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The Mitigating Effects of Phosphatidylcholines on Bile Salt- and Lysophosphatidylcholine-induced Membrane Damage

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Abstract—The effects, at pH 7·0, of a series of 0·2 mM phosphatidylcholines (PC), namely dicaproyl-PC (DCPC), didecanoyl-PC (DDPC), dilauroyl-PC (DLaPC), dimyristoyl-PC (DMPC), dipalmitoyl-PC (DPPC), dioleoyl-PC (DOPC) and dilinoleoyl-PC (DLPC) and a series of 0·2 mM fatty acid salts (namely sodium myristate, palmitate, stearate, oleate and linoleate) upon the erythrocyte haemolysis induced by 2 mM sodium taurodeoxycholate (STDC) were determined. The influence of egg PC and dihexadecyl phosphate (DHDP) concentration upon the haemolysis induced by 1·4 mM sodium deoxycholate (SDC), 2 mM STDC and 0·1 mM lysophosphatidylcholine (LPC) were also established. A bile salt : egg PC mole ratio of 0·5 virtually abolished the haemolysis induced by SDC and STDC, whereas the same ratio of LPC : egg PC only reduced haemolysis from 65 to 40% (maximum haemolysis). DHDP had no effect on the haemolytic action of SDC or STDC. The salts of the fatty acids were non-haemolytic, and when mixed with STDC did not affect the level of haemolysis induced by the bile salt. In contrast, DDPC and DLaPC enhanced the haemolysis of STDC and DCPC had no effect, whereas DMPC, DPPC, DSPC, DOPC, DLPC and egg PC all reduced haemolysis. Maximum reduction was determined for DMPC and egg PC. The mixed micelle preparation temperature (either room or 60°C) and temperature of incubation (either 20°C for 30 min or 37°C for 5 min) had only minor effects on the net haemolysis induced by STDC. These findings may be of significance in understanding the aetiology of certain gastrointestinal diseases and in determining whether mixed bile salt micelles have a role as drug penetration enhancers.

The natural occurrence of bile salts and phospholipids in the intestinal tract, where they fulfil the physiological function of emulsifying fats and facilitating lipolysis before absorption, has prompted many studies investigating the applicability of such surfactants as potentially safe absorption promoters of drugs. It is now apparent, however, that the attained enhancement in absorption is at least in part the consequence of direct physical damage to the membrane induced by these surfactants. Moreover, the presence of elevated levels or changed composition of bile is implicated in a number of gastrointestinal disorders, including gastritis, gastric ulcer and colonic cancer (Martin et al 1992). Bile is well tolerated by the mucosal epithelia of the duodenum and small intestine, where its composition is complex and varies according to the health of the individual.

In addition to at least six bile salts, bile contains fatty acids, cholesterol and a number of phospholipids. The major phospholipid is phosphatidycholine (PC), although small amounts of phosphatidylethanolamine and lysophosphatidylcholine (LPC) are also present, the latter being formed with free fatty acids from PC in the intestine by hydrolysis catalysed by pancreatic phospholipase A. LPC has also been advocated as a possible adsorption promoter of drugs (Illum et al 1988) although it is demonstrably toxic to some membranes (Martin et al 1992). Fatty acids and mixed micelles (comprising bile salts and fatty acids) have also been employed in a number of studies as penetration enhancers (Muranishi 1985).

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A number of authors have shown PC to protect membranes from bile salt- and LPC-induced damage. For example, Coleman et al (1979) demonstrated that egg PC can protect erythrocytes from bile and bile salt-induced haemolysis and Gjone (1961) showed LPC-induced haemolysis to be decreased by PC. Martin & Marriott (1981) showed that egg PC will lessen damage to goldfish epithelia; Newbery et al (1984) and Martin et al (1985) showed egg PC mitigated the effects of bile salts on the rat gastric mucosa.

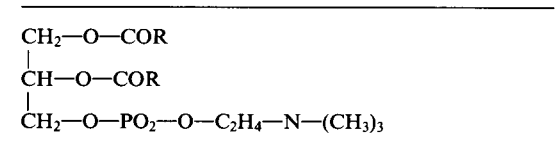
The mechanisms by which PC protects against bile salt- and LPC-induced membrane damage are still unclear. Martin & Marriott (1981) and Newbery et al (1984) have suggested that the formation of mixed micelles with bile salts or LPC is important, whereas Lichtenberger et al (1983) have proposed that PC protects membranes by being adsorbed as a monolayer onto the surface to create a hydrophobic barrier.

