Release of Phospholipids from Erythrocyte Membranes by Taurocholate Is Determined by Their Transbilayer Orientation and Hydrophobic Backbone†

Daniel Wüstner, Thomas Pomorski,‡ Andreas Herrmann, and Peter Müller*

Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Biologie/Biophysik, Invalidenstrasse 43, D-10115 Berlin, Germany

Received July 7, 1998; Revised Manuscript Received September 30, 1998

ABSTRACT: Bile salts mediate a specific release of phosphatidylcholine (PC) from the canalicular membrane into the bile fluid. We utilized human red blood cells (RBC) as a model system to study the release of endogenous phospholipids as well as phospholipid analogues from plasma membranes in the presence of the bile salt taurocholate (TC). Short- and long-chain fluorescent as well as spin-labeled analogues with various headgroups were chosen. RBC were labeled either on the exoplasmic or on the cytoplasmic leaflet with the analogues and incubated with various concentrations of TC. Analogues on the exoplasmic layer could be readily released by TC. Release was most efficient above the critical micellar concentration (CMC) of TC. Release was independent of the headgroup, but depended on the fatty acid chain length of the analogues; i.e., it was lower for long-chain than for short-chain labeled phospholipids. Analogues on the cytoplasmic leaflet were efficiently shielded from TC-mediated release. The preferential release of endogenous PC and sphingomyelin (SM) from the erythrocyte membrane above the CMC supports the conclusion that TC-mediated release of phospholipids occurs preferentially from the exoplasmic leaflet independent of their headgroup. However, the extent of release of endogenous phospholipids was significantly lower in comparison to that of analogues, endorsing the relevance of the hydrophobic backbone for bile salt mediated release of phospholipids. Implications for the mechanism of the release of PC from the canalicular membrane into the bile fluid are discussed.

Secretion of the bile is an important function of the liver. Bile components, as bile salts, phospholipids, and cholesterol, are secreted into the biliary canaliculus which is formed by the apical domain of the plasma membrane of adjacent hepatocytes, the so-called canalicular membrane. The concentration of bile salts in the lumen of the canaliculus attains (*1*) and presumably goes beyond the respective critical micellar concentration (CMC).[1] Their concentration in the hepatic bile is about 20 mM, well above the CMC for many bile salts (*2*). Together bile salts and phospholipids, mainly phosphatidylcholine (PC), enhance the solubility of cholesterol over several orders of magnitude [see (*3*)]. Thus, biliary phospholipid secretion is an essential step in bile formation and for cholesterol homeostasis.

Phospholipid secretion into the bile is specific. PC constitutes more than 95% of biliary phospholipids, whereas it contributes only to about 35% of the total phospholipid in the canalicular membrane [see (*4*−*6*)]. Although it is well accepted that bile salts mediate the secretion of biliary lipids from the canalicular membrane into the bile [for review, see (*7, 8*)], the molecular mechanisms of the specific enrichment of PC in the bile remain poorly understood. Recent investigations strongly support the hypothesis that a protein-mediated transport of PC across the canalicular membrane is involved. A candidate of those proteins has been identified: the mdr2 P-glycoprotein (Pgp) (*9, 10*) and its human homologue MDR3 Pgp (*11*). This protein is capable to transport specifically PC to the exoplasmic leaflet (*10*−*13*) and most likely accounts for the supply of biliary PC to the luminal leaflet of the canaliculus. However, other flippases not yet identified have been proposed to play a

† This work was supported by grants from the Deutsche Forschungsgemeinschaft (Mu 1017/1-4 to P.M. and GRK 268/97-1 to A.H.). D.W. is a recipient of a fellowship within the Graduiertenkolleg "Dynamics and Evolution of Cellular and Macromolecular Processes".

* To whom correspondence should addressed at Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Biologie/Biophysik, Invalidenstrasse 42, D-10115 Berlin, Germany. Tel/Fax: 49-30-20938830/8585. E-mail: Peter=Mueller@rz.hu-berlin.de.

‡ Present address: Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Monbijoustrasse 2, D-10117 Berlin, Germany.

[1] Abbreviations: C5-BODIPY-PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; C5-BODIPY-SM, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3pentanoyl)sphingosylphosphocholine; C12-BODIPY-PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; C12-BODIPY-SM, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosylphosphocholine; BSA, bovine serum albumin; CMC, critical micellar concentration; C6-NBD-PC, -PS, 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine, -phosphatidylserine; C12-NBD-PC, -PS, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine, -phosphatidylserine; DFP, diisopropyl fluorophosphate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; EPR, electron paramagnetic resonance; mdr, MDR, multidrug resistance; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Pgp, P-glycoprotein; RBC, red blood cell(s); SL-PC, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine; SL-PS, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; SL-PL, spin-labeled phospholipid; SM, sphingomyelin; TC, taurocholate; TDHC, taurodehydrocholate.

10.1021/bi981608b CCC: $15.00
Published on Web 11/11/1998

role in PC transbilayer movement in the canalicular membrane (*14*−*16*). A main issue of the specific enrichment of PC in the bile is the site of its recruitment from the canalicular membrane. On one hand, it has been proposed that PC is secreted by bile salt induced vesicles formed from both leaflets of the canalicular membrane [for review, see (*2*)]. To explain the enrichment of PC, this pinching-off mechanism has to presume the formation of PC bilayer microdomains as well as a specific interaction of luminal bile salts with those domains. On the other hand, it has been suggested that only PC localized in the exoplasmic, canaliculi-oriented leaflet is released into the bile by micellation (*17*, *18*) and/or vesiculation (*19*). In that case, the enrichment of PC would require either a specific interaction between bile salts and PC on the exoplasmic leaflet or, alternatively, a preferential sequestration of PC in the exoplasmic leaflet with respect to other phospholipids.

To explore the interaction of bile salts with biological membranes and, more specifically, the bile salt-induced lipid release from membranes, model systems, in particular human erythrocytes, have been employed (*20*). The composition as well as the transverse orientation and dynamics of lipids in the plasma membrane of human erythrocytes are well-known and considered to be typical for the plasma membrane of other mammalian cells (*21*). Billington and Coleman (*22*) observed that upon incubation with the bile salt glycocholate above its CMC mainly the choline-containing phospholipids PC and sphingomyelin (SM) were released from the erythrocyte membrane. In contrast, released phospholipids were depleted of the aminophospholipids phosphatidylserine (PS) and -ethanolamine (PE) in comparison to the composition of the erythrocyte membrane. Two different models can explain the pattern of phospholipids released by bile salts. First, bile salts release specifically choline-containing phospholipids from the membrane, perhaps from both leaflets. Second, considering that PC and SM are preferentially oriented to the exoplasmic half while PS and PE are enriched on the cytoplasmic leaflet of the erythrocyte membrane (*23*), bile salts may induce the release of phospholipids mainly from the exoplasmic leaflet.

To distinguish between those models, it has to be known (i) from which membrane leaflet(s) bile salt mediated PC release occurs and (ii) whether a specific interaction between bile salts and phospholipid species exists. Selective labeling of plasma membrane leaflets with phospholipid analogues allows one to follow on a quantitative basis whether lipids are released only from the exoplasmic or from both leaflets, and whether bile salts interact in a headgroup-dependent manner with phospholipids. Here, we have investigated the leaflet-specific release of fluorescence- and spin-labeled phospholipid analogues from the erythrocyte membrane by taurocholate (TC). For that purpose, human erythrocytes were labeled with the analogues specifically either on the outer or on the inner half of this membrane. We found that incubation of the labeled red blood cells (RBC) with TC leads to a preferred release of those analogues localized in the outer membrane leaflet. Thus, the results support a model of bile salt mediated phospholipid secretion preferentially from the exoplasmic layer of membranes. The bile salt mediated release is independent of the lipid headgroup but depends on the length of the fatty acid chains.

MATERIALS AND METHODS

Chemicals. Fatty acid free bovine serum albumin (BSA), diisopropyl fluorophosphate (DFP), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), sodium dithionite, taurocholate (highest purity, Sigma-No. T 9034), glucose, SDS, NaCl, Na_2HPO_4, and NaH_2PO_4 were obtained from Sigma (Deisenhofen, Germany), taurodehydrocholate (TDHC) was from Calbiochem (Bad Soden, Germany), Hepes was from Serva (Heidelberg, Germany), and Tris was from Fluka (Neu-Ulm, Germany).

Phosphate-buffered saline (PBS) contained 150 mM NaCl and 5.8 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4). Hepes-buffered saline contained 137 mM NaCl, 3 mM KCl, 10 mM glucose, and 20 mM Hepes (pH 7.4). Stock solutions of TC (100 mM) were prepared in PBS (pH was adjusted to 7.4). Stock solutions of dithionite (1 M) in 100 mM Tris/HCl (pH 9.0) were freshly prepared and stored at 0 °C for no longer than 3 h.

