectives (Leitz, Wetzlar, Germany) and an argon/krypton ion laser emitting at 488 nm was used.

Measurements of Analogue Internalization in Cell Suspensions—Labeled cells were incubated at 37 or 14 °C. After various times aliquots were transferred into a fluorescence cuvette containing 2.4 ml of ice-cold PBS*. NBD fluorescence was monitored at 540 nm (λₐ = 470 nm) (Amino Bowman Series 2 spectrophotometer) at 4 °C while continuously stirring the suspension. Dithionite was added from a freshly prepared 1 m stock solution in 100 mM Tris (pH 9.5) to give a final concentration of 50 μM as described earlier (24, 25). Dithionite quenches the fluorescence by chemical reaction with the NBD group. Because dithionite permeates very slowly across membranes at low temperature (25; see "Results"), only the fluorescence of analogues on the exoplasmic leaflet is quenched. Indeed, upon addition of dithionite, we observed for labeled HepG2 cells an initial rapid decline of fluorescence intensity corresponding to reduction of the analogues on the exoplasmic leaflet (not shown). Subsequently, only a very slow fluorescence decline was observed which, very likely, is because of slow permeation of dithionite and reduction of analogues on the cytoplasmic side. Reduction of intracellular localized analogues did not exceed 2% during dithionite assay measurements, which regularly took about 5 min. After the fluorescence intensity was reduced by dithionite to a plateau value, Triton X-100 was added to a final concentration of 2%, making the NBD-phospholipids on the cytoplasmic side accessible to dithionite. The amount of phospholipids on the cytoplasmic side (PLₐ) was determined according to:

\[ Fₐ - Fₐ^{\ast} = \frac{p}{1 - p} \left( F - F^{\ast} \right) \]

with \( F \) being the fluorescence of the plateau after dithionite reduction, \( Fₐ \) the background fluorescence after the addition of Triton X-100, and \( Fₐ^{\ast} \) the fluorescence intensity before the addition of dithionite.

Fluorescence Microscopy—Labeled cells grown on cover glasses were analyzed with an inverted Axiovert 100 standard epifluorescence microscope (Carl Zeiss, Inc. Oberkochen, Germany) equipped with a Planaapo 100/1.3 numerical aperture objective and a green fluorescence filter set (BP 450–490 nm excitation filter, FT 510 nm dichroic mirror, and LP 515 nm emission filter) (Carl Zeiss) as described previously (7). Canaliculac vacuoles (BC) were identified by phase-contrast microscopy. The percentage of BC containing the fluorescent lipid analogue was quantified by counting labeled and nonlabeled BC. BC having a fluorescence intensity in the BC as low as cellular autofluorescence levels were defined as nonlabeled BC.

Localization of the fluorescent lipid analogues in the BC was confirmed by the addition of dithionite, which can diffuse into BC and react with NBD-labeled analogues in the lumen and on the luminal membrane leaflet of BC (22). The remaining fluorescence of the canalicular membrane originates of phospholipid analogues located on the cytoplasmic side of this membrane. Cells were fixed with 4% paraformaldehyde in PBS supplemented with 20 μmol/liter of PS containing the respective analogue. After incubation for 15 min at 4 or 37 °C, cells were washed with ice-cold PBS and observed by fluorescence microscopy.

Measurements of Metabolism of Diaeryl-NBD Analogues—To measure hydrolysis of cell-associated lipid analogues, cells in suspensions or in monolayers were labeled as described above. Subsequent to labeling, suspensions were incubated for 60 min, monolayers for 30 min at 37 °C (see above and "Results"). For lipid extraction, monolayers were scraped from the dish and resuspended in 1 ml of HBSS.

Lipids were extracted as described previously (26). Briefly, 3.2 ml of methanol/chloroform (2:1) was added to 1 ml of cells and incubated for 30 min at room temperature. After phase separation by adding 1 ml of
chloroform and 1 ml of 40 mM acetic acid, lipids in the chloroform phase were collected. The aqueous phase was washed with 1 ml of chloroform, the two combined chloroform phases were dried under a nitrogen stream, and the lipids were resuspended in a small volume of chloroform/methanol (1:1) and applied to a TLC plate. Plates were developed in two dimensions using chloroform:methanol:ammonia (13:5:1) as basic solvent and aceton:chloroform:methanol:glacial acetic acid:water (8:6:2:2:1) as acidic solvents. Spots on the dried TLC plates were analyzed using a Fluorescence Image Analyzer FLA-3000 (Raytest Isotope- nmessgera¨te GmbH, Germany) and AIDA image analysis software.