Experiments studying the protective effects of phospholipids have generally used egg PC which contains a mixture of PCs of different acyl chain lengths and different degree of saturation. The purpose of this study was to examine the effects of PC (Fig. 1) and fatty acid salt structure upon membrane damage (erythrocyte haemolysis) induced by bile salts. In some experiments, the PC was replaced by dihexadecyl phosphate (DHDP), a synthetic double acyl chain compound.

Materials and Methods

Materials

All materials were used as supplied. Dilauroylphosphatidylcholine (DLaPC), dilinoleoylphosphatidylcholine (DLPC),



PC	Abbreviation	Carbon chain length of R	Double bonds
Egg	Egg PC	13–23	
Dicaproyl	DCPC	5	0
Didecanoyl	DDPC	9	0
Dilauroyl	DLaPC	11	0
Dimyristoyl	DMPC	13	0
Dipalmitoyl	DPPC	15	0
Distearoyl	DSPC	17	0
Dioleoyl	DOPC	17	1
Dilinoleoyl	DLPC	17	2

FIG. 1. The molecular structure of the phosphatidylcholines employed in the study.

dimyristoylphosphatidylcholine (DMPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), egg phosphatidylcholine (Grade 1) (egg PC) and lysophosphatidylcholine (Grade 1) (LPC) were obtained from Lipid Products, Nutfield, Surrey, UK.

The structure of the phospholipids used in this study are shown in Fig. 1; the relative molecular masses for egg PC and LPC were taken as 800 and 525, respectively. Dicaproylphosphatidylcholine (DCPC), didecanoylphosphatidylcholine (DDPC), sodium linoleate (SL), sodium myristate (SM), sodium oleate (SO), sodium palmitate (SP), sodium stearate (SS), sodium taurodeoxycholate (STDC) and sodium deoxycholate (SDC) were obtained from Sigma Ltd, Poole, Dorset, UK. Dihexadecyl phosphate (DHDP) was obtained from Aldrich Chemical Company Ltd. Blood was collected from a female Caucasian subject.

Methods

The required amount of PC, DHDP or fatty acid was dissolved in approximately 2 mL of chloroform in a 100 mL round-bottomed flask. Using a vacuum rotary evaporator (Buchi RE 111, Switzerland), the solvent was evaporated at 20°C to leave a thin film of the lipid on the inside surface of the flask. Any remaining traces of chloroform were removed under a stream of nitrogen. The appropriate amount of bile salt or LPC was dissolved in 10% McIlvaine's buffer containing 134 mM sodium chloride at pH 7·0 and added to the flask. The resultant suspension was mixed and allowed to stand at room temperature (21°C) for 18 h before use. In the experiments employing PCs of different chemical structure, the PC–bile salt mixture was rotated in a water bath at either room temperature or at 60°C before cooling; it was then made up to volume and allowed to stand at room temperature for 18 h.

Incubation of mixtures with erythrocytes was carried out as described previously (Martin et al 1992), in triplicate on two to four batches of blood. Incubation was usually carried out at 20°C for 30 min or 37°C for 5 min, where indicated.

The two-tailed Mann-Whitney U-test (Wardlaw 1987) was used to analyse statistically the data obtained.

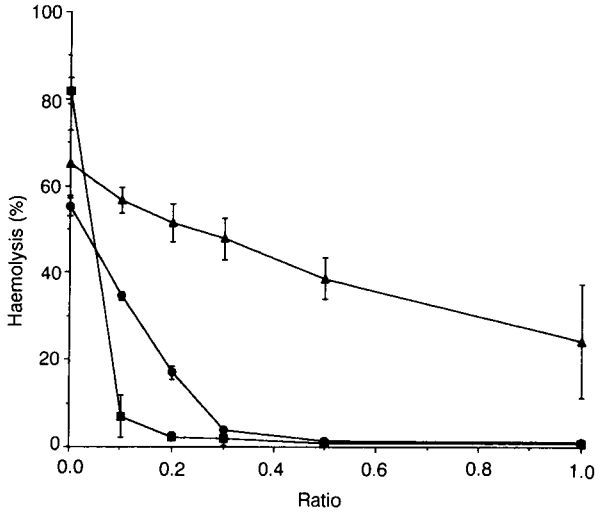


FIG. 2. The effect of mole ratio of egg phosphatidylcholine on the erythrocyte haemolysis induced by 1·4 mM SDC (■), 2 mM STDC (●) or 0·1 mM LPC (▲). (n = 6, bar lines: s.d.) See text for abbreviations.