The spin-labeled phospholipids (SL-PL) 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine (SL-PC) and -phosphatidylserine (SL-PS) were synthesized as described (*24*). The fluorescent phospholipids 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine (C6-NBD-PC) and -phosphatidylserine (C6-NBD-PS) as well as 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine (C12-NBD-PC) and -phosphatidylserine (C12-NBD-PS) were obtained from Avanti Polar Lipids (Birmingham, AL). 2-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (C12-BODIPY-PC), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (C5-BODIPY-PC), *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosylphosphocholine (C12-BODIPY-SM), and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosylphosphocholine (C5-BODIPY-SM) were purchased from Molecular Probes (MoBiTec, Göttingen, Germany). C12-fluorescent labeled phospholipids are called 'long-chain' analogues, all other analogues 'short-chain' analogues.

Preparation of Human Erythrocytes. Citrate-stabilized blood was obtained from the blood bank Berlin-Lichtenberg. Erythrocytes were washed twice in PBS (10 min, 2000*g*) at 4 °C and finally resuspended to a hematocrit of 30%. To diminish hydrolysis of labeled phospholipid analogues, erythrocytes were incubated with 5 mM DFP for 5 min at 37 °C prior to labeling (see below) (*25*, *26*).

Release of Endogenous Lipids. Two volumes of erythrocytes (hematocrit 30%) were mixed with 1 volume of TC solution of various concentrations. After incubation for 5 min at 25 °C, the suspension was centrifuged (1 min, 16000*g*). The phospholipids in the supernatant were extracted according to (*27*). Phospholipid species were separated by thin-layer chromatography and subsequently quantified by phosphorus determination (*28*). In parallel, phospholipids from RBC not treated with TC were isolated and quantified.

Labeling with Short-Chain Phospholipid Analogues. RBC were specifically labeled either on the exoplasmic or on the cytoplasmic leaflet with phospholipid analogues bearing the

label moiety on a short fatty acid chain at the *sn*-2 position. As has been shown, short-chain spin-labeled (*29*) as well as fluorescent (*25*) phospholipid analogues incorporate readily into the exoplasmic leaflet of human erythrocytes. The amount of analogues on either leaflet was determined by the back-exchange of those analogues localized in the exoplasmic leaflet to BSA (*25, 26, 30*). For that purpose, cells were incubated for 5 min with 2% (w/v) BSA (final concentration) on ice. After centrifugation, supernatant and pellet were treated as described below.

(A) Labeling of the Exoplasmic Leaflet. Appropriate amounts of fluorescent (C6-NBD- or C5-BODIPY-phospholipids) or spin-labeled (SL-PC) phospholipid analogues, respectively, in chloroform/methanol (1:1 v/v) were transferred to a glass tube and dried under nitrogen. Analogues were dispersed in PBS by vortexing. In the case of C6-NBD-phospholipids, a small quantity of ethanol was added prior to addition of PBS (final ethanol concentration was below 1%). The dispersion of SL- or C6-NBD-analogues was added to the erythrocyte suspension (30% final hematocrit, final analogue concentration was about 2 mol % of endogenous RBC phospholipids), incubated on ice for 15 min, and washed twice with ice-cold PBS to remove nonincorporated analogues. About 90% of the total analogues became incorporated.

Labeling of erythrocytes with C5-BODIPY-analogues was performed as described *(31)*. Erythrocytes (final hematocrit 30%) were incubated with a C5-BODIPY-phospholipid dispersion (1.2 mol % of endogenous phospholipids) containing 0.1% (w/v) BSA (final concentration) for 30 min on ice and washed twice with ice-cold PBS to remove nonincorporated analogues. About 95% and 70% of total C5-BODIPY-SM and -PC, respectively, became incorporated.

(B) Labeling of the Cytoplasmic Leaflet. The exoplasmic leaflet of RBC membranes was labeled with SL-PS, C6-NBD-PC, or C6-NBD-PS as described above. Aminophospholipid analogues become easily enriched on the cytoplasmic membrane leaflet of RBC owing to the activity of the aminophospholipid translocase (*25, 30, 32, 33*). As has been shown, the translocase transports SL-PS and C6-NBD-PS with half-times of 2−3 min (*26*) and about 10−15 min (*33, 34*), respectively, to the inner leaflet at 37 °C. Therefore, after labeling, erythrocytes (30% final hematocrit) were incubated for 30 min (SL-PS) or 2 h (C6-NBD-PS) at 37 °C to ensure accumulation of these analogues in the inner leaflet. Indeed, after incubation, about 80% of C6-NBD-PS as well as of SL-PS was transported to the cytoplasmic monolayer as measured by the back-exchange assay (data not shown).

Since PC and the corresponding analogues traverse the RBC membrane only by slow passive diffusion (*25, 26, 33*), labeling of the inner leaflet with C6-NBD-PC was performed by a 14 h incubation under aseptic conditions at 37 °C (*34*). About 50% of C6-NBD-PC became oriented to the cytoplasmic layer (data not shown). Exclusive labeling of the cytoplasmic leaflet was achieved by removal of the analogues from the exoplasmic leaflet using the back-exchange method (see above). Any residual BSA was removed by two washes with ice-cold PBS.

Labeling with Long-Chain Fluorescent Phospholipid Analogues. (A) Labeling with C12-NBD-Phospholipids. The incorporation of long-chain labeled phospholipids into membranes is less efficient compared with that of short-chain labeled analogues. Therefore, to enhance incorporation, C12-NBD-phospholipids were suspended in PBS as described above for short-chain labeled analogues but with the addition of BSA (*31*). Erythrocytes (final hematocrit 30%) were incubated with a C12-NBD-phospholipid dispersion (2 mol % of endogenous phospholipids) containing 0.1% (w/v) BSA (final concentration) for 1 h at 37 °C (except for labeling of the exoplasmic leaflet with C12-NBD-PS, see below). Subsequently, cells were washed 3 times with ice-cold PBS to remove nonincorporated analogues as well as BSA. About 75% of the analogues became incorporated. This was determined by lipid extraction with 2-propanol (500 μL) of RBC (100 μL) before and after washing (see below). Labeling of RBC by the ethanol injection method (*35*) or by supply of analogues from donor vesicles (*36, 37*) was less efficient (data not shown).

(B) Labeling of the Exoplasmic Leaflet. The exoplasmic leaflet was labeled either with C12-NBD-PC or with C12-NBD-PS. In the case of C12-NBD-PC, about 15−20% of the incorporated analogues redistributed to the cytoplasmic leaflet during labeling as verified by the back-exchange as well as the dithionite assay (see below). To reduce inward translocation due to the action of the aminophospholipid translocase, labeling of the outer leaflet with C12-NBD-PS was performed with the following modifications. After addition of a dispersion of C12-NBD-PS (3 mol % of endogenous phospholipids) in PBS containing 0.1% (w/v) BSA (final concentration), erythrocytes were incubated for 1 h on ice. About 40% of the analogues became incorporated. Approximately 40% of the incorporated analogues redistributed to the cytoplasmic layer during labeling, which was verified as described below.

(C) Labeling of the Cytoplasmic Leaflet. Erythrocytes were labeled with C12-NBD-PS as described, washed 3 times with ice-cold PBS to remove nonincorporated analogues, and incubated further for 1 h at 37 °C to allow redistribution of label to the cytoplasmic leaflet owing to the action of the aminophospholipid translocase. Afterward the cells were incubated 3 times with 10% (w/v) BSA at 25 °C for 10 min to remove those analogues oriented to the exoplasmic leaflet. Remaining C12-NBD-PS was localized in the cytoplasmic layer as probed by the back-exchange as well as the dithionite assay. Finally, RBC were washed 3 times with ice-cold PBS to remove BSA.

(D) Transbilayer Distribution of NBD-Analogues. The transbilayer distribution of incorporated long-chain NBD-analogues was estimated (i) by back-exchange to BSA (*38*) and (ii) by using the dithionite assay (*35, 39, 40*). *(i) Back-exchange.* To extract C12-NBD-phospholipids from the exoplasmic leaflet, 2 volumes of RBC (hematocrit 30%) were 2-fold incubated with 1 volume of 30% (w/v) BSA at 25 °C for 10 min. After centrifugation, supernatant and pellet were treated as described below. *(ii) Dithionite assay.* Labeled erythrocytes were incubated with 250 μM DIDS (final concentration in cell suspension; stock solution 25 mM DIDS in DMSO) for 10 min at 37 °C. It has been shown that DIDS prevents permeation of dithionite into RBC (*40*). Subsequently, RBC were incubated in the absence or presence of 100 mM dithionite for 10 min at 2 °C. Aliquots of dithionite-treated as well as nontreated labeled erythrocytes were washed 3 times with ice-cold PBS. The cell pellets

(30 μL) were solubilized by the addition of 500 μL of 2-propanol. After centrifugation (1 min, 16000*g*), the supernatants containing the extracted lipids were transferred to a fluorescence cuvette containing 1.5 mL of PBS with 0.2% Triton X-100. Fluorescence intensities were measured as described below. All incorporated analogues became accessible to dithionite after hemolysis of RBC in aqua dest containing 1.3% (v/v) Triton X-100 and 100 mM dithionite.