RESULTS

To identify and characterize APLT activity in the canalicular membrane, we used diacyl and diether analogues of PS and PC and a diacyl analogue of PE, each bearing the fluorescent NBD moiety at the short sn2 fatty acid chain (C6). These analogues differ in their affinity to and transport by APLT which is primarily determined by the head group as well as the glycerol backbone of phospholipids (13). As shown for various mammalian cells, PC analogues are not transported by APLT (20, 23, 25, 27). In contrast to PC, diacyl-NBD-PS and, to a lesser extent diacyl-NBD-PE, are transported by APLT. However, the transport of the diether analogue of PS by APLT is very slow compared with diacyl-NBD-PS (20).

First, we characterized the transbilayer redistribution of these analogues across the plasma membrane of suspended HepG2 cells to verify that the respective analogues behave in a manner similar to that found for other mammalian cells (see above). Subsequently, we studied the enrichment of aminophospholipid analogues in BC of polarized HepG2 cells in monolayer cultures.

Internalization of Diacyl-Lipid Analogues in Suspended HepG2 Cells—In Fig. 1 the kinetics of internalization of NBD-lipid analogues in suspended HepG2 cells is shown. To differentiate between uptake by endocytic activity and by transbilayer movement from the exo- to the cytoplasmic leaflet, internalization was measured not only at 37 °C (Fig. 1A and B), but also at 14 °C (Fig. 1C and D), where endocytosis is strongly reduced (28, 29). During the labeling procedure a low amount of NBD analogues was lost from the exoplasmic leaflet, indicated by the offset at \( t_11005 \) of the respective plots.

At 37 °C the internalization of diacyl-NBD-PS was rapid, about 75% of the analogue was internalized with a half-time of about 5 min (Fig. 1A). We also observed a rather rapid uptake of the analogue with respect to the other analogues at 14 °C (Fig. 1B). Although the internalization of diacyl-NBD-PE was slower compared with diacyl-NBD-PS, it was still faster with respect to the PC analogue (see below). The internalization of diacyl-NBD-PS and -PE across the plasma membrane was diminished by preincubation of the cells with suramin, an inhibitor of APLT (Fig. 1, A and C); the plateau of redistribution kinetics was about 3–4-fold lower compared with that in the absence of suramin. These results are consistent with an APLT activity in HepG2 cells.

The fraction of diacyl-NBD-PC localized in the exoplasmic leaflet of the plasma membrane was much higher compared with that of the diacylaminophospholipid analogues (Fig. 1A). At 37 °C only about 20% of the analogue was internalized with a half-time of about 5 min (Fig. 1A). We also observed a rather rapid uptake of the analogue with respect to the other analogues at 14 °C (Fig. 1B). Although the internalization of diacyl-NBD-PE was slower compared with diacyl-NBD-PS, it was still faster with respect to the PC analogue (see below). The internalization of diacyl-NBD-PS and -PE across the plasma membrane was diminished by preincubation of the cells with suramin, an inhibitor of APLT (Fig. 1, A and C); the plateau of redistribution kinetics was about 3–4-fold lower compared with that in the absence of suramin. These results are consistent with an APLT activity in HepG2 cells.

The fraction of diacyl-NBD-PC localized in the exoplasmic leaflet of the plasma membrane was much higher compared with that of the diacylaminophospholipid analogues (Fig. 1A). At 37 °C only about 20% were found nonaccessible to dithionite after 1 h of incubation. Again, the internalization was significantly reduced upon lowering the temperature (cf. Fig. 1C). At 14 °C, about 10% of PC analogues redistributed to cytoplasmic side within 1 h. Preincubation of cells with suramin did not affect the transbilayer dynamics of diacyl-NBD-PC (data not shown).
The basolateral membrane of HepG2 cells was labeled with 4 μm diacyl (A, B, E, and F) or diether NBD-phospholipid analogues (C, D, G, and H) for 20 min on ice. After washing and incubation for 30 min at 37 °C, cells were again washed and twice incubated with 5% (w/v) BSA (in HBSS) for 10 min at room temperature to remove remaining label from the exoplasmic leaflet of the basolateral cell membrane (see Ref. 7). B and F, fluorescence microscopy of diacyl-NBD-PC and -PS labeled cells, respectively. A and E are phase-contrast images of the same field as shown in B and F, respectively. For diacyl-NBD-PC, enrichment in the BC indicated by bright labeling of this structure and punctuate staining of vesicular structures were observed (B). For diacyl-NBD-PS, a diffuse cytoplasmic labeling was detected but no enrichment of the analogue in the BC. D and H, fluorescence microscopy of diether NBD-PC- and -PS-labeled cells, respectively. C and G are phase-contrast images of the same field as shown in D and H, respectively. Both analogues enriched in the BC, although it was more pronounced for diether NBD-PC. Labeled BC are indicated by white arrows, nonlabeled BC by white arrowheads. Bar, 20 μm.