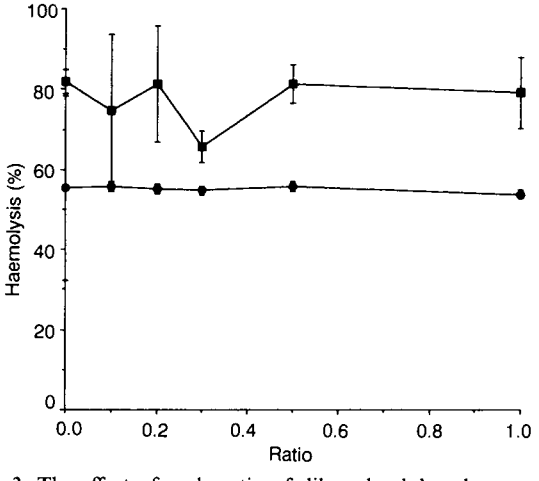


FIG. 3. The effect of mole ratio of dihexadecylphosphate on the erythrocyte haemolysis induced by 1·4 mM SDC (■) and 2 mM STDC (●). (n = 6, bar lines: s.d.) See text for abbreviations.

Results

When PC was added to a solution of bile salt or LPC, the haemolytic action of that solution was decreased. Fig. 2 shows that PC reduced the toxicity of bile salts more markedly than it reduced LPC toxicity when employed at the same molar ratios. At a molar ratio of 1:0·1 for SDC:PC, and 1:0·3 for STDC:PC, the haemolytic effect was reduced to below 10%. However, LPC toxicity was only reduced from 65 to 24·3% at a molar ratio of 1:1 with egg PC. DHDP had no effect on the haemolytic action of either SDC or STDC (Fig. 3).

The salts of fatty acids (SM, SP, SS, SO and SL) had little intrinsic haemolytic activity. At a molar concentration of 0·2 mM, maximum haemolysis was seen with SL which produced 1·2% haemolysis. These fatty acid salts, in molar ratios of 1:10 with 2 mM STDC, did not affect the haemolysis produced by 2 mM STDC alone.

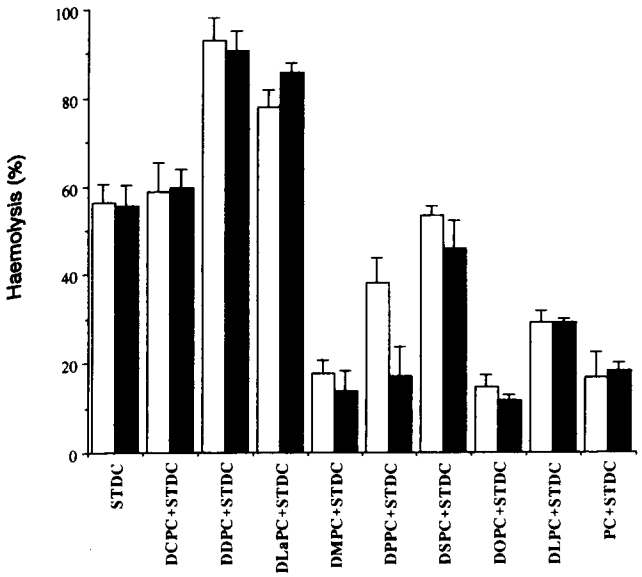


FIG. 4. The effect of 0·2 mM phospholipid on the erythrocyte haemolysis induced by 2 mM STDC. Open bars represent samples prepared at room temperature and incubated at 20°C; solid bars represent samples prepared at 60°C and incubated at 20°C (n = 6, bar lines: s.d.) See text and Fig. 1 for abbreviations.

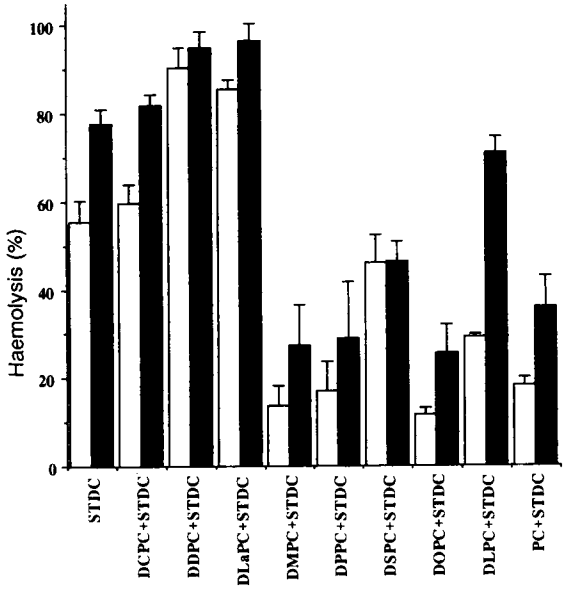


FIG. 5. The effect of 0·2 mM phospholipid on the erythrocyte haemolysis induced by 2 mM STDC. Open bars represent samples prepared at 60°C and incubated at 20°C for 30 min; solid bars represent samples prepared at 60°C and incubated at 37°C for 5 min (n = 6, bar lines: s.d.) See text and Fig. 1 for abbreviations.