(E) Labeling with BODIPY-Analogues. RBC (hematocrit 30%) were incubated in the presence of C12-BODIPY-PC or -SM (1.2 mol % of endogenous phospholipids) dissolved in DMSO [final DMSO concentration 1% (v/v)] for 1 h at 37 °C. The amount of incorporated analogues corresponded to 0.3 mol % of endogenous phospholipids. Subsequently, the cells were washed 3 times with PBS to remove nonincorporated analogues. Only about 8% of the incorporated analogues could be extracted after 3 times of back-exchange with 10% (w/v) BSA at 25 °C. A similar observation has been reported for short-chain labeled BODIPY-analogues (*41*). Therefore, the transverse distribution of C12-BODIPY-phospholipids in the erythrocyte membrane could not be assayed by the back-exchange method. However, in analogy to the other analogues of PC and SM (NBD- as well as SL-labeled lipids), an almost exclusive localization of the C12-BODIPY-phospholipids on the exoplasmic layer was assumed.

In one set of experiments, RBC were preincubated with short-chain SL-PS for 10 min at 37 °C prior to incubation with C12-BODIPY-SM for 1 h at 37 °C. Afterward, those labeled cells were incubated twice with 2% (w/v) BSA on ice for 15 min to remove remaining SL-PS from the outer leaflet. Residual BSA and nonincorporated analogues were removed by three washes with ice-cold PBS.

Release of Phospholipid Analogues from Red Blood Cells by Bile Salts. Two volumes of exoplasmic and cytoplasmic leaflet-labeled erythrocytes (hematocrit 30%), respectively, were mixed with 1 volume of TC or TDHC solution of various concentration. The suspension was incubated at 25 °C for 5 min. After centrifugation (1 min, 16000*g*), supernatant and pellet were treated as described below. For each time point, the amount of released analogues was normalized to the total amount of cell-associated analogue (supernatant plus pellet). To investigate the kinetics of TC-mediated phospholipid release, RBC labeled either on the inner or on the exoplasmic leaflet with NBD-phospholipids were incubated at 25 °C in the presence of 7 mM TC. After various times, aliquots were taken and centrifuged (1 min, 16000*g*), and the amount of analogues was determined from the supernatant and pellet as described below. Data were fitted to a single-exponential function using SigmaPlot 2.0 (Jandel Scientific, Erkrath, Germany).

EPR and Fluorescence Measurements. (A) Spin-Labeled Cells. Five hundred microliters of 2-propanol was added to the supernatant (100 μL) and to the pellet (30 μL), respectively. Both samples were centrifuged (5 min, 16000*g*), and the organic solvent of the supernatant was evaporated in a vacuum centrifuge (Univapo 100 H, Martinsried, Germany). Remaining lipids were resuspended in 50 μL of PBS containing 8% (v/v) Triton X-100, and the amount of spin-labeled phospholipids was determined from the intensities of their EPR spectra in the presence of the reoxidizing agent potassium hexacyanoferrate (10 mM). EPR spectra were recorded with a Bruker ECS 106 spectrometer (Karlsruhe, Germany) (microwave power 20 mW, modulation amplitude 2.5 G, modulation frequency 100 kHz).

(B) Fluorescent Labeled Cells (Short-Chain as Well as Long-Chain Analogues). The supernatant (100 μL) was transferred to a fluorescence cuvette containing 1.5 mL of PBS, 500 μL of 2-propanol, and 0.2% (v/v) Triton X-100, and the fluorescence intensities were measured (see below). The cell pellet (30 μL) was solubilized by addition of 500 μL of 2-propanol and centrifuged (1 min, 16000*g*). The supernatant (500 μL) containing the extracted lipids was transferred to a fluorescence cuvette containing 1.5 mL of PBS with 0.2% (v/v) Triton X-100. Fluorescence intensities were measured at an excitation/emission wavelength of 470 nm/540 nm and 490 nm/515 nm for NBD- and BODIPY-labeled analogues, respectively, with a slit width of 4 nm/8 nm and 4 nm/4 nm, respectively, using an Aminco Bowman Series 2 spectrofluorometer (Urbana, IL).

Measurement of Hemolysis. TC-mediated hemolysis of nonlabeled and labeled RBC was measured. Cells were incubated in the presence of TC for 5 min at 25 °C. After centrifugation (1 min, 16000*g*), the absorbance of the supernatant was measured at 540 nm using a Shimadzu UV-1202 spectrophotometer (Kyoto, Japan). Complete hemolysis (100%) was determined by incubation of erythrocytes in aqua dest. containing 4% (v/v) Triton X-100.

Data are presented as mean ± standard error of estimate (SE), if not stated otherwise.

RESULTS

Release of Endogenous Phospholipids. First, we determined the amount and composition of endogenous phospholipids released by TC. To this end, erythrocytes were incubated in the presence of various concentrations of TC up to 20 mM and, subsequently to centrifugation, the total amount of phospholipids in the supernatant was determined. As shown in Figure 1A, the amount of endogenous phospholipids released after 5 min at 25 °C increased continuously with the concentration of TC. At >15 mM TC, data suggest a plateau of released phospholipids corresponding to about 9% of total erythrocyte phospholipid.

Likewise, the relative composition of extracted phospholipids depended on the concentration of TC. A preference of TC-released phospholipids for the choline-containing lipids SM and PC became evident, in particular at higher TC concentration (Figure 1B). When compared with the composition of the RBC membrane, the portion of the aminophospholipids PS and PE on released phospholipids decreased while that of the choline-containing phospholipids increased (PC) or remained unaffected (SM) (Figure 1B). This preference for the choline-containing phospholipids also became obvious when relating the released amount of a lipid species to its total amount in the erythrocyte plasma membrane: about 10 and 14% of PC and SM, respectively, but only about 4% of PS and PE were released above 10 mM TC (Figure 1C).

Our results on the release of endogenous phospholipids in the presence of TC are in agreement with those obtained upon incubation of RBC with the bile salt glycocholate reported previously (*22*). The composition of released

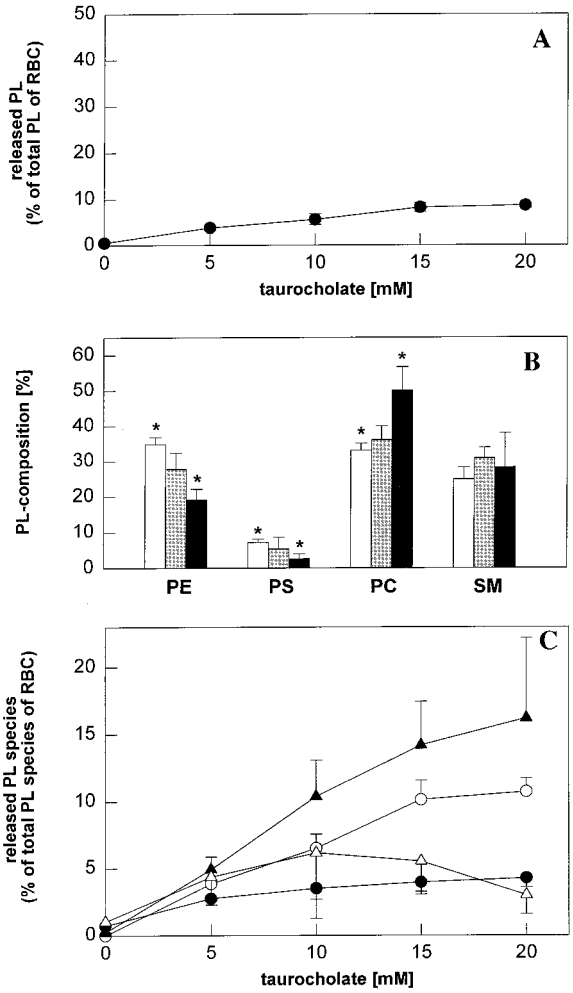

FIGURE 1: Release of endogenous phospholipids of RBC by taurocholate. Erythrocytes were incubated for 5 min at 25 °C with various concentrations of TC. After centrifugation, lipids in the supernatant were determined as described under Materials and Methods. The data represent the mean ± SE of at least four (0 and 5 mM TC) and five experiments, respectively. (A) Amount of released phospholipids normalized to the total phospholipid content of control erythrocytes (no incubation with TC). (B) Phospholipid composition of the supernatant after incubation with 5 mM TC (gray bars) and with 20 mM TC (black bars); for comparison, the phospholipid composition of the erythrocyte membrane is shown (white bars). Significant differences between the phospholipid composition of erythrocyte membranes and released phospholipids were found at 20 mM TC (PE, PC, and PS) (unpaired *t*-test $P < 0.05$) (indicated by asterisks). (C) TC-released amount of lipid species normalized to its amount in nontreated erythrocyte plasma membrane: PE (●), PC (○), SM (▲), and PS (△).

endogenous phospholipids at higher concentration of TC indeed reflects qualitatively that of the exoplasmic leaflet of the human erythrocyte membrane (*21*, *23*). However, the data do not allow one to conclude that phospholipids preferentially originate from the exoplasmic leaflet. A release of phospholipids from both leaflets by TC cannot be precluded.