**Fig. 2.** Transport of diacyl- and diether NBD-phospholipid analogues to the BC of polarized HepG2 cells at 37 °C. The basolateral membrane of HepG2 cells was labeled with 4 μm diacyl- (A, B, E, and F) or diether NBD-phospholipid analogues (C, D, G, and H) for 20 min on ice. After washing and incubation for 30 min at 37 °C, cells were again washed and twice incubated with 5% (w/v) BSA (in HBSS) for 10 min at room temperature to remove remaining label from the exoplasmic leaflet of the basolateral cell membrane (see Ref. 7). B and F, fluorescence microscopy of diacyl-NBD-PC and -PS labeled cells, respectively. A and E are phase-contrast images of the same field as shown in B and F, respectively. For diacyl-NBD-PC, enrichment in the BC indicated by bright labeling of this structure and punctate staining of vesicular structures were observed (B). For diacyl-NBD-PS, a diffuse cytoplasmic labeling was detected but no enrichment of the analogue in the BC. D and H, fluorescence microscopy of diether NBD-PC- and -PS-labeled cells, respectively. C and G are phase-contrast images of the same field as shown in D and H, respectively. Both analogues enriched in the BC, although it was more pronounced for diether NBD-PC. Labeled BC are indicated by white arrows, nonlabeled BC by white arrowheads. Bar, 20 μm.

**Internalization of Diether Lipid Analogues in Suspended HepG2 Cells**—Internalization kinetics of diether NBD-PC was very similar to that of the diacyl analogue (Fig. 1). However, the internalization of diether NBD-PS was slower with respect to the diacyl analogue (Fig. 1, B and D), which is consistent with our previous observation on fibroblasts and red blood cells, indicating a very low affinity of APLT for diether NBD-PS. In agreement with this, suramin only had a marginal effect on the internalization of diether NBD-PHPS (Fig. 1). However, the amount of internalized diether NBD-PS was still higher compared with both PC analogues (Fig. 1, B and D).

**Enrichment of Fluorescent NBD-Phospholipid Analogues to the Basolateral Cell Membrane of HepG2 Cells**—We next compared the enrichment of various fluorescent lipid analogues in BC of polarized HepG2 cells by fluorescence microscopy. Subsequent to labeling of the basolateral membrane on ice, the accumulation of lipid analogues in the BC was monitored after incubation of cells for 30 min at 37 °C (Fig. 2) or at 14 °C (see Fig. 4). For diacyl-NBD-PC, an extensive labeling of BC was found at 37 °C (Fig. 2, A and B). About 80% of the BC were labeled with the analogue (Fig. 3). The punctate intracellular staining observed originates from endocytotic uptake of the analogue (7, 30). We found the same pattern of intracellular labeling for diether NBD-PC, in particular the enrichment of the analogue in the BC (Fig. 2, C and D). The amount of labeled BC was similar to that of diacyl-NBD-PC (Fig. 3).

The pattern of intracellular fluorescence was very different between the PC and diacylaminophospholipid analogues. For the latter, we observed a diffusive distribution in the cytoplasm (Fig. 2, E and F; only shown for diacyl-NBD-PS), rather than the punctuate staining of endocytic vesicles as detected for the PC analogues. The amount of BC labeled by diacylaminophospholipid analogues was low (Fig. 2, E and F). Only about 20% of the BC were labeled with diacyl-NBD-PS after 30 min at 37 °C (Fig. 3). In these BC the fluorescence intensity was lower than that of those labeled with PC analogues. This indicates a reduced enrichment of the PS analogue compared with PC. Similarly, diacyl-NBD-PE was excluded from the BC but less rigorously as diacyl-NBD-PS. Enrichment of the PE analogue was found in about 30% of the BC (see Fig. 3).

Unlike diacyl-NBD-PS, diether NBD-PS became enriched in the BC (Fig. 2, G and H). The percentage of labeled BC was almost identical to that of PC analogues (Fig. 3). However, compared with PC analogues, the punctate staining within the cytoplasm was less pronounced, and we noted a somewhat diffusive fluorescence in the cytoplasm.

