Head group-independent interaction of phospholipids with bile salts: a fluorescence and EPR study

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Abstract **Bile salts are essential for phospholipid secretion into the bile. To study the relevance of the structure of phospholipids for their interaction with bile salts, we used spin-labeled or fluorescent phospholipid analogues of different head groups and acyl chain length. Those analogues form micelles in aqueous suspension. Their solubilization by bile salts resulting in the formation of mixed micelles was followed by the decrease of spin-spin interaction of spin-labeled analogues or by the relief of fluorescence self-quenching of (7-nitro-2-1,3-benzooxadiazol (NBD))-labeled analogues. Solubilization of analogue micelles occurred at and above the critical micellar concentration (CMC) of the bile salts. As revealed by stopped-flow technique, solubilization of NBD-analogues was very rapid with half times as low as 0.1 sec above the CMC of taurocholate. Both kinetics and extent of solubilization were independent of the phospholipid head group, but were significantly affected by the fatty acid chain length. Furthermore, using vesicles with varying phospholipid composition and different types of analogues in self-quenching concentrations, we could show that bile salt-mediated vesicle solubilization depended on the fatty acid chain length of phospholipids. In contrast, neither for phospholipids nor for analogues could an influence of the lipid head group on the solubilization process be observed. These findings support a head group-independent mechanism of bile salt-mediated enrichment of specific phospholipids in the bile fluid.**—Wüstner, D., A. Herrmann, and P. Müller. **Head group-independent interaction of phospholipids with bile salts: a fluorescence and EPR study.** ***J. Lipid Res.*** **2000.** 41: **395–404.**

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Bile salts are amphiphilic molecules that aggregate in aqueous solution above their critical micellar concentration (CMC). The number of hydroxyl groups and their stereospecific position in the molecule determine the hydrophobicity of bile salts and, thus, their CMC (1). Bile salts can solubilize phospholipids and form with them thermodynamically stable aggregates, micelles and/or vesicles (2, 3). Due to the ability to solubilize phospholipids, bile salts *i)* support the absorption of nutritionary lipids in the intestine and *ii)* mediate the secretion of phospholipids from hepatocytes into the bile (4, 5).

Formation of the bile, which consists mainly of bile salts, phospholipids, and cholesterol, occurs at the canalicular membrane, a specialized domain of the plasma membrane of hepatocytes. The concentration of bile salts in the lumen of the canaliculus attains (6) and presumably goes beyond the respective CMC. Their concentration in the hepatic bile is about 20 mm, well above the CMC for many bile salts (7). There is strong evidence that interaction of bile salts with phospholipids is mandatory for phospholipid release from the canalicular membrane into the bile fluid (1, 7). Remarkably, phosphatidylcholine (PC) becomes enriched in the bile fluid. It comprises about 95% of the total phospholipid content in the biliary fluid, whereas it contributes only about 35% of phospholipids in the canalicular membrane (8–10). The basis of this enrichment is not known. In principal, phospholipid head group and/or acyl chain composition could be determinants of a specific bile salt–phospholipid interaction. It has been shown that the acyl chain composition of phospholipids influences the interaction of bile salts with phospholipids (11). However, while some models for specific PC enrichment in the bile fluid may imply a phospholipid head group specific interaction of bile salts with phospholipids (12, 13), this has not been proven.

Abbreviations: CMC, critical micellar concentration; C6-(C12-) NBD-PL, short (long) chain NBD-labeled phospholipid; C16/C6-NBD-PC, -PS, -PE, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzooxadiazol-4-yl)amino] caproyl]-*sn*-glycero-3-phosphatidylcholine, -phosphatidylserine, -phosphatidylethanolamine; C16/C12-NBD-PC, -PS, -PE, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzooxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphatidylcholine, -phosphatidylserine, -phosphatidylethanolamine; C14/C12-NBD-PC, 1-myristoyl-2-[12-[(7-nitro-2-1,3-benzooxadiazol-4-yl)amino-dodecanoyl]-*sn*-glycero-3-phosphatidylcholine; C6-Pyr-PC, -PE, 1-hexadecanoyl-2-(1-pyrenehexanoyl)-*sn*-glycero-3-phosphatidylcholine, -phosphatidylethanolamine; DOPC, DOPS, dioleoyl-phosphatidylcholine, -phosphatidylserine; EPR, electron paramagnetic resonance; EY-PC, egg yolk-phosphatidylcholine; mdr, multi-drug resistance; PBS, phosphate–buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Pgp, P-glycoprotein; SL-PC, -PS (1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylcholine, -phosphatidylserine; SL-PL, spin-labeled PL; TC, taurocholate; TDC, taurodeoxycholate; TDHC, taurodehydrocholate; GC, glycocholate; PL, phospholipids

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In this study the relevance of the head group and the fatty acid chain length of phospholipids for their interaction with bile salts is investigated. Aside from various (non-labeled) phospholipids, spin-labeled and fluorescent phospholipid analogues of different *i)* head groups and *ii)* fatty acid chain lengths in the *sn*1- and *sn*2-positions were used. Those analogues are known to form micelles in aqueous solution above their CMC as extensively studied for spin-labeled analogues (14). The solubilization of micelles of the analogues by bile salts leading to the formation of mixed micelles allows us to investigate the influence of phospholipid structure on the interaction with bile salts. Furthermore, we have investigated the interaction of bile salts with large unilamellar vesicles (LUV) of varying lipid composition labeled with fluorescent phospholipid analogues in self-quenching concentration. Both analogue micelles and LUV were solubilized by bile salts at and above the CMC of the bile salts. The solubilization was independent of the phospholipid head group. However, the disruption of micelle and vesicle integrity depended on the fatty acid chain length of phospholipids and phospholipid analogues. Our results support the view that the enrichment of PC in the bile is not determined by a head group-specific interaction of bile salts with this phospholipid.

MATERIALS AND METHODS

Chemicals

NaCl, Na_2HPO_4, NaH_2PO_4, and the sodium salts of taurocholate (TC) (Cat. No. T 9034), taurodeoxycholate (TDC) (Cat. No. T 0557), and glycocholate (GC) (Cat. No. G 7132) as well as the phospholipids l-α-dioleoyl-phosphatidylcholine (DOPC), l-α-dioleoyl-phosphatidyl-l-serine (DOPS), and egg yolk phosphatidylcholine (EY-PC) were obtained from SIGMA (Deisenhofen, Germany). Taurodehydrocholate (TDHC) (Cat. No. 580219) was obtained from CalBiochem (Bad Soden, Germany). Stock solutions of bile salts (100 mm or 200 mm) were prepared in phosphate-buffered saline (PBS: 150 mm NaCl, 5.8 mm Na_2HPO_4/NaH_2PO_4 (pH 7.4)). Spin-labeled phospholipid analogues (SL-PL) 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylcholine (SL-PC), -phosphatidylserine (SL-PS), and phosphatidylethanolamine (SL-PE) were synthesized as described by Fellmann, Zachowski, and Devaux (15). Pyrene-labeled phospholipids 1-hexadecanoyl-2-(1-pyrenehexanoyl)-*sn*-glycero-3-phosphatidylcholine (C6-Pyr-PC) and -phosphatidylethanolamine (C6-Pyr-PE) were obtained from Molecular Probes (Leiden, The Netherlands). Fluorescent phospholipids bearing the NBD-group on a 6-carbon fatty acid chain in the *sn2*-position (C6-NBD-PL) (1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzooxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine (C16/C6-NBD-PC), -phosphatidylethanolamine (C16/C6-NBD-PE), and -phosphatidylserine (C16/C6-NBD-PS) as well as analogues bearing the NBD-group on a 12-carbon fatty acid chain in the *sn*2-position (C12-NBD-PL) (1-myristoyl-2-[12-[(7-nitro-2-1,3-benzooxadiazol-4-ylamino]dodecanoyl]-*sn*-glycero-3-phosphatidylcholine (C14/C12-NBD-PC), 1-palmitoyl-2-[12-[(7-nitro-2-1, 3-benzooxadiazol-4-ylamino]dodecanoyl]-*sn*-glycero-3-phosphatidylcholine (C16/C12-NBD-PC), -phosphatidylethanolamine (C16/C12-NBD-PE), and -phosphatidylserine (C16/C12-NBD-PS) were obtained from Avanti Polar Lipids (Birmingham, AL). All measurements except those of the dithionite assay (see below) were done at 25°C.