To determine the site, i.e., the leaflet(s), from which phospholipids are released by TC, we have investigated the TC-mediated release of various fluorescent and spin-labeled phospholipid analogues incorporated initially into the outer or the inner layer of RBC (see Materials and Methods). In view (i) of the asymmetric transbilayer arrangement of endogenous phospholipids in the RBC membrane, (ii) of their TC-mediated release, and (iii) of the specific phospholipid composition of the bile, we have selected analogues of PC, SM, and PS.

Release of SL- and C6-NBD-Phospholipid Analogues by Taurocholate. First, the exoplasmic leaflet was labeled either with the PC analogues C6-NBD-PC, or SL-PC, with the SM analogue C6-NBD-SM or with the PS analogue C6-NBD-PS. Within the time course of the subsequent experiments (see below), these analogues did not significantly redistribute to the cytoplasmic leaflet since (i) transbilayer movement of PC- and SM-analogues in the erythrocyte membrane occurs only by slow passive diffusion (*26*, *30*, *33*, *34*) and (ii) the aminophospholipid translocase mediated transport of C6-NBD-PS to the cytoplasmic leaflet at 25 °C proceeds only with a half-time of about 25 min (T. Pomorski, personal communication). Indeed, 5 min after labeling about 95% and 90% of incorporated PC- and PS-analogues, respectively, remained in this layer at 25 °C as verified by analogue back-exchange to BSA. The spin-labeled analogue of PS could not be employed for those studies since even at 25 °C the analogue is efficiently transported to the cytoplasmic leaflet of the RBC membrane (half-time 5 min; T. Pomorski, personal communication).

Bile salt mediated release of the analogues was measured after incubation of RBC for 5 min in the presence of TC and TDHC at 25 °C. At a concentration of ≥5 mM, TC mediates an efficient release of short-chain phospholipid analogues from the exoplasmic leaflet. This was deduced from an increase and a concomitant decrease of analogue amount in the supernatant (Figures 2 and 3; filled symbols; C6-NBD-SM not shown) and in the pellet (only shown for C6-NBD-PC; see inset Figure 2A, filled triangles), respectively. The release of labeled phospholipids from the exoplasmic layer displays a sigmoidal dependence on the TC concentration, which was similar for all analogues used. A steep increase of released analogues was found above 5 mM TC, reaching a plateau at >10 mM TC. Independent of the lipid headgroup about 80−90% of C6-NBD-phospholipids were extracted from the outer leaflet of RBC at 15−20 mM TC (Figure 2A,B). A similar result was obtained when employing a different analogue of PC, the spin-labeled PC. More than 90% of SL-PC was solubilized at this TC concentration (Figure 3; filled circles). The data indicate that the TC-mediated release is determined neither by the phospholipid headgroup nor by the label moiety. In contrast, the non-micelle-forming bile salt TDHC could not trigger any phospholipid release from the RBC membrane (figure not shown).

Next, we labeled the cytoplasmic leaflet of RBC with C6-NBD-PC or with the PS analogues C6-NBD-PS and SL-PS, respectively (see Materials and Methods). Within the experimental time course, analogues remained essentially confined to the cytoplasmic leaflet due to slow passive transbilayer diffusion. Less than 5% of analogues from the cytoplasmic leaflet redistributed to the exoplasmic leaflet. We could not employ the analogue SL-PC since significant labeling of the cytoplasmic leaflet would require prolonged incubation even at 37 °C. However, under those conditions the paramagnetic NO moiety would be reduced by intracellular redox systems (*42*).

Lipid analogues localized in the cytoplasmic leaflet are less efficiently released by TC as those oriented to the

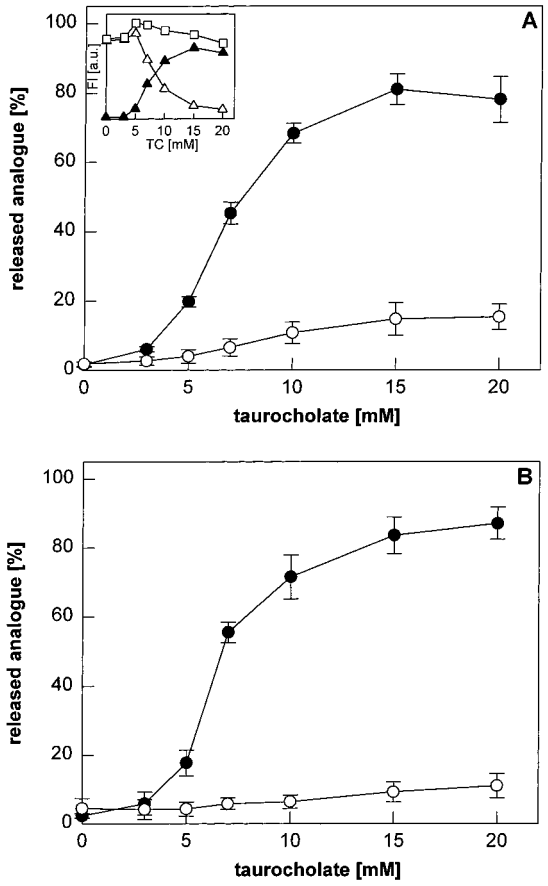

FIGURE 2: Release of short-chain fluorescent phospholipid analogues (C6-NBD-phospholipids) from erythrocyte membranes by taurocholate. Erythrocytes were labeled with fluorescent phospholipid analogues specifically on the exo- (●) or cytoplasmic leaflet (○). The release of analogues at various concentrations of TC after 5 min incubation at 25 °C was determined as described under Materials and Methods. The amount of released analogues was normalized to the total amount of analogues in the supernatant and in the pellet. (A) C6-NBD-PC; (B) C6-NBD-PS. The data represent the mean ± SE of three experiments. The inset of panel A shows a typical experiment. Erythrocytes were labeled on the exoplasmic leaflet with C6-NBD-PC. The absolute amount of analogues (expressed as fluorescence intensities in a.u.) in the supernatant (▲) and in the pellet (△) as well as the sum of both (□) is given.

exoplasmic one. Only 15% of C6-NBD-PC, 11% of C6-NBD-PS, and 6% of SL-PS could be released from the cytoplasmic layer in the presence of above 10 mM TC (Figures 2 and 3, hollow circles). Comparison of the results for the fluorescent analogues C6-NBD-PC and C6-NBD-PS reveals that the TC-mediated release from the cytoplasmic leaflet is independent of the headgroup, too.

Release of C12-NBD-Analogues by Taurocholate. To examine the influence of the fatty acid chain length on TC-induced lipid release, we performed similar experiments with NBD-analogues bearing a long-chain (C12) at the *sn*-2 position. Release of C12-NBD-phospholipids localized in the exoplasmic leaflet followed also a sigmoidal dependence on bile salt concentration (Figure 4). Approximately 60% of total C12-NBD-PC and 45% of total C12-NBD-PS could be released at ≥15 mM TC. However, in the case of the long-chain NBD-analogues, a significant amount of analogues redistributed to the cytoplasmic leaflet during the labeling procedure (15% and 40% for C12-NBD-PC and C12-NBD-PS, respectively; see Materials and Methods).