**Fig. 3.** Percentage of BC labeled with NBD-phospholipid analogues. The basolateral membrane of HepG2 cells was labeled with 4 μm of various NBD-phospholipid analogues and, subsequently, treated as described in the legend to Fig. 2 and under “Experimental Procedures.” The amount of NBD-positive BC in the absence (white bars) and in the presence of 200 μM suramin (filled bars) was determined as described under “Experimental Procedures.” Data are expressed as the mean ± S.E. of more than 10 experiments.

**Endocytosis and Transcytosis of Vesicles from the Basolateral to the Apical Membrane**—Endocytosis and transcytosis of vesicles from the basolateral to the apical membrane in polarized cells are temperature-dependent and are strongly reduced at 14 °C (31). However, also under this condition bright labeling exclusively of the BC was found for both PC analogues and diether NBD-PS (Fig. 4). In contrast, BC were not labeled by diacyl-NBD-PS. Similar to our observation at 37 °C, we found a bright and diffuse cytoplasmic staining for the PS analogue.

To confirm the difference in localization between diacyl-NBD-PS and diether NBD-PS as well PC analogues, cells were double labeled with NBD-phospholipid analogues and a diacyl-β-BODIPY-PC analogue. We have shown previously that β-BODIPY-PC is specifically enriched in BC (7). In agreement with that, β-BODIPY-PC redistributed rapidly to the lumen of the BC (Fig. 5), which was not observed for diacyl-NBD-PS. In
To this end, cells were labeled with suramin after internalization of the PS analogue seen for PC analogues. And with diether NBD analogues nor the punctuate staining affected neither the number of labeled BC with diacyl-NBD-PC observed for diacyl-NBD-PE (data not shown). Suramin affected the order of about 15 min. A comparable effect of suramin was with the inhibitor (Fig. 7). The half-time of the process was in decreased to a value found for cells that had not been treated with the presence of DFP.

In the presence of suramin, we found a significant enrichment of diacyl-NBD-PS in BC. Suramin treatment increased the amount of BC labeled by diacyl-NBD-PS to about 50% (Fig. 5A). Interestingly, punctuate intracellular staining was observed, indicating that endocytic pathways contribute significantly to the uptake of the PS analogue under those conditions. When suramin was washed out, the amount of labeled BC decreased to a value found for cells that had not been treated with the inhibitor (Fig. 7). The half-time of the process was in the order of about 15 min. A comparable effect of suramin was observed for diacyl-NBD-PE (data not shown). Suramin affected neither the number of labeled BC with diacyl-NBD-PC and with diether NBD analogues nor the punctuate staining seen for PC analogues.

Diacyl-NBD-PS was also enriched in BC when cells were treated with suramin after internalization of the PS analogue (see "Experimental Procedures"). To this end, cells were labeled with diacyl-NBD-PS and incubated for 30 min at 37 °C to allow internalization of the analogue. At this point there was only low labeling of BC (see above). Subsequent addition of suramin led to a bright labeling of BC (data not shown).

As shown by CLSM, diacyl-NBD-PS became enriched in the lumen of BC after suramin treatment (Fig. 8, A–E). The labeling of BC was very similar to that observed for diacyl-NBD-PC in (nontreated) HepG2 cells (see above). When the inhibitor was washed out and cells were incubated for 30 min at 37 °C, a labeling pattern of BC similar to the one seen in Fig. 6 was observed (not shown).

Metabolism of the Lipid Analogues—The intracellular hydrolysis of the diacyl-NBD analogues and their metabolic conversion to other fluorescent lipids are summarized in Table I. For cells in suspension, between 10 and 20% of diacyl analogues were hydrolyzed after incubation at 37 °C for 60 min. In monolayers of polarized cells, 6–8% of diacyl-NBD-PE and -PC and about 17% of diacyl-NBD-PS were hydrolyzed after incubation for 30 min at 37 °C. In the presence of DFP, hydrolysis was reduced to a level of 2–4% of total analogues. We were not able to treat polarized cells with DFP to study intracellular localization of analogues because of impaired labeling of BC in the presence of DFP.

For both cells in suspension and polarized cells a metabolic conversion of diacyl-NBD-PS to PE and PA was observed which was not affected by DFP (Table I).

As expected, no breakdown of diether analogues was observed.