Preparation of aqueous dispersions of phospholipid analogues

Appropriate amounts of fluorescent or spin-labeled phospholipid analogues, respectively, in chloroform–methanol 1:1 (v/v) were transferred to a glass tube, dried under nitrogen, and vortexed with a volume of PBS to give a concentration of 1 μm and 80 μm of fluorescent and spin-labeled phospholipid analogues, respectively. Fluorescent phospholipid analogues were resolubilized in ethanol prior to the addition of PBS (final ethanol concentration was below 1% v/v).

Preparation and characterization of large unilamellar vesicles

Phospholipids were dissolved in chloroform in a glass tube. NBD-labeled phospholipids in chloroform–methanol 1:1 (v/v) were added at 20 mol% of total phospholipid content to ensure efficient self-quenching of analogues in the vesicles. The mixture was dried under nitrogen and then hydrated by vortexing with PBS to give a final lipid concentration of 1 mm. To prepare LUV, the resulting aqueous phospholipid dispersion was subjected to five freeze–thaw cycles and was then extruded 10 times through two 0.1-μm polycarbonate filters (extruder from Lipex Biomembranes Inc.,Vancouver, Canada; filters from Costar, Nucleopore GmbH, Tübingen, Germany). Vesicle diameter was routinely checked by photon correlation spectroscopy (PCS) using a Coulter N4 Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL). The mean diameter of LUV was about 100 ± 20 nm independent of the vesicle composition. Unilamellarity of vesicles was tested by using the dithionite assay (16, 17). Dithionite, which is non-permeable to lipid membranes, destroys the fluorescence by chemical reaction with the NBD moiety. Briefly, 100 μm of the vesicle suspension (final concentration) was transferred into a cuvette containing a magnetic bar. Under continuous stirring, dithionite was added from a freshly prepared stock solution of 1 m in 100 mm Tris/HCl (pH 9.0) to give a final dithionite concentration of 25 mm. Fluorescence intensities of analogues were measured at 4°C with λ_{ex}= 470 nm and λ_{em}= 540 nm on a Shimadzu RF53001 (Tokyo, Japan). Upon addition of dithionite to labeled LUV a rapid fluorescence decrease to about 46–50% of the initial intensity was found (not shown). This corresponds to the expected symmetric analogue distribution indicating their unilamellarity. Upon disruption of vesicle integrity by addition of Triton X-100 (0.5% v/v), fluorescence was completely abolished.

Determination of the critical micellar concentration of fluorescent and spin-labeled phospholipids

To estimate the CMC of NBD-labeled analogues, self-quenching of fluorescent phospholipids in micelles was used (18). Stock solutions of NBD-PL were prepared (see above) and titrated into a cuvette containing 2 ml PBS (25°C) stirred with a magnetic bar. The fluorescence was continuously monitored using an Aminco Bowman Series 2 spectrofluorometer (Urbana, IL) with λ_{ex}= 470 nm, λ_{em}= 540 nm and an excitation/emission slit of 4 nm and 8 nm, respectively. We observed a bi-phasic rise of the fluorescence intensity with increasing analogue concentration typical for the formation of micelles: initially a steep enhancement of the intensity followed by a low increase of the fluorescence at higher analogue concentration (not shown). The latter is characteristic for the formation of micelles. Both phases could be approximated by linear functions. From the cross point of both functions, the CMC was determined: 0.116 μm, 0.076 μm, and 0.165 μm for C16/C6-NBD-PC, C16/C6-NBD-PE, and C16/C6-NBD-PS, respectively, and 0.05 μm for C14/C12-NBD-PC. The CMC of the analogues C16/C12-NBD-PC, C16/C12-NBD-PE, and C16/C12-NBD-PS could not be determined by this approach, as we found only a single linear dependence of the fluorescence intensity up

to an analogue concentration as low as 0.01 μm. Below this analogue concentration the signal/noise ratio was too low for accurate measurements. Therefore, we surmise that the CMC of those analogues is below 0.01 μm.

For spin-labeled analogues, the CMC was inferred from the EPR spectrum following the approach of King and Marsh (14) and Schmidt, Gähwiller, and Planta (19). For that, the intensity of the mid-field peak was measured and plotted versus the analogue concentration. From the intercept of this biphasic curve, the CMC was calculated: 6 μm and 13.2 μm for SL-PC and SL-PS, respectively. The CMC of SL-PE is lower than 3.5 μm. Below this analogue concentration the signal/noise ratio of the EPR spectrum was too low for accurate measurements. For parameters of EPR measurements see next paragraph.

Measurement of EPR spectra of spin-labeled phospholipids in the presence of bile salts (TC)

The dispersion of SL-PL (see above) was incubated in the presence of various concentrations of either TC or TDHC (final concentration of SL-PL 40 μm). After 5 min, EPR spectra were recorded using a Bruker ECS 106 spectrometer (Karlsruhe, Germany) with the following parameters: microwave power 20 mW, modulation amplitude 1.0 G, modulation frequency 100 kHz. Incubation for 10 min and 15 min revealed identical spectra. From the EPR spectra the intensity of the mid field peak was measured and normalized (I_{norm}) according to:

$$I_{norm} = (I - I_0)/(I_{max} - I_0) \qquad \textit{Eq. 1}$$

where I_0 is the signal intensity of the label dispersion without bile salt and I_{max} is the signal intensity at the highest bile salt concentration employed (20 mm). Note that the signal intensity was independent of the TC concentration above 15 mm (see Results).

Fluorescence dequenching of NBD-labeled phospholipids by bile salts

Two ml of either C12-NBD-PL or C6-NBD-PL (1 μm in PBS, see above) was added to a fluorescence cuvette. The solution was stirred with a magnetic bar and fluorescence intensity was monitored continuously as described above. Bile salts were added step-wise from a stock solution (100 mm) to the label dispersion by injection with a Hamilton microliter syringe (Bonaduz, Switzerland). Upon addition of an aliquot of bile salt solution, fluorescence was measured until a plateau was established. The time required to reach the plateau depended on the analogue (short or long chain) as well as on the bile salt (see Results; last paragraph). Therefore, the fluorescence intensity measured after 5 min and after 15 min was taken for short (C6-NBD-PL) and for long chain analogues (C12-NBD-PL), respectively. No fluorescence bleaching occurred in the time course of the experiment. Longer incubation did not alter the fluorescence spectrum, e.g., measuring the intensity after 45 min of bile salt addition revealed the same values. Except for TDHC, the intensity of the fluorescence signal was normalized according to equation 1 with I_0 being the intensity in the absence of bile salts and I_{max} the intensity at the highest bile salt concentration used. For TDHC, I_{max} corresponds to the intensity in the presence of Triton X-100 (0.5% v/v). To test whether the step-wise addition of bile salts caused a systematic error, we have performed a different experimental protocol. For that, NBD-PL analogues were added to a cuvette (final concentration 1 μm) containing the bile salts at the respective concentration. The change of fluorescence in dependence on bile salt concentration was identical for both protocols.