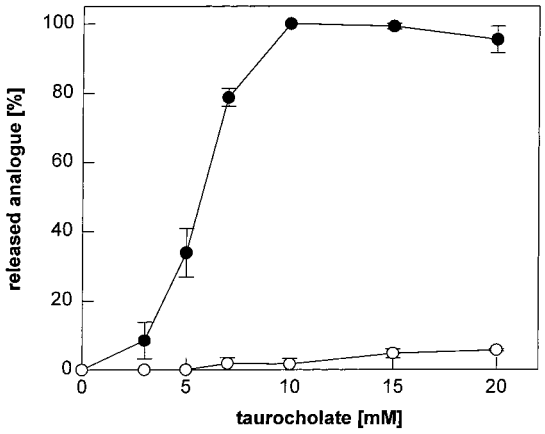

FIGURE 3: Release of spin-labeled phospholipid analogues from erythrocyte membranes by taurocholate. Erythrocytes were labeled with phospholipid analogues specifically on the exo- (SL-PC; ●) or cytoplasmic leaflet (SL-PS; ○). The release of analogues at various concentrations of TC after 5 min incubation at 25 °C was determined as described under Materials and Methods. The amount of released analogues was normalized to the total amount of analogues in the supernatant and in the pellet (see legend to Figure 2). The data represent the mean ± SE of three (SL-PC) and two (SL-PS) experiments.

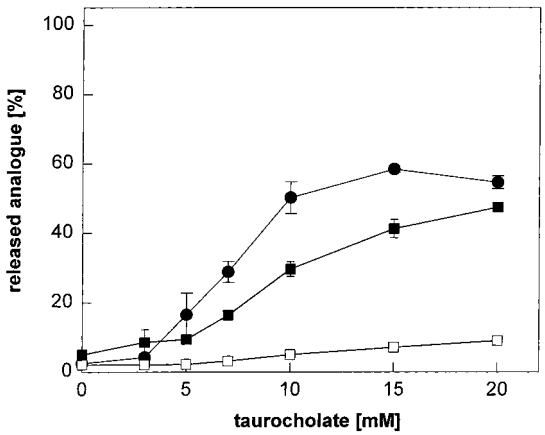

FIGURE 4: Release of long-chain fluorescent phospholipid analogues (C12-NBD-phospholipids) from erythrocyte membranes by taurocholate. Erythrocytes were labeled with fluorescent phospholipid analogues specifically on the exo- (C12-NBD-PC, ●; C12-NBD-PS, ■) or cytoplasmic leaflet (C12-NBD-PS; □). The release of analogues at various concentrations of TC after 5 min incubation at 25 °C was determined as described under Materials and Methods. The amount of released analogues was normalized to the total amount of analogues in the supernatant and in the pellet (see legend to Figure 2). The data represent the mean ± SE of two experiments.

Normalizing the amount of analogues to that of the exoplasmic leaflet, about 70% and 75% of C12-NBD-PC and C12-NBD-PS, respectively, were released by TC. Even after correction the amount of released analogues from the exoplasmic layer is lower for long-chain than that for short-chain NBD-analogues (about 80−90%, see above).

This normalization is only valid if TC-mediated release of analogues oriented to the cytoplasmic leaflet is low. To test this, erythrocytes were exclusively labeled on the cytoplasmic leaflet with C12-NBD-PS (see Materials and Methods). Indeed, only 9% of C12-NBD-PS localized on the inner leaflet of RBC were released at 20 mM TC (Figure 4), indicating again an efficient shielding of phospholipids from the inner leaflet against bile salt induced extraction.

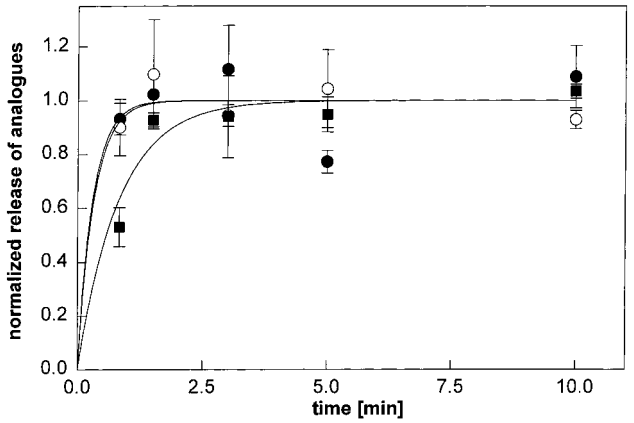

FIGURE 5: Kinetics of release of fluorescent phospholipid analogues from the exoplasmic leaflet of erythrocyte membranes by taurocholate. Erythrocytes were labeled with fluorescent phospholipid analogues specifically on the exoplasmic leaflet. The release of analogues at 7 mM TC after various incubation times at 25 °C was determined as described under Materials and Methods. The data were fitted to a single-exponential function and normalized by setting the amplitude parameter of this function to 1. The half-time was determined to 12.5 s for C6-NBD-PC (●), 13.4 s for C6-NBD-PS (○), and 36.5 s for C12-NBD-PC (■). The data represent the mean ± SE of three or two (only C12-NBD-PC) experiments.

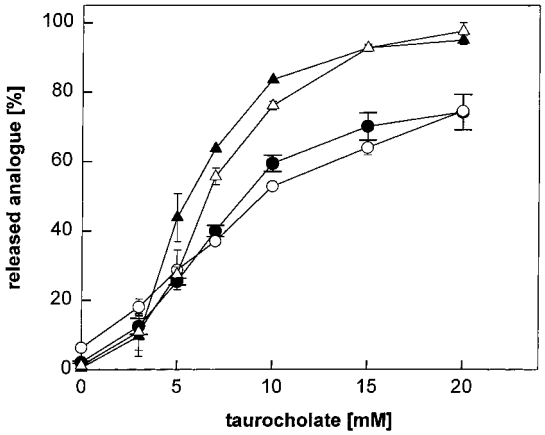

FIGURE 6: Release of short- and long-chain BODIPY-phospholipid analogues from erythrocyte membranes by taurocholate. Erythrocytes were labeled with C5- or C12-BODIPY phospholipid analogues, and the release of analogues at various concentrations of TC after 5 min incubation at 25 °C was determined as described under Materials and Methods. The amount of released analogues was normalized to the total amount of analogues in the supernatant and in the pellet (see legend to Figure 2). C5-BODIPY-PC (△), C5-BODIPY-SM (▲), C12-BODIPY-PC (○), C12-BODIPY-SM (●). The data represent the mean ± SE of two (C5-BODIPY) or three (C12-BODIPY) experiments.

In conclusion, the difference of TC-mediated release of long-chain analogues between exo- and cytoplasmic monolayer is similar to that found for short-chain labeled lipids (see Figure 2). The data support a specific release of analogues from the exoplasmic leaflet which is determined by the length of their fatty acid chain but is independent of the lipid headgroup.

Kinetics of Bile Salt Induced Release of Phospholipid Analogues. The influence of the headgroup and of the length of the fatty acid chain on the kinetics of TC-mediated release of lipids was measured. For this purpose, the time dependence of the release of the fluorescent phospholipid analogues C6-NBD-PC, C6-NBD-PS, and C12-NBD-PC from the exoplasmic leaflet of erythrocytes was followed at 7 mM TC (Figure 5). Half-times of kinetics were deduced from a single-exponential fit of data.

The kinetics observed for the short-chain analogues of PC and PS were too rapid in order to be well resolved by the experimental setup. The estimated half-time for both analogues was almost identical (about 13 s). In contrast, we found a much slower release of the long-chain analogue C12-NBD-PC with a half-time of about 37 s. The data on the kinetics confirm the above conclusions: the TC-mediated release of phospholipid analogues (i) does not depend on the lipid headgroup, but (ii) does depend on the length of the fatty acid chain, at least in the *sn*-2 position.

We have also measured the kinetics of the release of analogues from the cytoplasmic leaflet of RBC in the presence of 7 mM TC which was slower in comparison to that from the exoplasmic layer. For example, the half-time of release was 28.3 s (SE = 2.9, $n = 3$) and 42.9 s (SE = 8.6, $n = 3$) for C6-NBD-PC and C6-NBD-PS, respectively (figure not shown).

Release of BODIPY-Analogues by Taurocholate. The transbilayer distribution of NBD-analogues can be easily and rapidly assessed by various assays. However, a disadvantage of NBD-analogues is that due to the bulky polar NBD group the *sn*-2 chain loops back to the membrane surface disturbing the membrane structure (*43*). BODIPY analogues mimic better the arrangement of endogenous lipids in the membrane than NBD-analogues (*41*). They are less polar and show in comparison to NBD-analogues a negligible spontaneous monomeric transfer from the membrane into the aqueous environment. This was first reported for short-chain labeled C5-BODIPY-analogues, explaining the resistance of those lipids toward back-exchange to BSA (*41*). Indeed, the resistance of C12-BODIPY-analogues toward the back-exchange to BSA (see Materials and Methods) underlines their rigid anchoring to the membrane excluding the determination of their transbilayer distribution by the corresponding assay.