Transport of Fluorescent Bile Salts to the BC of Monolayer Cultures of Polarized HepG2 Cells—To confirm that HepG2 cells were functionally polarized we examined whether cells are

![Fig. 4](image1)

**Fig. 4.** Transport of diacyl- and diether NBD-PC and -PS to the BC of polarized HepG2 cells at 14 °C. The basolateral membrane of HepG2 cells was labeled with 4 μM diacyl- (A, B, E, and F) or diether NBD-phospholipid analogues (C, D, G, and H) for 20 min on ice. After washing and incubation for 30 min at 14 °C, cells were again washed and twice incubated with 5% (w/v) BSA (in HBSS−) for 10 min on ice to remove remaining label from the exoplasmic leaflet of the basolateral cell membrane (see Ref. 7). B and F, fluorescence microscopy of diacyl-NBD-PC- and -PS-labeled cells, respectively. A and E are phase-contrast images of the same field as shown in B and F, respectively. Bright labeling almost exclusively of BC was found for diacyl-NBD-PC (arrow), punctuate staining was strongly reduced compared with 37 °C (Fig. 2B). For diacyl-NBD-PS, a diffuse cytoplasmic labeling was detected, but no enrichment of the analogue in the BC. D and H, fluorescence microscopy of diether NBD-PC- and -PS-labeled cells, respectively. C and G are phase-contrast images of the same field as shown in D and H, respectively. Both analogues enriched in BC, although it was more pronounced for diether NBD-PC. Labeled BC are indicated by white arrows, nonlabeled BC by white arrowheads. Bar, 20 μm.

![Fig. 5](image2)

**Fig. 5.** Colocalization of diacyl-NBD-PS and β-BODIPY-PC analogues. Polarized cells on cover glasses were double labeled with diacyl-NBD-PS (B) and β-BODIPY-PC (C) (see “Experimental Procedures”) and incubated further at 37 °C for 30 min. A bright labeling of BC by β-BODIPY-PC occurred (C), whereas diacyl-NBD-PS was absent from BC (B). A, phase-contrast to B and C.
FIG. 6. Enrichment of diacyl-NBD-PS in the canalicular membrane of polarized HepG2 cells studied by confocal laser scanning microscopy. HepG2 cells were labeled with diacyl-NBD-PS on ice for 20 min. After washing, the cells were incubated for 30 min at 14 °C. Subsequently, cells were washed and further incubated twice with 5% (w/v) BSA for 10 min to remove remaining label from the outer leaflet of the basolateral membrane. Labeled cells were scanned with the plane of focus in the center of the BC. A and B, diacyl-NBD-PS clearly labeled the canalicular membrane (arrowheads) of three adjacent cells (numbered 1–3) (B, phase-contrast image to A). C and D, the top BC in A and B was zoomed to demonstrate the location of diacyl-NBD-PS: fluorescence staining (C) exactly matched the canalicular membrane visualized in the corresponding phase-contrast image (dark ring around a bright center in D). E and F, image (E) and line profile (F) of a BC labeled with diacyl-NBD-PS. The line scan starts and ends at brightly labeled organelles (arrowheads) and crosses the BC. Staining of the canalicular membrane indicated by a fluorescence peak (arrow in F) is only about 50% of fluorescence of diacyl-NBD-PS in intracellular organelles located in proximity to the BC (left and right maximum of intensity in the line scan). Pixel position 0 of F corresponds to the upper arrowhead of the line scan in E, whereas pixel position 32 corresponds to the lower arrowhead. Fluorescence of diacyl-NBD-PS in the lumen of the BC is only about 20% of the intensity in the canalicular membrane. The other side of the canalicular membrane cannot be resolved as an individual peak because of bright intracellular fluorescence of diacyl-NBD-PS adjacent to the BC. Bar, 20 μm except C and D, where the bar is 5 μm.

able to transport bile salts into the BC. To this end, we used the fluorescent bile salt analogues CGamF and NBD-cholan. Although HepG2 cells do not express all bile salt transporter proteins identified in the basolateral membrane of hepatocytes (32), we observed bright staining of BC after labeling of the cells for 15 min at 37 °C (Fig. 9, A and B; only shown for NBD-cholan). About 80% of BC were labeled by the bile salt analogues (Fig. 9C). Incubation of the cells at 4 °C significantly reduced the number of labeled BC (Fig. 9). Preincubation of cells with the inhibitor PSC 833 (5 μM) according to Cantz et al. (33) reduced the percentage of labeled BC in either case to less than 30% (Fig. 9). These results indicate a vectorial transport of fluorescent bile acid analogues from the basolateral membrane toward the BC in HepG2 cells. The inhibition by PSC 833 suggests that ABC proteins are involved in the enrichment of the analogues in the BC. It has been shown that PSC 833 inhibits the ABC transporter MDR1 Pgp, MRP2, and BSEP (bile salt export pump) (34). Taken together, these data prove the functional polarity of HepG2 cells used in our experiments. The identification of the transporters involved in bile salt analogue enrichment in HepG2 cells is beyond the aim of this study and warrants further investigations.