Solubilization of vesicles by taurocholate

Labeled LUV were incubated in a cuvette under continuous stirring with a magnetic bar. TC was added step-wise from a 200 mm stock solution and the suspension was incubated for 10 min. Subsequently, the excitation shutter was opened and the fluorescence intensity was measured at 25°C with λ_{ex} = 470 nm and λ_{em} = 540 nm, excitation and emission slit 4 nm. Fluorescence intensities were normalized and plotted according to equation 1 and as described for micelles of fluorescent phospholipid analogues (see above).

Static light-scattering intensity was taken as an independent measure of solubilization of LUV upon addition of TC. To this end, the same samples as described for fluorescence dequenching were used, and the intensities were measured at $\lambda_{ex} = \lambda_{em}$ = 450 nm, excitation slit 4 nm and emission slit 8 nm (20).

Stopped flow measurement

The kinetics of interaction between TC and micelles of C6-NBD-PL or C12-NBD-PL or LUV labeled with 20 mol% NBD-lipids were measured using a thermostatted stopped-flow device (RX 1000 Rapid Kinetics, Applied Photophysics, Surrey, UK) connected to an Aminco Bowman spectrometer series 2. The two chambers of the stopped-flow device were filled with 2.5 ml of an aqueous solution with various TC concentrations and 2.5 ml of either the label dispersion (final concentration of analogues 1 μm) or fluorescently labeled LUV in PBS (final concentration of total phospholipid 50 μm). Fluorescence intensities were measured at 25°C with λ_{ex}= 470 nm and λ_{em}= 540 nm, excitation and emission slit 8 nm and 4 nm for analogue micelles and for labeled vesicles, respectively. The dead time of the stopped-flow apparatus is ≤10 msec. For resolving the kinetics of the process properly, the time resolution of the measurement was adapted in the range between 5 ms and 40 ms. At least five measurements were accumulated in order to improve the signal to noise ratio.

RESULTS

Solubilization of phospholipid analogue micelles by bile salts

Spin-labeled phospholipids. Lipid analogues bearing a spin label moiety on the short fatty acid chain in the *sn*2-position form micelles in aqueous solutions (14, 21). This can be inferred from their composite EPR spectrum (**Fig. 1A**). As shown for SL-PC dispersed at 40 μm in PBS, the EPR spectrum is composed of two components: *i)* an isotropic signal with three narrow lines arising from the free tumbling of monomers in the dispersion, and *ii)* a broad component caused by strong spin-spin interaction of analogues organized in micelles (upper panel of Fig. 1A). Addition of TC to an aqueous dispersion of SL-PL resulted *i)* in a decrease of the broad component and *ii)* in an appearance of a rather narrow triplet signal (see Fig. 1A, middle and lower panel, only shown for SL-PC). The decrease of the broad component is due to solubilization of SL-PC micelles by TC resulting in the formation of mixed micelles consisting of both, TC and SL-PC. Within these micelles, SL-PC experiences a rather high degree of mobility indicated by the narrow component.[2] The same pattern of spectral alterations upon addition of TC was observed for SL-PE and SL-PS (not shown). In order to

[2] Note that this spectrum indicates some motional restrictions precluding its origin from monomers tumbling freely in the aqueous phase.

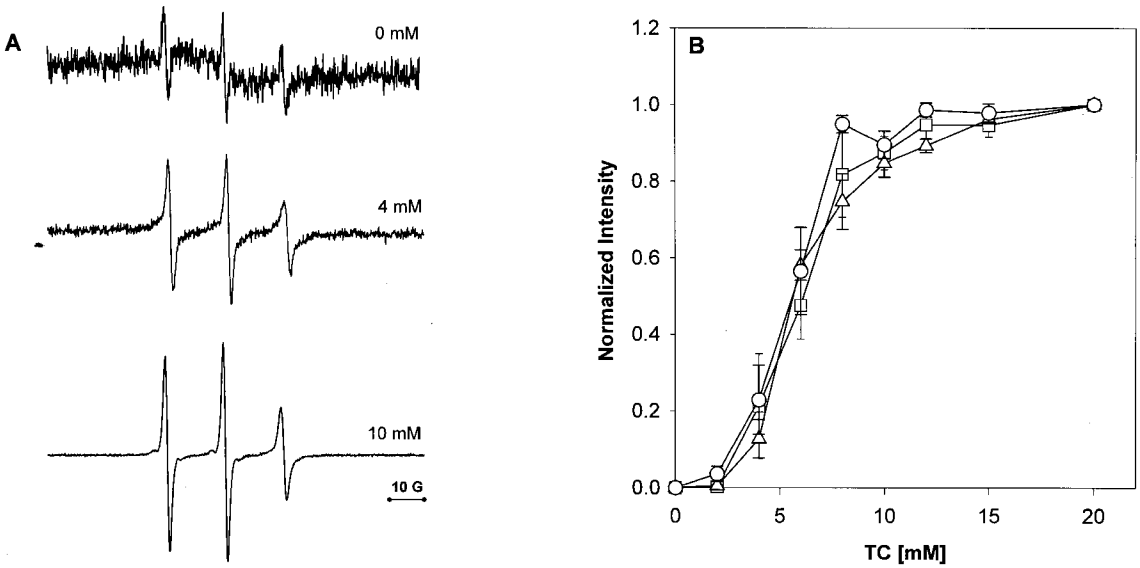

Fig. 1. Interaction of spin-labeled phospholipid analogues with taurocholate. Spin-labeled phospholipids (final concentration 40 μm) in PBS were incubated with various concentrations of TC for 5 min at 25°C. Subsequently, EPR-spectra were recorded at 25°C. A: EPR spectra of SL-PC at 0, 4, and 10 mm TC. Note that the scaling of the spectrum at 4 mm TC and 10 mm TC is 4-fold and 20-fold, respectively, reduced compared to that in the absence of TC. B: Intensity of the middle field peak of the EPR spectra in dependence on the concentration of TC: SL-PE (△), SL-PC (○), SL-PS (□). Intensities were normalized as described in Materials and Methods. The data represent the mean ± SE of three experiments.

compare the solubilization of micelles of different spin-labeled analogues by TC, the intensity of the mid-field peak of the triplet signal was measured and normalized as described in Materials and Methods. The dependence of the normalized intensity on the concentration of TC for different phospholipid analogues is shown in Fig. 1B. The intensity of the mid-field peak shows a sigmoid dependence on the concentration of TC. Up to 2 mm almost no alteration of the EPR spectrum was found. A steep increase of signal intensity was observed (see Fig. 1B) in a concentration range that coincides with the CMC of TC (4–6 mm) (22, 23). All spin-labeled analogues used showed a similar dependence of the signal intensity on the TC concentration. No further increase of EPR intensity was detected for TC concentrations ⩾15 mm. Similarly, line shapes of the spectra for all three phospholipid analogues were identical (not shown). Remarkably, addition of TDHC up to 20 mm did not affect the EPR spectra of analogues. This bile salt does not form micelles in aqueous solution. Taken together, the results argue for an interaction between bile salts and phospholipids which is mainly determined by the presence of bile salt micelles, but independent of the phospholipid head group.