The outer leaflet of RBC was labeled with C12-BODIPY-PC and C12-BODIPY-SM, respectively. Upon incubation of C12-BODIPY-labeled erythrocytes with TC for 5 min at 25 °C, we found that about 74% of the incorporated analogues could be extracted at 15−20 mM TC (Figure 6). We found no significant difference between C12-BODIPY-PC and -SM, supporting that the TC-mediated release is independent of the headgroup. To confirm that the release of phospholipid analogues depended on the fatty acid chain length (see above for NBD-labeled analogues), we have done similar experiments with the short-chain analogues C5-BODIPY-PC and C5-BODIPY-SM, respectively. Indeed, we observed a higher release of those analogues by TC in comparison to the long-chain labeled phospholipids (Figure 6). Again, no headgroup specificity was found.

One may argue that the efficient release of analogues from the exoplasmic leaflet by TC is related to the excess of lipids in this leaflet with respect to the cytoplasmic leaflet upon labeling. To test this notion, we performed a double-labeling of the cells with SL-PS (2 mol % of endogenous phospholipids) on the cytoplasmic and C12-BODIPY-SM (0.3 mol % of endogenous phospholipids) on the exoplasmic leaflet of the erythrocyte membrane. Upon incubation with TC,

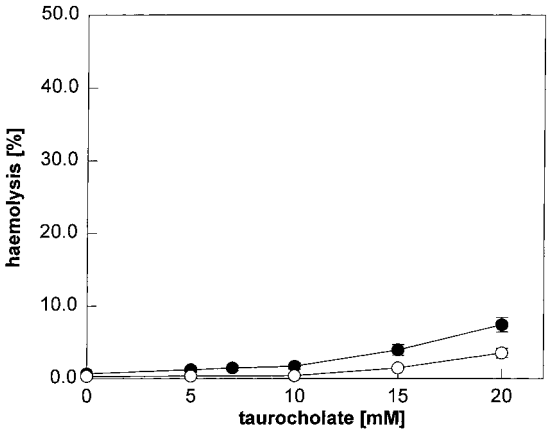

FIGURE 7: Taurocholate-induced hemolysis. Erythrocytes (nonlabeled, ○; labeled, ●) were incubated for 5 min at 25 °C with various concentrations of TC. Hemolysis was measured from the absorbance of the supernatants at 540 nm (as described under Materials and Methods). Hemolysis of cells labeled with various analogues was determined in parallel to the measurements shown in Figures 2−4. The data represent the mean ± SE of 13 experiments in the case of labeled RBC and 3 experiments for nonlabeled cells.

we found essentially the same release for both analogues as shown in Figures 3 and 6, respectively. Thus, we can preclude that the preferential release of analogues from the exoplasmic leaflet by TC is caused by an excess of lipids in this leaflet.

Bile Salt Induced Hemolysis of Erythrocytes. The influence of TC on the cell integrity of RBC was estimated by measuring the hemolysis. Incubation of erythrocytes in the presence of TC was performed under conditions similar to those for determination of lipid analogue release. A low level of hemolysis was found for >10 mM TC, reaching 4% at 20 mM TC (Figure 7). To clarify whether labeling affects TC-mediated hemolysis, we compared nonlabeled and labeled erythrocytes. However, we found only a slight enhancement of hemolysis upon labeling. Even at 20 mM TC, hemolysis did not exceed 8% for labeled RBC (Figure 7). In conclusion, the extensive release of analogues from the exoplasmic monolayer at those TC concentrations cannot be explained by hemolysis (Figures 2, 3, and 4). We cannot preclude that the low amount of analogues released from the cytoplasmic leaflet is associated with hemolysis, too.

DISCUSSION

TC-Mediated Release of Phospholipids from the Human Erythrocyte Membrane. In the present study we have investigated the leaflet and phospholipid specificity of the interaction of the bile salt taurocholate with erythrocyte membranes. Selective incorporation of phospholipid analogues of PC, SM, and PS into the exo- or cytoplasmic leaflet of the erythrocyte membrane permitted us to address whether the release of phospholipids from the erythrocyte membrane by TC depends on the phospholipid headgroup and occurs preferentially only from one of the two leaflets. We have used spin-labeled and various fluorescent phospholipid analogues to preclude that the reporter moiety determines the interaction with TC. To assess the relevance of fatty acid residues for TC-mediated release of analogues, we have employed short- as well as long-chain analogues with different fluorescent moieties. We found that in the presence of TC mainly analogues from the exoplasmic, but not from the cytoplasmic, leaflet are released. Interaction of TC with the membrane does not trigger an enhanced movement of phospholipid analogues between both leaflets of erythrocytes. The release of the analogues is not determined by the phospholipid headgroup. However, the chain length of their fatty acid residues is an important determinant of the efficiency of TC-mediated release from the membrane.

Short- as well as long-chain phospholipid analogues localized in the exoplasmic leaflet could be readily released from the erythrocyte membrane by TC. The extent of release followed a sigmoidal dependence on the bile salt concentration. The onset of substantial analogue release from erythrocyte membranes by TC coincides with the CMC of the bile salt, which is about 4−5 mM [see (*44*)]. In contrast, the bile salt TDHC, which is unable to form aggregates in aqueous solution (*2*), could not mediate the release of phospholipid analogues from the RBC membrane. Thus, micellation of the bile salt is a precondition for effective release of lipids from the erythrocytes, supporting previous studies (*2*, *20*). Above the CMC, more than 80% of the short-chain analogues on the outer leaflet are released independent of the headgroup and the label moiety. As quantified for C6-NBD-lipids, release was rapid with a half-time below 13 s for both the PC and the PS-analogue. However, analogues localized on the cytoplasmic leaflet are efficiently shielded against bile salt activity. Only about 10% are released by incubation with TC at a concentration above its CMC. Again, this was independent of the headgroup and the label moiety. Notably, it has been shown for lipid membranes that the transbilayer movement of taurine-conjugated bile salts in the ionized state is on the order of several hours (*45*, *46*). Thus, it is very unlikely that under our conditions analogues from the cytoplasmic leaflet are released into the cytoplasm by intracellular enrichment of TC. For C12-NBD-phospholipids, a similar picture as found for short-chain analogues emerged: analogues on the exoplasmic leaflet were efficiently released independent of their headgroup while analogues oriented to the cytoplasmic leaflet remained associated with the erythrocyte membrane. However, in comparison to the short-chain analogues, we observed that the extent of released analogues from the outer leaflet was reduced. Moreover, the half-time of release increased by a factor of about 3.

We can preclude that TC causes a significant and fast passive flip-flop of phospholipid analogues between both membrane leaflets in the time course of our experiments. In the latter case, a continuous removal of analogues from the cytoplasmic leaflet of the erythrocyte membrane by TC should be expected, which was not seen. With a half-time of less than 1 min, the low plateau of analogues released from the cytoplasmic leaflet was achieved even above the CMC of TC. Nevertheless, we cannot preclude that this low plateau is caused by a transient accelerated transbilayer movement after the addition of TC by a perturbation of the bilayer arrangement either due to intercalation of TC and/or by a release of lipids from the exoplasmic leaflet of erythrocytes. Other reasons may contribute to the release of analogues from the cytoplasmic leaflet in the presence of TC. Hemolysis could lead to an exposure of the cytoplasmic leaflet to TC. However, even at high concentration of TC,

hemolysis was below 8% Thus, it can only partly account for that, if it all. We also have to consider that the bile salt could trigger a limited vesiculation of RBC involving both leaflets of the plasma membrane. Such a mechanism is indicated by the results of TC-mediated release of endogenous phospholipids. With increasing concentration of TC, we observed for endogenous phospholipids a preferential release of those species residing typically in the exoplasmic leaflet, PC and SM. However, at lower concentration of TC (5 mM), the relative amount of released PS and PE was only moderately reduced with respect to its relative abundance in the erythrocyte plasma membrane. Since both aminophospholipids are oriented mainly to the cytoplasmic leaflet of the RBC, this result implies that TC could also trigger a vesiculation involving both leaflets of the plasma membrane. However, at higher TC concentration, release of phospholipids from the exoplasmic leaflet dominates. These data are in accordance with those of Billington and Coleman (*22*), who found a microvesicle fraction with similar lipid composition as the plasma membrane of RBC mainly at 6 mM glycocholate but, additionally, an enrichment of PC at high bile salt concentration (i.e., 12.7 mM).