DISCUSSION

The present study was aimed at providing experimental evidence for a mechanism that explains the absence of aminophospholipids from the bile. In particular, we investigated whether the access of the aminophospholipids PS and PE to the luminal side of the BC is prevented by an APLT activity in the canalicular membrane by analyzing the redistribution of various fluorescent analogues to the BC lumen. The main result of our study is that aminophospholipid analogues that are not (efficiently) transported by APLT are enriched in BC, whereas analogues that represent suitable substrates for APLT are exported from the lumen of the canalicular membrane, where they accumulated only when APLT activity is inhibited. We conclude that the canalicular membrane harbors an APLT activity essential for preserving the specific phospholipid composition of the bile. This APLT activity is sufficient to explain the virtual absence of aminophospholipids from the bile.

It may be asked whether the fluorescent analogues used are faithful reporters of natural phospholipid movements because they may locally perturb the lipid bilayer. The great differences in translocation for various lipid analogues demonstrate selectivity to the head group/glycerol backbone and not the fluorescent NBD moiety. Indeed, the active transport of aminophospholipids in red cells first discovered using spin-labeled lipids (8) was confirmed with fluorescent probes (35) as used here as well as with radiolabeled long chain lipids (12). Thus, the same conclusions could be drawn employing different families of lipid probes. Therefore, we are confident that in HepG2 cells study-
Polarized cells on cover glass were preincubated with 200 μM suramin for 30 min at 37 °C and labeled with 4 μM diacyl-NBD-PS (see “Experimental Procedures”). Nonbound label was removed, and the cells were incubated further at 37 °C for 30 min. Subsequently, cells were back exchanged to 5% BSA twice. The inhibitor was present during all steps. CLSM revealed a bright labeling of BC (B, arrows) (A, phase-contrast to B). The lower BC was scanned with a higher resolution (D; C is phase-contrast to D). A line scan along the white line shown in D demonstrates that the analogue is enriched in the lumen of BC (E).

**TABLE I**

| Analogue and spot | Polarized cells (monolayers) | Cells in suspension |
|-------------------|-----------------------------|---------------------|
|                   | −DFP | +DFP | −DFP | +DFP |
| Diacyl-NBD-PC     |       |       |       |       |
| Intact            | 93.6 ± 0.4 | 97.4 ± 1.6 | 87.8 ± 2.5 | 96.9 ± 1.0 |
| FA                | 6.4 ± 0.4 | 2.6 ± 1.6 | 12.2 ± 2.5 | 3.1 ± 1.0 |
| Diacyl-NBD-PS     |       |       |       |       |
| Intact            | 71.2 ± 2.3 | 80.2 ± 1.0 | 60.2 ± 0.7 | 72.7 ± 2.3 |
| FA                | 16.9 ± 2.3 | 31.6 ± 2.5 | 17.3 ± 2.0 | 18.8 ± 1.0 |
| Fluorescent PE<sup>a</sup> | 6.6 ± 0.8 | 10.6 ± 5.5 | 19.2 ± 1.5 | 19.4 ± 2.1 |
| Fluorescent FA<sup>a</sup> | 5.3 ± 0.8 | 6.1 ± 2.0 | 3.4 ± 0.1 | 6.1 ± 2.5 |
| Diacyl-NBD-PE     |       |       |       |       |
| Intact            | 92.1 ± 1.3 | 96.8 ± 2.5 | 79.2 ± 7.5 | 95.9 ± 1.3 |
| FA                | 7.9 ± 1.8 | 3.2 ± 2.5 | 20.8 ± 7.5 | 4.1 ± 1.3 |

<sup>a</sup> Spots correspond to those observed for diacyl-NBD-PE and diacyl-NBD-phosphatidic acid.

The enrichment of the respective diacyl analogue was significantly lower. This confirms previous studies on the plasma membrane of human fibroblasts and red blood cells showing a low transport activity of APLT for diether PS (20). It is also consistent with the fact that the glycerol backbone of phospholipids affects transport of lipids by APLT (13).