Fluorescent phospholipids. Next, we have investigated the interaction of NBD-labeled phospholipids above their CMC (1 μm, see Materials and Methods) with bile salts. The solubilization of analogue micelles upon addition of various bile salts was followed by measuring the relief of self-quenching, i.e., the increase of the fluorescence intensity. As shown in **Fig. 2 A-C** solubilization of micelles of C16/C6-NBD-PE, -PC, and -PS by TC, GC, or TDC depended on the concentration of the particular bile salt in a sigmoid fashion similar to the results with SL-PL for TC (see above). The dependence of the fluorescence intensity on the concentration of the various bile salts was similar for the different analogues, arguing for a lipid head group-independent solubilization process. The onset of a steep fluorescence increase coincides with a bile salt concentration close to the respective CMC. The CMC has been reported to be 4–6 mm, 6.2 mm, 1.5 mm for TC, GC, and TDC, respectively (22, 23). Up to concentrations close to the CMC only a slight fluorescence increase was observed. For example, at 3.5 mm TC the intensity was only about 7% of the maximum intensity (Fig. 2A). Addition of Triton X-100 (0.5% v/v) at the highest bile salt concentration used did not result in any further increase of fluorescence intensity, suggesting a complete solubilization of analogue micelles. The relevance of the ability of bile salts to form micelles for solubilization of phospholipid analogue micelles is underlined by the results using the non-micelle-forming bile salt TDHC. Addition of TDHC did not cause solubilization of C6-NBD-PL micelles as no change of the fluorescence intensity was observed (Fig. 2D).

We can rule out that the NBD-moiety determines the interaction of bile salts with phospholipid analogues, as we found a similar behavior for NBD- and spin-labeled analogues. To support this conclusion we have investigated the interaction of TC with micelles from phospholipid analogues bearing a pyrene group on the short fatty acid chain in the *sn*2-position (C6-Pyr-PC, C6-Pyr-PE). To this end we followed the excimer/monomer ratio which decreased upon solubilization of analogue micelles. The dependence of this ratio on the TC concentration (data

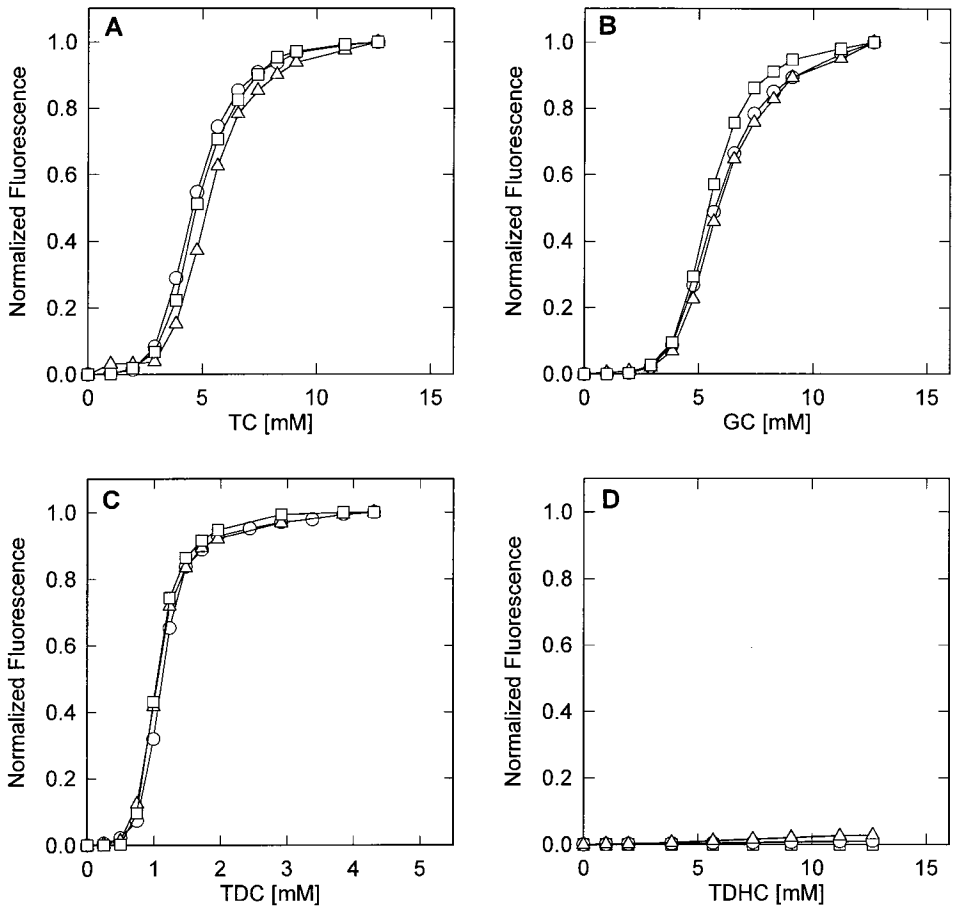

Fig. 2. Interaction of bile salts with short-chain NBD-labeled phospholipid (C6-NBD-PL) analogues. Dispersions of 1 μm of C16/C6-NBD-PE (△), -PC (○), or -PS (□), respectively, in PBS were incubated in a fluorescence cuvette at 25°C. The bile salts TC (A), GC (B), TDC (C), and TDHC (D) were added step-wise from a stock solution. The fluorescence increase was measured and normalized as described in Materials and Methods. A representative experiment is shown. Note the different scaling of the abscissa (bile salt concentration).

not shown) paralleled that of the dequenching of C6-NBD-PL (see above) with a steep increase of the monomer signal around the CMC of TC and no influence of the head group of the lipid analogues.

In order to elucidate the relevance of the fatty acid chain length of phospholipids for the solubilization of analogue micelles, we investigated the bile salt-mediated solubilization of micelles of analogues with the NBD-group on a longer (12 carbon atoms) fatty acid chain in the *sn*2-position and different chain lengths in the *sn*1-position (C16 or C14). Upon addition of TC or TDC, the fluorescence of those analogues dispersed in PBS increased in a sigmoidal fashion depending on the concentration of the bile salt (**Fig. 3**). Similar to C6-NBD-PL, the relief of self-quenching for C16/C12-NBD-PC, -PE, and -PS depended on the CMC of the particular bile salt but was independent of the phospholipid head group. However, the solubilization of micelles of fluorescent phospholipids by bile salts depended on the fatty acid chain length of the phospholipids. Compared with C6-NBD-PL, the sigmoid dependence of fluorescence intensity on the bile salt concentration was less pronounced for phospholipid analogues bearing the reporter moiety on a longer fatty acid chain in the *sn*2-position (compare Figs. 2 and 3 and see inset of Fig. 3A). The more efficient solubilization of C14/C12-NBD-PC with respect to C16/C12-NBD-PC demonstrates that the fatty acid chain length in the *sn*1-position also affects the interaction of phospholipids with bile salts in a similar manner (inset to Fig. 3A). Furthermore, it indicates that the length of the *sn*2-chain determines the TC-mediated solubilization rather than the location of the NBD-moiety.