How can we explain the differences of the extent of TC-mediated release between endogenous phospholipids and analogues? Our results show that the hydrophobic backbone, more specifically the fatty acid chain length, but not the headgroup of the analogue significantly affects the release of phospholipid analogues from the erythrocyte membrane. Several previous studies have already pointed out the importance of the hydrophobicity of phospholipids for bile salt mediated release from liposomal (*47*) as well as canalicular membranes (*5*). The hydrophobicity of phospholipids is mainly determined by the length and the degree of saturation of the fatty acid residues (*48*). Vesicles containing phospholipids with shorter and/or unsaturated fatty acids were more easily solubilized by bile salts than those consisting of lipids with longer and/or saturated residues (*47*). Very likely, the much lower amount of TC-mediated release of endogenous phospholipids in comparison to that of the analogues can be explained by their different hydrophobicity. Typically, the chain length of endogenous phospholipids is longer even than that of our long-chain analogues. In addition, for the human RBC membrane, the exoplasmic leaflet is enriched in saturated fatty acid residues with respect to the cytoplasmic monolayer (*49, 50*). Approximately 9% of the total phospholipids of erythrocytes were only released at 15−20 mM TC compared to about 70% of the long-chain labeled analogues. However, the clear preference for the endogenous phospholipid species, PC and SM, typical for the exoplasmic leaflet is in accordance with the results obtained for the lipid analogues.

Although our data provide evidence that the interaction of TC with the erythrocyte membrane causes release of phospholipids and respective analogues specifically from the exoplasmic leaflet, they do not allow one to conclude on the mechanism of this bile salt induced lipid extraction. The efficient release by TC but low extractability by BSA of C12-BODIPY-analogues from the erythrocyte membrane provides an indication that the bile salt induced lipid release cannot simply be conceived by analogue monomers, which only upon spontaneous diffusion from the membrane interact with TC preventing their re-incorporation into the membrane. Notably, Hägerstrand et al. (*51*) showed that bile salts such as TC induce spherical exovesicles from the erythrocyte membrane even at 13 mM TC. Interestingly, those vesicles were often hemoglobin-depleted in contrast to exovesicles induced by other membrane-active amphiphiles. Depletion of intracellular content may indicate that vesicles are recruited from lipids only of the outer leaflet, but not formed by a pinching-off mechanism involving both leaflets.

Physiological Relevance. Our data support a model in which PC is mainly secreted from the exoplasmic leaflet of the canalicular membrane (or luminal leaflet of the canaliculus), but not by a pinching-off mechanism involving both leaflets of the canalicular membrane (*2*). The release from the outer leaflet by a micellation process (*18, 52*) has been anticipated. It has also been suggested that the presence of vesicles in the bile fluid is consistent with an exclusive recruitment of biliary phospholipids from the exoplasmic leaflet of the canalicular membrane (*19, 53*). We have no indication that the interaction of bile salts, at least of TC, could mediate neither a substantial release of lipids from the cytoplasmic leaflet nor an enhanced transbilayer movement of phospholipids between leaflets. Thus, it seems very unlikely that bile salts cause a rapid flip-flop of lipids, providing a continuous supply of PC to the luminal site of the canaliculus necessary to restore secreted PC. However, the mdr2 glycoprotein typical for the canalicular membrane is thought to act as a phospholipid translocase transporting PC from the cytoplasmic to the exoplasmic leaflet of the canalicular membrane (*10, 13, 17, 18*). Indeed, it has been shown that NBD-analogues of PC are moved by the human homologue of mdr2, the MDR3, to the exoplasmic leaflet of the apical domain of MDCK cells (*12*). Thus, most likely the activity of the mdr2/MDR3 accounts for the supply of biliary PC to the luminal leaflet of the canaliculus. Notably, previous studies have shown that TC stimulates mdr2-mediated PC flipping (*13, 18*).

Our study strongly suggests that the preferential secretion of PC into the bile lumen does not relate to a specific interaction of bile salts (TC) with phospholipid headgroups. Other mechanisms have to account for the specific release of PC into the bile fluid. Several nonexclusive mechanisms can be put forward: (1) The structure and properties of the fatty acid residues of phospholipids affect the secretion into the bile. This is indicated by our results on the influence of the chain length on TC-mediated release of phospholipid analogues from the erythrocyte membrane (see above). More strikingly, it has been shown that the fatty acid residue composition of biliary PC is different from that of the canalicular membrane. Biliary PC is enriched in unsaturated fatty acid residues, predominantly C16:0/C18:2 and C16:0/C18:1 [(*5*); see (*54*)]. (2) It has been proposed that PC in the canalicular membrane organizes in fluid domains which resemble a target for bile acids (*2*). However, so far no evidence for those domains in the canalicular membrane is available. (3) As in plasma membranes of other mammalian cells, PC is enriched in the exoplasmic leaflet of the canalicular membrane. It has been concluded from isolated membranes that both PC and SM are the major phospholipids located on the exoplasmic leaflet of the hepatocyte canalicular membrane. The aminophospholipid PS is exclusively oriented to the cytoplasmic leaflet of the hepatocyte canalicular membrane (*55*). It is not known how this pronounced

asymmetry is maintained in the canalicular membrane. Although the mdr2 protein has been suggested to permit a continuous supply of PC to the luminal leaflet of the canaliculus, it is not clear how other phospholipids, e.g., PS, can maintain their asymmetric distribution. For a variety of mammalian cells, it has been shown that phospholipids adopt a similar transverse asymmetric arrangement in the plasma membrane [see (*21*, *56*)]. The maintenance of the phospholipid asymmetry has been ascribed to the activity of an aminophospholipid translocase pumping at the expense of ATP specifically the aminophospholipids PS and PE to the cytoplasmic leaflet but not PC and SM (*35*, *57*−*61*). It remains to be elucidated whether an aminophospholipid translocase activity is responsible, perhaps in concert with the mdr2 protein, for maintaining transverse asymmetric distribution of phospholipids in the canalicular membrane under the constraint of a continuous supply of PC and cholesterol to the bile lumen. Recently, we have found a pronounced aminophospholipid translocase activity in the plasma membrane of the hepatocytic like cell line HepG2 (*62*). Moreover, we established an aminophospholipid translocase activity in the apical as well as in the basolateral domain of the epithelial cell lines MDCK-II and Caco2 (T. Pomorski, A. Herrmann, P. Müller, G. van Meer, and K. Burger, unpublished experiments). In this respect, we have to stress that erythrocytes lack membrane transport proteins which are typical for the canalicular membrane and necessary for secretion of biliary constitutents including phospholipids [for review, see (*63*−*65*)]. We do not know how these proteins affect the bile salt mediated release of phospholipids from the canalicular membrane into the bile fluid.

Remarkably, we found a TC-mediated release of endogenous SM from the erythrocyte membrane comparable to that of endogenous PC. Likewise we found no difference in the release neither between short-chain NBD-analogues, between short-chain BODIPY-analogues, nor between long-chain BODIPY-analogues of SM and PC. Since SM is enriched in the exoplasmic leaflet of the canalicular membrane, but not in the bile fluid, a mechanism must exist which prevents its presence in the bile fluid. One explanation could be provided by the proposed high glycolipid content of the apical membrane of hepatocytes (*8*). Association of SM in glycosphingolipid−cholesterol-rich domains might hamper the release of SM into the bile and could prevent the destruction of the canalicular membrane in the presence of bile salts at lytic concentration in the canalicular lumen (*8*, *66*). On the other hand, studies on model systems revealed that segregation of SM in such cholesterol-rich domains is lost in the presence of bile salts (*67*). These authors argue that an alkaline sphingomyelinase in the bile (*68*) cleaves released SM to ceramide, which becomes reabsorbed by the bile duct epithelial cells. Further studies are warranted to clarify this issue.

ACKNOWLEDGMENT

We thank Dr. M. Fuchs (Medizinische Universität Lübeck) for helpful discussions and for critical reading of the manuscript and Mrs. S. Schiller (Humboldt-Universität zu Berlin) for the synthesis of spin-labeled phospholipid analogues.