Compared with diacyl-NBD-PS and -PE, internalization of diacyl-NBD-PC and diether NBD-PC from the exoplasmic leaflet was much lower. Nevertheless, we have shown recently that diacyl-NBD-PC is internalized in HepG2 cells at 37 °C by two routes, by endocytic uptake and by transbilayer movement facilitated by a yet unknown transporter (7). Upon endocytosis diacyl-NBD-PC was also transported into a recycling compartment containing transferrin (7). Because of subsequent exposure of PC analogues to the plasma membrane, the fraction of analogues internalized at 37 °C is underestimated.

Taken together, we found that the inward redistribution of various NBD-lipid analogues for the plasma membrane of HepG2 cells is very similar to that of other mammalian cells. In particular, a high affinity of APLT to diacyl-PS, a slightly lower affinity to diacyl-PE, and a very low affinity of the transporter to diether NBD-PS were observed.

**Presence of an APLT Activity in the Canalicular Membrane**—Having established this pattern of inward redistribution, we studied the labeling of BC by fluorescence microscopy after incorporation of fluorescent analogues into the basolateral membranes of polarized HepG2 cells. We found a strong correlation between a low degree of BC labeling and APLT-mediated internalization of the analogue (Table II). Analogues that were not or only inefficiently transported by APLT such as PC analogues and diether NBD-PS were rapidly enriched in the BC. For the aminophospholipid analogues diacyl-NBD-PS and -PE that were shown to be transported efficiently by an APLT activity in HepG2 cells (see above), only a low percentage of labeled BC was found (see bold entries in Table II). Importantly, the PE analogue was less rigorously excluded from the BC. This can be explained by the lower affinity of APLT to PE (see above), which can also rationalize the small amount of PE found in the bile fluid (4).

Labeling of BC by diacyl-NBD-PS increased upon treatment of cells with suramin an inhibitor of APLT. Suramin did not influence the labeling pattern of PC analogues and their enrichment in BC, indicating that the transport pathways of PC (7) are not affected by the inhibitor. These results strongly suggest that low labeling of BC by diacyl-NBD-aminophospholipids is related to an APLT activity in the canalicular membrane pumping aminophospholipids from the luminal to the cytoplasmic leaflet and that inhibition of this APLT activity, e.g., by suramin, leads to labeling of BC by the analogues.

One may wonder whether the rapid inward movement of diacyl-NBD-PS and -PE by an APLT activity on the basolateral
membrane may specifically prevent the access of the analogues to the BC. First of all, we would like to emphasize that tight junctions prevent lateral diffusion of analogues from the basolateral to the apical membrane on the exoplasmic leaflet and thereby access of the analogues to the BC by extra-cellular aqueous space (7, 36, 37). Therefore, analogues have to be delivered to the BC via intracellular pathways or at least by accessing the cytoplasmic leaflet of the plasma membrane. We found a bright diffuse intracellular staining for both diacyl-NBD-PS and -PE but no labeled endocytic vesicles. This shows that the major route of intracellular uptake of these analogues is the rapid transport from the exo- to the cytoplasmic leaflet by APLT, whereas the endocytic pathway plays only a minor role. Once on the inner leaflet of the plasma membrane, both analogues equilibrate rapidly with the cytoplasmic leaflet of subcellular membranes presumably by monomer diffusion (9). Thus, these analogues have access to the canalicular/apical membrane by lateral diffusion in the cytoplasmic leaflet of the plasma membrane and/or by diffusion through the cytoplasm. Indeed, CLSM confirmed the localization of aminophospholipid analogues to the canalicular membrane as shown for diacyl-NBD-PS. The diffuse intracellular labeling observed in the case of diether NBD-PS suggests that this analogue may have access to the canalicular membrane in a similar manner.

It might be argued, that enrichment of diacylaminophospholipid analogues in BC upon treatment with suramin is related solely to an inhibition of APLT activity in the basolateral membrane but does not argue for an APLT activity in the canalicular membrane. This concern is ruled out by the following observations. First, when cells were treated with suramin after internalization of diacyl-NBD-PS or -PE, the original low labeling of BC (see above) changed to a bright labeling of BC, indicating strongly that APLT activity in the canalicular membrane was inhibited. Second, bright labeling of BC by diacylaminophospholipid analogues of suramin-treated HepG2 cells was reversed upon removal of suramin, suggesting that the restored APLT activity in the canalicular membrane caused a redistribution of the analogues from the luminal to the cytoplasmic leaflet of the BC.