Kinetics of the solubilization of phospholipid analogue micelles by TC

To characterize further the relevance of phospholipid structure for the interaction with bile salts, we have measured the kinetics of bile salt-mediated solubilization of NBD-analogue micelles using stopped-flow fluorescence spectroscopy (24). Phospholipid analogues were mixed with TC and the kinetics of fluorescence increase were measured (**Fig. 4**, only shown for PC). Various concentrations of TC were chosen, above (12 mm), around (7 mm), and below (3.5 mm) the concentration range for which a steep fluorescence increase was found (see Figs. 2 and 3). In Fig. 4 the kinetics of fluorescence dequenching upon

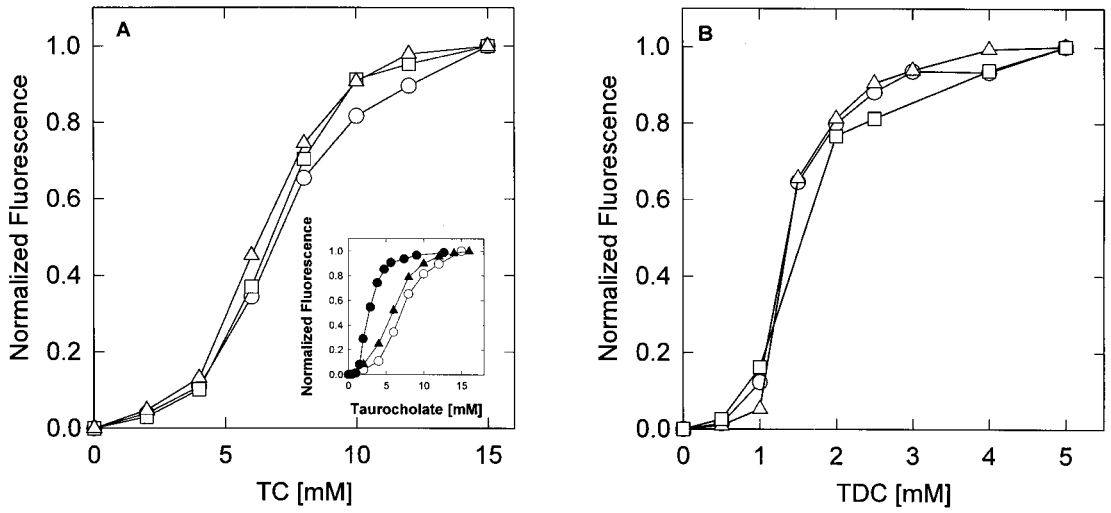

Fig. 3. Interaction of bile salts with long-chain NBD-labeled phospholipid analogues. Dispersions of 1 μm of C16/C12-NBD-PE (△), -PC (○) or -PS (□), respectively, in PBS were incubated in a fluorescence cuvette at 25°C. The bile salts TC (A) or TDC (B) were added step-wise from a stock solution. The fluorescence increase was measured and normalized as described in Materials and Methods. A representative experiment is shown. Inset of A shows solubilization of C14/C12-NBD-PC (▲) by TC (mean ± SEM, n = 3; SEM is of the size of symbols) and for comparison solubilization of C16/C6-NBD-PC (●, compare Fig. 2A) and C16/C12-NBD-PC (○). Note the different scaling of the abscissa.

rapid mixing with the dispersion of TC are shown for C16/C6-NBD-PC (Fig. 4, left panel) and C16/C12-NBD-PC (Fig. 4, right panel). Note the different scaling of the axis for fluorescence intensity. The final intensities depended on the TC concentrations as expected from the experiments above (see Fig. 2 and 3).

Increasing the concentration of TC accelerated the dequenching of all phospholipid analogues (Fig. 4). For C16/C6-NBD-PC, at 12 and 7 mm of TC (Fig. 4A and C), we observed a rapid increase of the fluorescence intensity with a half-time (t½) of 0.03 ± 0.001 sec and 0.05 ± 0.01 sec (mean ± standard error of estimate), respectively (see **Fig. 5**). In contrast, below the CMC of TC (Fig. 4E; 3.5 mm TC) the half time of the initial rapid dequenching was much larger with 0.24 ± 0.035 sec and 0.25 ± 0.02 sec for C16/C6-NBD-PS and -PC, respectively. In addition, subsequent to the rise in fluorescence, a small decline was found which levelled off after about 60 sec. This points to the formation of intermediate, non-equilibrium structures during interaction of bile salts with phospholipid analogue micelles. The formation of those structures depends not only on the bile salt concentration but presumably also on the presence of phospholipid micelles. The latter is supported by the fact that we did not observe the slow intensity decline when the concentration of C16/C6-NBD-PC was below the analogue CMC (Fig. 4G; 0.05 μm C16/C6-NBD-PC, 3.5 mm TC). The same observation was made for C16/C6-NBD-PS (not shown).

For a given TC concentration, the fluorescence dequenching for analogues with longer fatty acid chains was slower than for those with shorter chains (Fig. 4, right panel; Fig. 5). The half-time of the kinetics of micelle solubilization increased in the order C16/C6-NBD-PC < C14/C12-NBD-PC < C16/C12-NBD-PC. Thus, the kinetics of solubilization of NBD-analogue micelles is affected by the fatty acid chain length of phospholipid analogues in the *sn*1- as well as in the *sn*2-position. The difference in half-time of analogues with varying fatty acid chain length in the *sn*1-position indicates again that the fatty acid chain length in the *sn*2-position, and not the location of the reporter moiety, affects the solubilization of micelles by TC.

The phospholipid head group has no significant influence on the kinetics of analogue micelle solubilization by TC as assessed by an unpaired *t*-test ($P < 0.1$) (see legend to Fig. 5). Taken together, the results demonstrate that the kinetics of fluorescence increase depended *i)* on the concentration of the bile salt, and *ii)* on the fatty acid chain length of the analogue but were independent of the analogue head group.

Solubilization of fluorescently labeled LUV by TC

To study the interaction of bile salts with phospholipids and respective analogues being organized in a bilayer, we have used LUV of varying lipid composition. As for analogue micelles (see above), a sigmoid dependence of fluorescence dequenching of analogues on bile salt concentration was found for LUV consisting of EY-PC and of EY-PC with 20 mol% brain PS, respectively (**Fig. 6**). Fluorescence dequenching reflects the solubilization of vesicles as indicated by a concomitant decrease of the light scattering intensity (not shown). Although analogues contributed to only 20 mol% of total lipid content of those LUV, we found that the length of the fatty acid residues in the *sn*1- and *sn*2-positions of analogues affects vesicle solubilization by TC. The efficiency of solubilization decreased in the order C16/C6-NBD-PC > C14/C12-NBD-PC > C16/C12-NBD-PC for LUV containing EY-PC or EY-PC/brain PS (Fig. 6; C14/C12-NBD-PC is not shown for the sake of clarity). However, the following observations strongly indicate a head group-independent interaction of TC with vesicles: *i)* EY-PC LUV containing C16/C6-NBD-PS showed the