REFERENCES

1. Möckel, G. M., Gorti, S., Tandon, R. K., Tanaka, T., and Carey, M. C. (1995) *Am. J. Physiol. 269*, G73−G84.
2. Coleman, R., and Rahman, K. (1992) *Biochim. Biophys. Acta 1125*, 113−133.
3. Gilat, T., and Sömjen, G. J. (1996) *Biochim. Biophys. Acta 1286*, 95−115.
4. Rigotti, A., Marzolo, M. P., and Nervi, F. (1994) *Curr. Top. Membr. 40*, 579−615.
5. Yousef, I. M., and Fisher, M. M. (1976) *Can. J. Biochem. 54*, 1040−1046.
6. Meier, J., Sztul, E. S., Reuben, A., and Boyer, J. L. (1984) *J. Cell Biol. 98*, 991−1000.
7. Verkade, H. J., Vonk, R. J., and Kuipers, F. (1995) *Hepatology 21*, 1174−1189.
8. Elferink, R. P. J. O., Tytgat, G. N. J., and Groen, A. K. (1997) *FASEB J. 11*, 19−28.
9. Smit, J. J., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar, E., vanDeemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993) *Cell 75*, 451−462.
10. Ruetz, S., and Gros, P. (1994) *Cell 77*, 1071−1081.
11. Smith, A. J., Timmermanshereijgers, J. L. P. M., Roelofsen, B., Wirtz, K. W. A., Vanblitterswijk, W. J., Smit, J. J. M., Schinkel, A. H., and Borst, P. (1994) *FEBS Lett. 354*, 263−266.
12. vanHelvoort, A., Smith, A. J., Sprong, H., Fritzsche, I., Schinkel, A. H., Borst, P., and vanMeer, G. (1996) *Cell 87*, 507−517.
13. Nies, A. T., Gaitman, Z., and Arias, I. M. (1996) *J. Lipid Res. 37*, 1125−1136.
14. Berr, F., Meier, P. J., and Stieger, B. (1993) *J. Biol. Chem. 268*, 3976−3979.
15. KullakUblick, G. A., Gerloff, T., Hagenbuch, B., Berr, F., Meier, P. J., and Stieger, B. (1996) *Hepatology 23*, 1254−1259.
16. Cohen, D. E., and Fuchs, M. (1996) *Hepatology 24*, 1546−1548.
17. Higgins, C. F. (1994) *Cell 79*, 393−395.
18. Ruetz, S., and Gros, P. (1995) *J. Biol. Chem. 270*, 25388−25395.
19. Crawford, J. M., Mockel, G. M., Crawford, A. R., Hagen, S. J., Hatch, V. C., Barnes, S., Godleski, J. J., and Carey, M. C. (1995) *J. Lipid Res. 36*, 2147−2163.
20. Graham, J. M., and Northfield, T. C. (1987) *Biochem. J.*, 825−834.
21. Zachowski, A. (1993) *Biochem. J. 294*, 1−14.
22. Billington, D., and Coleman, R. (1978) *Biochim. Biophys. Acta 509*, 33−47.
23. Roelofsen, B., and Op den Kamp, J. A. F. (1994) in *Cell Lipids* (Hoekstra, D., Ed.) pp 7−46, Academic Press Inc., San Diego, CA.
24. Fellmann, P., Zachowski, A., and Devaux, P. F. (1994) in *Biomembrane Protocols II* (Graham, J. M., and Higgins, J. A., Eds.) pp 161−175, Humana Press Inc., Totowa, NJ.
25. Colleau, M., Herve, P., Fellmann, P., and Devaux, P. F. (1991) *Chem. Phys. Lipids 57*, 29−37.
26. Morrot, G., Herve, P., Zachowski, A., Fellmann, P., and Devaux, P. F. (1989) *Biochemistry 28*, 3456−3462.
27. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
28. Rouser, G., Siakotos, A. N., and Fleisher, S. (1966) *Lipids 1*, 85−86.
29. Marx, U., Lassmann, G., Wimalasena, K., Müller, P., and Herrmann, A. (1997) *Biophys. J. 73*, 1645−1654.
30. Calvez, J.-Y., Zachowski, A., Herrmann, A., Morrot, G., and Devaux, P. F. (1988) *Biochemistry 27*, 5666−5670.
31. Pagano, R. E., and Martin, O. C. (1994) in *Cell Biology—A Laboratory Handbook* (Celis, J. E., Ed.) Vol. 2, pp 387−393, Academic Press Inc., San Diego, CA.
32. Seigneuret, M., and Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751−3755.

33. Connor, J., Gillum, K., and Schroit, A. J. (1990) *Biochim. Biophys. Acta 1025*, 82−86.
34. Connor, J., Pak, C. H., Zwaal, R. F. A., and Schroit, A. J. (1992) *J. Biol. Chem. 267*, 19412−19417.
35. Pomorski, T., Müller, P., Zimmermann, B., Burger, K., Devaux, P. F., and Herrmann, A. (1996) *J. Cell Sci. 109*, 687−698.
36. Pagano, R. E., and Sleight, R. G. (1985) *Science 229*, 1051−1057.
37. Tanaka, Y., and Schroit, A. J. (1983) *J. Biol. Chem. 258*, 11335−11343.
38. Haest, C. W. M., Plasa, G., and Deuticke, B. (1981) *Biochim. Biophys. Acta 649*, 701−708.
39. McIntyre, J. C., and Sleight, R. G. (1991) *Biochemistry 30*, 11819−11827.
40. Pomorski, T., Herrmann, A., Zachowski, A., Devaux, P. F., and Müller, P. (1994) *Mol. Membr. Biol. 11*, 39−44.
41. Martin, O. C., and Pagano, R. E. (1994) *J. Cell Biol. 125*, 769−781.
42. Seigneuret, M., Zachowski, A., Herrmann, A., and Devaux, P. F. (1984) *Biochemistry 23*, 4271−4275.
43. Chattopadhyay, A., and London, E. (1987) *Biochemistry 26*, 39−45.
44. Coello, A., Meijide, F., Nunez, E. R., and Tato, J. V. (1996) *J. Pharm. Sci. 85*, 9−15.
45. Cabral, D. J., Small, D. M., Lilly, H. S., and Hamilton, J. A. (1987) *Biochemistry 26*, 1801−1804.
46. Donovan, J. M., and Jackson, A. A. (1997) *Biochemistry 36*, 11444−11451.
47. Cohen, D. E., Angelico, M., and Carey, M. C. (1990) *J. Lipid Res. 31*, 55−70.
48. Heikinheimo, L., and Somerharju, P. (1998) *J. Biol. Chem. 273*, 3327−3335.
49. Hullin, F., Bossant, M.-J., and Salem, N., Jr. (1991) *Biochim. Biophys. Acta 1061*, 15−25.
50. Ways, P., and Hanahan, D. J. (1964) *J. Lipid Res. 5*, 318−328.
51. Hägerstrand, H., and Isomaa, B. (1992) *Biochim. Biophys. Acta 1109*, 117−126.
52. Higgins, C. F. (1994) *Curr. Biol. 4*, 259−260.
53. Crawford, A. R., Smith, A. J., Hatch, V. C., Elferink, R. P. J. O., Borst, P., and Crawford, J. M. (1997) *J. Clin. Invest. 100*, 2562−2567.
54. Crawford, J. M. (1996) *Comp. Biochem. Physiol. B 115*, 341−354.
55. Higgins, J. A., and Evans, W. H. (1978) *Biochem. J. 174*, 563−567.
56. Devaux, P. F. (1991) *Biochemistry 30*, 1164−1173.
57. Calvez, J.-Y., Zachowski, A., Herrmann, A., Morrot, G., and Devaux, P. F. (1988) *Biochemistry 27*, 5666−5670.
58. Heinrich, R., Brumen, M., Jaeger, A., Müller, P., and Herrmann, A. (1997) *J. Theor. Biol. 185*, 295−312.
59. Gudi, S. R. P., Kumar, A., Bhakuni, V., Gokhale, S. M., and Gupta, C. M. (1990) *Biochim. Biophys. Acta 1023*, 63−72.
60. Middelkoop, E., van der Hoek, E. E., Bevers, E. M., Comfurius, P., Slotboom, A. J., op den Kamp, J. A. F., Lubin, B. H., Zwaal, R. F. A., and Roelofsen, B. (1989) *Biochim. Biophys. Acta 981*, 151−160.
61. Bruckheimer, E. M., and Schroit, A. J. (1996) *J. Leukocyte Biol. 59*, 784−788.
62. Müller, P., Pomorski, T., Porwoli, S., Tauber, R., and Herrmann, A. (1996) *Hepatology 24*, 1497−1503.
63. Meier, P. J. (1996) *J. Hepatol. 24 (Suppl. 1)*, 29−35.
64. Keppler, D., and König, J. (1997) *FASEB J. 11*, 509−516.
65. Müller, M., and Jansen, P. L. (1998) *J. Hepatol. 28*, 344−354.
66. Schroeder, R., London, E., and Brown, D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12130−12134.
67. van Erpecum, K. J., and Carey, M. C. (1997) *Biochim. Biopys. Acta 1345*, 269−282.
68. Duan, R. D., Hertervig, E., Nyberg, L., Hauge, T., Sternby, B., Lillienau, J., Farooqi, A., and Nilsson, A. (1996) *Dig. Dis. Sci. 41*, 1801−1806.

BI981608B