Notably, the repartition of PC analogues, of diether NBD-PS and, in the presence of suramin, of diacyl-NBD-aminophospholipids to the BC lumen is in agreement with our previous observation that solubilization of analogues by bile salts is independent of their head group (5, 6). Thus, low labeling of BC by diacylaminophospholipid analogues in the absence of suramin (control) cannot be explained by a failure of bile components to solubilize these analogues from the luminal leaflet. Does Labeling of BC Correlate with Metabolic Conversion of Analogues?—Our data preclude that labeling of BC is caused by products of metabolic conversion of analogues, but not by the analogues themselves. In particular, we can rule out that labeling of BC corresponds to the enrichment of the hydrolyzed

| Analogue       | APLT-mediated transport | Percentage of labeled BC |
|----------------|-------------------------|--------------------------|
| Diacyl-NBD-PS  | ++                      | +                        |
| Diacyl-NBD-PS  |                         | +                        |
| suramin treatment |                        |                          |
| Diacyl-NBD-PE  | ++                      | +                        |
| Diether NBD-PS |                         | ++                       |
| Diacyl-NBD-PC  |                         | ++                       |
| Diether NBD-PC |                         | ++                       |
sn2 fatty acid residue of the analogues carrying the NBD moiety. First, if the latter was the case, the highest degree of BC labeling would be expected when using diacyl-NBD-PS (see Table I); however, the opposite was found. We can also discard that fluorescence in BC arises from NBD-labeled lysolipids. We never observed a metabolic conversion of analogues to fluorescent lysolipids. Second, when incubating polarized cells with the free NBD-labeled fatty acid according to the protocol used for lipid analogues we did not find a stable enrichment of the fatty acid in the BC. Although BC-associated fluorescence in the case of PC and diether analogues or of diacyl-NBD-PS upon treatment with suramin was not affected by washing cell monolayers, for free NBD-labeled fatty acid, BC-associated fluorescence was removed after washing (not shown). This is consistent with a rapid transbilayer movement of this short NBD-labeled fatty acid chain as probed by the dithionite assay in liposomes (not shown). Third, the strong labeling of BC by diether analogues, which are resistant to hydrolytic cleavage of the sn2 chain (e.g. by endogenous phospholipases; see “Results”), argues also against such an explanation.

We can eliminate the possibility that the metabolic conversion of diacyl-NBD-PS (Table I) accounts for the low degree of BC labeling. Even after incubation for 30 min at 37 °C, about 70% of internalized diacyl-NBD-PS were not metabolized. Furthermore, addition of suramin at this point of incubation lead to a strong labeling of BC.

CONCLUSIONS

We have provided evidence for an APLT activity in the canalicular/apical membrane of polarized HepG2 cells. In the light of the finding, that solubilization of phospholipids from the membrane by bile salts is independent of the phospholipid head group, and, thus, does not offer a mechanism to ensure the specific phospholipid composition of the bile fluid (see Introduction), the presence of an APLT activity in the canalicular membrane may provide an alternative explanation for the absence of aminophospholipids in bile fluid. This activity excludes aminophospholipids from the luminal leaflet of the BC in a very efficient manner. Remarkably, this activity is even sufficient to prevent BC enrichment of diacyl-NBD-PS, which can be solubilized by bile salts much more efficiently than endogenous PS having two long fatty acid chains (6).

Further studies are warranted to clarify which proteins of the canalicular membrane are involved in the secretion of diether NBDC-PC and -PS, and, visible upon suramin treatment, of diacyl-NBD-PS and -PE. The Fic1 gene, which is mutated in patients with progressive familial intrahepatic cholestasis 1, was shown to code for a P-type ATPase (38), and hence it was supposed to encode for APLT in the canalicular membrane. Recently, Ujhazy et al. (39) have demonstrated a Fic1-mediated PS translocation in transfected cells. However, because the authors did not investigate the translocation of non-aaminophospholipids, it remains to be established whether Fic1 is a transporter specific for aminophospholipids. Also, several ABC transporters are localized in the canalicular membranes (40). ABC transporters, e.g. MDR1 Pgp and MDR3 (see above), have been shown to transport phospholipids including PC and PE as well as ether lipids (31, 41, 42). Recently, we found that MDR1 Pgp is also able to transport diacyl-NBD-PS and endogenous PS (43). However, the transport activity of MDR1 Pgp was much lower compared with that of APLT.