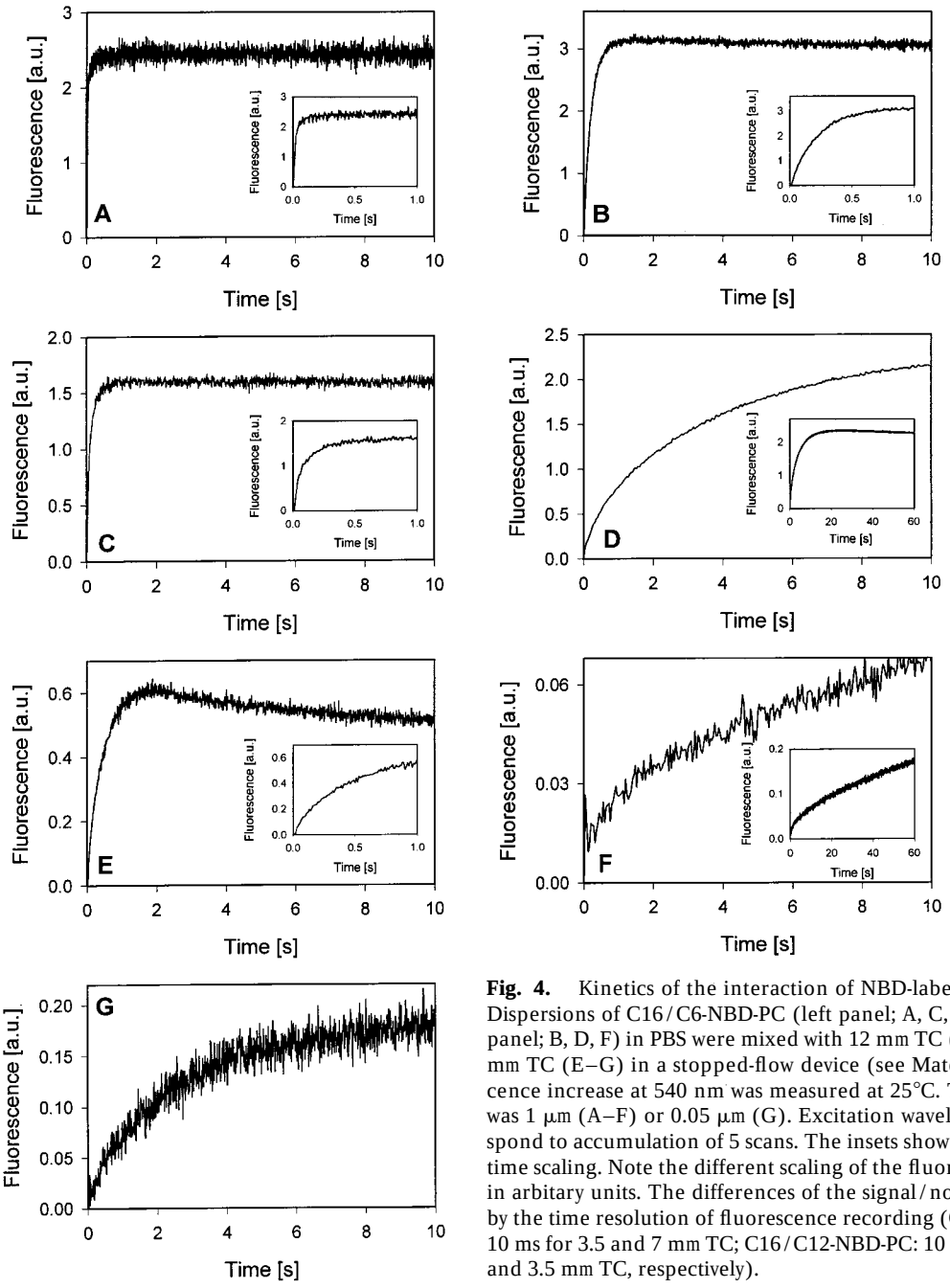

Fig. 4. Kinetics of the interaction of NBD-labeled PC-analogues with taurocholate. Dispersions of C16/C6-NBD-PC (left panel; A, C, E, G) or of C16/C12-NBD-PC (right panel; B, D, F) in PBS were mixed with 12 mm TC (A and B), 7 mm TC (C and D), or 3.5 mm TC (E–G) in a stopped-flow device (see Materials and Methods) and the fluorescence increase at 540 nm was measured at 25°C. The final concentration of analogues was 1 μm (A–F) or 0.05 μm (G). Excitation wavelength was 470 nm. The traces correspond to accumulation of 5 scans. The insets show the same kinetics but with a different time scaling. Note the different scaling of the fluorescence intensity (see Results) shown in arbitary units. The differences of the signal/noise between the kinetics were caused by the time resolution of fluorescence recording (C16/C6-NBD-PC: 5 ms for 12 mm TC, 10 ms for 3.5 and 7 mm TC; C16/C12-NBD-PC: 10 ms, 20 ms and 40 ms for 12 mm, 7 mm and 3.5 mm TC, respectively).

same solubilization profile as those with C16/C6-NBD-PC (not shown); no difference existed in the solubilization profile *ii)* between LUV of EY-PC and LUV of EY-PC and brain PS (20 mol%) for the same type of analogue (Fig. 6), and *iii)* between LUV of DOPC and LUV of DOPC and 20 mol% DOPS labeled with C16/C12-NBD-PC (not shown).

The kinetics of vesicle solubilization was studied also by stopped-flow fluorescence spectroscopy. Using EY-PC LUV labeled with C16/C6-NBD-PC, the half-time of fluorescence dequenching was about 13 ms, whereas for EY-PC LUV with C16/C12-NBD-PC or -PS the half-time was 19 and 22 ms, respectively, at 12 mm TC (kinetics not shown). Although the half-times support the conclusion that interaction of bile salts with phospholipid analogues is determined by the fatty acid chain length of the lipids but is independent of the lipid head group, we note that the measured half-times are close to the time resolution of our experimental setup (about 10 ms), and the kinetics are too fast to be resolved properly.

DISCUSSION

In the present study we have investigated the role of the phospholipid structure, i.e., of the head group and of the length of fatty acid residues, for the interaction of bile salts with phospholipids. We have used spin-labeled and fluorescent analogues of phospholipids with different

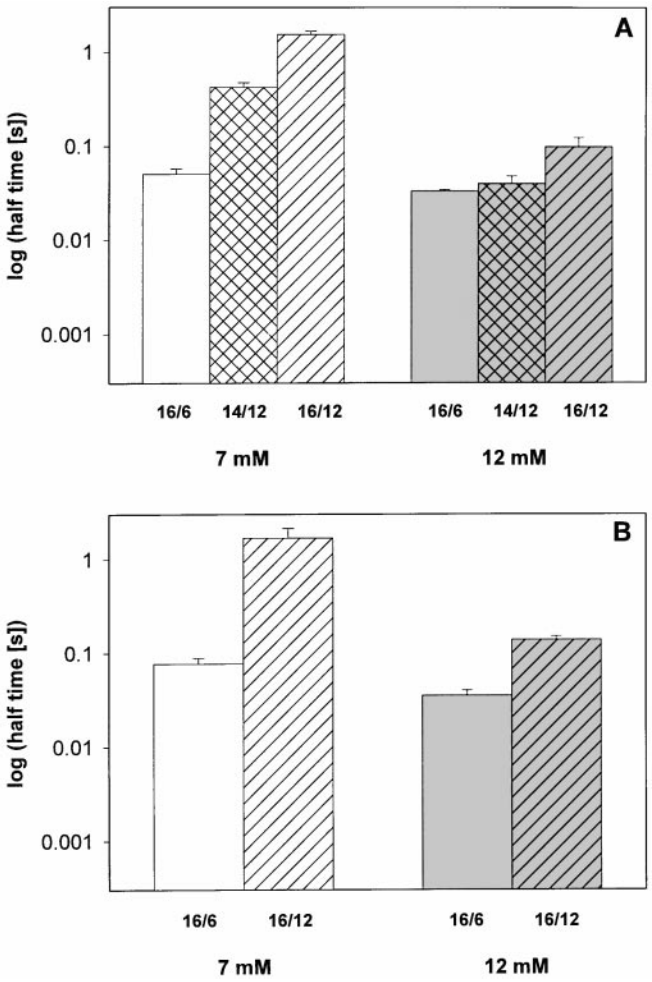

Fig. 5. Half-time of the fluorescence dequenching of NBD-labeled phospholipids upon mixing with taurocholate. The half-time of fluorescence dequenching which corresponds to 50% of maximum fluorescence intensity upon mixing of the NBD-analogue dispersion with the bile salt dispersion was obtained from stopped-flow fluorescence measurements as described in Fig. 4. Values of the half-time are given for short-chain (C16/C6) labeled (open bars) and for long-chain (C14/C12) (cross-hatched) and (C16/C12) (hatched bars) labeled analogues of PC (A) and PS (B) in the presence of 7 mm TC (white bars) and 12 mm TC (grey bars), respectively. Data are the mean ± SE of three to six measurements. The results were statistically assessed using an unpaired *t*-test. No significant differences were detected between the half times of C16/C6-NBD-PC and -PS and between those of C16/C12-NBD-PC and -PS, respectively, at the respective concentration of TC ($P < 0.05$, except for C16/C6-NBD-PC and -PS at 7 mm TC, $P = 0.094$). Note the logarithmic scaling of the half-time.

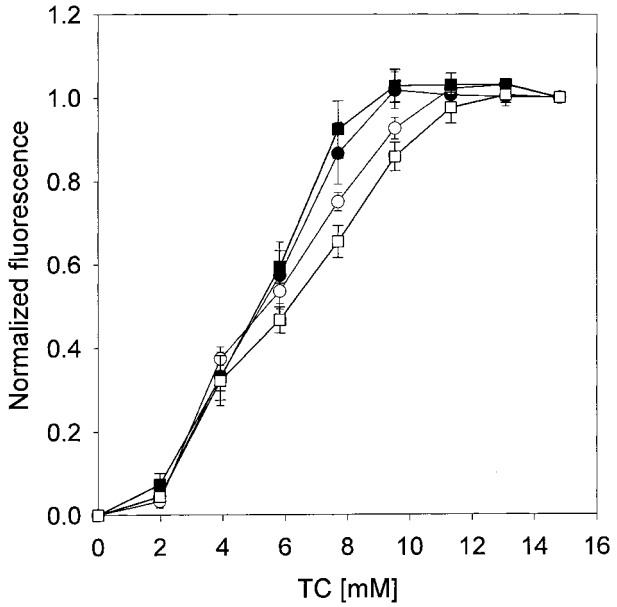

Fig. 6. Solubilization of fluorescently labeled vesicles by taurocholate. LUV consisting either of EY-PC (circles) or of EY-PC with 20 mol% brain PS (squares) were labeled with 20 mol% of C16/C6-NBD-PC (filled symbols) or C16/C12-NBD-PC (open symbols) (see Material and Methods). TC was added step-wise from a stock solution. The fluorescence increase was measured and normalized as described in Materials and Methods. The data represent the mean ± SE of three experiments.

head groups and length of the acyl chains either organized in micelles or incorporated into LUV of various phospholipid composition. We have followed both the extent as well as the kinetics of the bile salt-mediated solubilization of micelles and LUV. By this approach, we could show that the solubilization of analogue micelles and LUV is determined by the fatty acid chain length of phospholipids but not by their lipid head group.

The characteristics of the bile salt-mediated solubilization of micelles were similar for fluorescent and spin-labeled analogues. Thus, we can preclude that the reporter moiety determines the interaction of bile salts with the analogues and would suppress any influence of the phospholipid head groups on the process of micelle solubilization. We found that the solubilization of phospholipid analogue micelles depends on the concentration and the specific CMC of the bile salt. This was indicated by the bile salt-induced decrease of spin-spin interaction of spin-labeled analogues and by the fluorescence dequenching of short- as well as of long-chain NBD-labeled phospholipids. Up to concentrations of 0.5 mm (TDC) and 2 mm (TC, GC) almost no fluorescence increase was detected, although the analogue concentration was 500- and 2000-fold lower, respectively, in comparison to that of the bile salt. Micelles of phospholipid analogues only became solubilized by bile salts in a concentration range that is similar to or above the CMC of bile salts. For example, at a concentration of about 1 mm TDC, about 50% of the maximum fluorescence intensity was attained for the NBD-analogues. At this concentration, the fluorescence quenching was almost unaffected in the presence of TC and GC, which have a 4-fold higher CMC in comparison to TDC (22). However, when reaching their CMC, TC and GC were also able to solubilize analogue micelles. Because the CMC of GC and TC as well as the solubilization of micelles of fluorescent lipid analogues by both bile salts were similar, we can exclude that the chemical structure of the conjugated amino acid of the bile salts influences the interaction with phospholipids. The role of the ability to form micelles for solubilization is underlined by the non-micelle-forming bile

salt TDHC which did not solubilize fluorescent phospholipids in the concentration range investigated.

Remarkably, we have found no influence of the phospholipid head group on the bile salt-mediated solubilization of micelles from phospholipid analogues. Two lines of evidence support this conclusion. *i)* Neither for spin-labeled analogues nor for short- or long-chain NBD-labeled analogues was the sigmoid increase of the EPR-intensity and fluorescence intensity, respectively, affected by the head group. *ii)* The kinetics of formation of mixed micelles between NBD-labeled analogues and TC was independent of the head group. Therefore, we argue for a minor role of the lipid head group, if at all.

However, the formation of mixed micelles depends on the length of the fatty acid chains of the phospholipids. Increasing the length of the fatty acids at the *sn*1- and/or *sn*2-position caused a less sharp sigmoid dependence of the fluorescence dequenching on bile salt concentration. This observation indicates that shorter fatty acid residues promote solubilization of analogue micelles by bile salts. Strong support for this conclusion was given by stopped-flow measurements on the formation of mixed micelles between NBD-labeled analogues and TC. The increase in fluorescence upon mixing with TC was faster for analogues bearing a shorter fatty residue in the *sn*1- or in the *sn*2-position. For example, the half time of solubilization at 7 mm TC was more than 3-fold higher for analogues of PC with palmitoylic acid (C16/C12-NBD-PC) compared to those with myristoylic acid (C14/C12-NBD-PC) in the *sn*1-position. For analogues with varying chain length in the *sn*2-position, the difference was even more pronounced (see Fig. 5).

Similar conclusions on the relevance of phospholipid structure for interaction with bile salts can be drawn from the TC-mediated solubilization of LUV which was independent of the lipid head group of (non-labeled) phospholipids and phospholipid analogues. However, the solubilization was facilitated when phospholipid analogues with a shorter fatty acid residue in the *sn*1- or *sn*2-position were incorporated in liposomal membranes. Taken together, the solubilization of phospholipid assemblies, micelles and vesicles, by bile salts is independent of the lipid head group but is largely affected by the fatty acid composition. The latter result is supported by Cohen, Angelico, and Carey (11) who measured the bile salt-mediated solubilization of small unilamellar vesicles and of multilamellar vesicles consisting of various species of PC by quasielastic light scattering and turbidimetry. For example, a solubilization of vesicles with PC bearing short fatty acid residues was more efficient compared to those with long fatty acid residues in the *sn*1-position (11).[3]

Our results are of importance for understanding the physiologically relevant interaction of bile salts with phospholipids, i.e., in the bile. PC is enriched in the bile by a bile salt-mediated release from the canalicular membrane. Given a head group-independent interaction of bile salts with phospholipids, as shown in the present work, the high amount of PC in the bile cannot be explained by a head group-specific interaction of this phospholipid with bile salts and subsequent release from the canalicular membrane of hepatocytes. Indeed, using human erythrocytes as a model system, we have recently shown that TC, but not TDHC, induces the release of phospholipid analogues from the erythrocyte membrane independent of the head group of those analogues (26). Therefore, other mechanisms must be considered for explaining the specific enrichment of PC in the bile.

One possible explanation is an asymmetric distribution of phospholipids between both halves of the canalicular membrane. PC might be enriched in the outer, i.e., canalicular-facing, leaflet due to the action of PC transporting proteins, e.g., mdr2 (13, 27), which transport PC from the inner to the outer leaflet. Moreover, a specific protein-mediated transport of aminophospholipids in the opposite direction, i.e., from the outer to the inner monolayer, would result in a depletion of those lipid species in the external leaflet. Recently, we characterized such an aminophospholipid-specific transport in the plasma membrane of the hepatocytic-like cell line HepG2 (28). A preferred localization of PC in the outer leaflet of the canalicular membrane and a selective extraction of lipids by bile salts from this leaflet would explain an enrichment of PC in the bile. This view is supported by our recent observation on erythrocyte membranes that bile salts such as TC induce the release of phospholipids mainly from the exoplasmic leaflet (26). Moreover, the PC-species found in the bile fluid are generally more hydrophilic than those in the canalicular membrane. This is in accordance with a preferential interaction of bile salts with more hydrophilic phospholipid species as shown in the present study. PC species found in the bile contain mainly palmitic acid in the *sn*1- and linoleic acid in the *sn*2-position (29). They are less hydrophobic than PC species remaining in the canalicular membrane which have a more hydrophobic composition of fatty acid residues (9, 10).

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[3] We note that the interaction of bile salts with phospholipids (analogues) might be affected by the supramolecular organization of the latter. Nichols and co-workers (18, 25) investigated the interaction of bile salts with various head group NBD-labeled lyso-PE at submicellar concentrations of those fluorescent lysolipids. Under those conditions the interaction with bile salts was promoted by increasing the length of the fatty acid residue of the lysolipid analogue.

REFERENCES

1. Elferink, R. P. J. O., G. N. J. Tytgat, and A. K. Groen. 1997. The role of mdr2 P-glycoprotein in hepatobiliary lipid transport. *FASEB. J.* **11:** 19–28.

2. Mazer, N. A., M. C. Carey, R. F. Kwasnick, and G. B. Benedek. 1980. Quasielastic light scattering studies of aqueous biliary lipid systems. Size, shape and thermodynamics of bile salt micelles. *Biochemistry.* **18:** 3064–3075.
3. Long, M. A., E. W. Kaler, and S. P. Lee. 1994. Structural characterization of the micelle-vesicle transition in lecithin-bile salt solutions. *Biophys. J.* **67:** 1733–1742.
4. Shiau, Y-F. 1987. Lipid digestion and absorption. *In* Physiology of the Gastrointestinal Tract. L. R. Johnson, editor. Raven Press, New York. 1527–1546.
5. Verkade, H. J., R. J. Vonk, and F. Kuipers. 1995. New insights into the mechanism of bile acid-induced biliary lipid secretion. *Hepatology.* **21:** 1174–1189.
6. Möckel, G. M., S. Gorti, R. K. Tandon, T. Tanaka, and M. C. Carey. 1995. Microscope laser light-scattering spectroscopy of vesicles within canaliculi of rat hepatocyte couples. *Am. J. Physiol.* **269:** G73–G84.
7. Coleman, R., and K. Rahman. 1992. Lipid flow in bile formation. *Biochim. Biophys. Acta.* **1125:** 113–133.
8. Rigotti, A., M. P. Marzolo, and F. Nervi. 1994. Lipid transport from the hepatocyte into the bile. *Curr. Top. Membr.* **40:** 579–615.
9. Yousef, I. M., and M. M. Fisher. 1976. In vitro effect of free bile acids on the bile canalicular membrane phospholipids in the rat. *Can. J. Biochem.* **54:** 1040–1046.
10. Meier, J., E. S. Sztul, A. Reuben, and J. L. Boyer. 1984. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. *J. Cell Biol.* **98:** 991–1000.
11. Cohen, D. E., M. Angelico, and M. C. Carey. 1990. Structural alterations in lecithin-cholesterol vesicles following interactions with monomeric and micellar bile salts: physical-chemical basis for subselection of biliary lecithin species and aggregative states of biliary lipids during bile formation. *J. Lipid Res.* **31:** 55–70.
12. Crawford, J. M., G. M. Mockel, A. R. Crawford, S. J. Hagen, V. C. Hatch, S. Barnes, J. J. Godleski, and M. C. Carey. 1995. Imaging biliary lipid secretion in the rat: ultrastructural evidence for vesiculation of the hepatocyte canalicular membrane. *J. Lipid Res.* **36:** 2147–2163.
13. Ruetz, S., and P. Gros. 1995. Enhancement of Mdr2-mediated phosphatidylcholine translocation by the bile salt taurocholate. Implications for hepatic bile formation. *J. Biol.Chem.* **270:** 25388–25395.
14. King, M. D., and D. Marsh. 1987. Head group and chain length dependence of phospholipid self-assembly studied by spin-label electron spin-resonance. *Biochemistry.* **26:** 1224–1231.
15. Fellmann, P., A. Zachowski, and P. F. Devaux. 1994. Synthesis and use of spin-labeled lipids for studies of the transmembrane movement of phospholipids. *In* Methods in Molecular Biology, Biomembrane Protocols II. J. M. Graham and J. A. Higgins, editors. Humana Press Inc. 161–175.
16. McIntyre, J. C., and R. G. Sleight. 1991. Fluorescence assay for phospholipid asymmetry. *Biochemistry.* **30:** 11819–11827.
17. Pomorski, T., A. Herrmann, B. Zimmermann, A. Zachowski, and P. Müller. 1995. An improved assay for measuring the transverse redistribution of fluorescent phospholipids in plasma membranes. *Chem. Phys. Lipids.* **77:** 139–146.
18. Shoemaker, D. G., and J. W. Nichols. 1990. Hydrophobic interaction of lysophospholipids and bile salts at submicellar concentrations. *Biochemistry.* **29:** 5837–5842.
19. Schmidt, D., C. Gähwiller, and C. Planta. 1981. Thermodynamics and kinetics of micellization in aqueous solutions of some spin-labeled short-chain lecithins. *J. Colloid Interfac. Sci.* **83:** 191–198.
20. Shoemaker, D. G., and J. W. Nichols. 1992. Interaction of lysophospholipid/taurodeoxycholate submicellar aggregates with phospholipid bilayers. *Biochemistry.* **31:** 3414–3420.
21. Seigneuret, M., A. Zachowski, A. Herrmann, and P. F. Devaux. 1984. Asymmetric lipid fluidity in human erythrocyte membrane: new spin label evidence. *Biochemistry.* **23:** 4271–4275.
22. Coello, A., F. Meijide, E. R. Nunez, and J. V. Tato. 1996. Aggregation behaviour of bile salts in aqueous solution. *J. Pharmac. Sci.* **85:** 9–15.
23. Billington, D., C. E. Evans, P. P. Godfrey, and R. Coleman. 1980. Effects of bile salts on the plasma membranes of isolated rat hepatocytes. *Biochem. J.* **188:** 321–327.
24. Soltys, C. E., and M. F. Roberts. 1994. Fluorescence studies of phosphatidylcholine micelle mixing: relevance to phospholipase kinetics. *Biochemistry.* **33:** 11608–11617.
25. DeLong, L. J., and J. W. Nichols. 1996. Time-resolved fluorescence anisotropy of fluorescent-labeled lysophospholipid and taurodeoxycholate aggregates. *Biophys. J.* **70:** 1466–1471.
26. Wüstner, D., T. Pomorski, A. Herrmann, and P. Müller. 1998. Release of phospholipids from erythrocyte membranes by taurocholate is determined by their transbilayer orientation and hydrophobic backbone. *Biochemistry.* **37:** 17093–17103.
27. Crawford, A. R., A. J. Smith, V. C. Hatch, R. P. J. O. Elferink, P. Borst, and J. M. Crawford. 1997 Hepatic secretion of phospholipid vesicles in the mouse critically depends on mdr2 or MDR3 P-glycoprotein expression: visualization by electron microscopy. *J. Clin. Invest.* **100:** 2562–2567.
28. Müller, P., T. Pomorski, S. Porwoli, R. Tauber, and A. Herrmann. 1996. Transverse movement of spin-labeled phospholipids in the plasma membrane of a hepatocytic cell line (HepG2): implications for biliary lipid secretion. *Hepatology.* **24:** 1497–1503.
29. Crawford, J. M. 1996. Intracellular traffic and plasma membrane secretion of small organic solutes involved in hepatocellular bile formation. *Comp. Biochem. Physiol. B Biochem. & Mol. Biol.* **115:** 341–354.