The resultant membrane damage induced by the bile salt, STDC, depended upon the structure of the PC present in the incubating mix (Fig. 4). DCPC had no effect on STDC-induced haemolysis. The presence of DDPC and DLaPC increased the haemolysis attained by STDC alone from 56·4 to 92·9 and 77·7%, respectively. DMPC, DDPC and DSPC all reduced the toxicity of STDC. The effect was greatest with DMPC and then was found to decrease with increasing chain length, i.e. STDC-induced haemolysis was decreased from 56·4%, with the bile salt alone, to 53·3 ($P < 0·05$), 38·0 ($P < 0·0001$) and 17·8% ($P < 0·0001$) when DSPC, DPPC or DMPC, respectively, was present in a ratio of 1:10 phospholipid:bile salt. When a *cis* double-bond was introduced into the acyl chain, the protective effect of the PC increased, but when a second *cis* double-bond was introduced, the effect was not as great, i.e. DOPC caused the percentage haemolysis induced by STDC to decrease from 56·4 to 14·6% ($P < 0·0001$) but with DLPC, haemolysis only decreased to 29·1% ($P < 0·0001$). When STDC was incubated with erythrocytes in the presence of egg PC, the egg PC produced a similar protection (16·9% haemolysis, $P < 0·0001$) to DMPC (17·8%) and DOPC (14·6%).

When these bile salt–PC samples were prepared at 60°C rather than at room temperature, the percentage haemolysis obtained was similar, except for DPPC (Fig. 4). The percentage haemolysis for the STDC-DPPC mixture decreased from 38·0 to 17·1% ($P < 0·001$). When samples prepared at 60°C were incubated at 37°C for 5 min, the percentage haemolysis obtained for each sample increased. The effect, however, was most marked with DLPC which showed greater than a 100% increase in the percentage haemolysis obtained (Fig. 5).

Discussion

The results suggest that at physiological ratios, egg PC is able to protect against bile salt-induced damage (Fig. 2). At the molar ratios used in this study, PC provided more protection to bile salt-induced haemolysis than LPC-induced haemolysis. However, since LPC is usually present in only relatively small amounts, it may be expected that at higher, more physiological, ratios than used in this study, LPC-induced membrane damage would be limited further. Since some of the membrane damage induced by STDC or LPC is likely to be attributable to micelles solubilizing membrane components, micellar saturation with PC may be expected to reduce or prevent this process occurring.

Bile salt structure has been shown to be an important factor in the solubilizing properties of the micelle. For example, in a study examining the ability of bile salts to solubilize cholesterol, Armstrong & Carey (1982) showed that micellar capacity was correlated inversely with bile salt hydrophilicity. These workers were able to show that bile salt solubility increased in the order SDC < SCDC < SC with the free bile salts being less soluble than the glycine conjugates and these, in turn, being less soluble than the taurine conjugates. In this study, it could be seen (Fig. 2) that PC protected erythrocyte membranes against SDC-induced damage to a greater extent than STDC-induced damage at the same ratio of bile salt: PC. Since SDC is able to solubilize phospholipid to a greater extent than STDC (Armstrong & Carey 1982) mixed micelle formation is likely to be important in mitigating membrane damage. Indeed, physiologically, bile salt hydrophilicity may influence the quantity of PC present in bile. Alvaro et al (1986) have observed that animal species which possess a high percentage of relatively more hydrophilic bile salts, e.g. dog and sheep, have lower amounts of PC per mole bile (i.e. a lower PC: bile salt molar ratio) than those species which have less hydrophilic bile

salts, e.g. pig and man. The hydrophobicities of bile salts is also related to their membrane perturbing effects (Martin et al 1992).

Gjone (1961) showed that PC from human serum could protect against human serum LPC-induced haemolysis. Matsuzaki et al (1988) showed that LPC-induced haemolysis was related to the acyl chain length and its degree of saturation. Hence, the haemolytic activity decreased as the LPC acyl chain was altered from stearoyl to palmitoyl to myristoyl to oleoyl to lauroyl. Reman et al (1969) demonstrated that short acyl chained PCs of 8 to 10 carbon atoms disrupt lipid layers whereas PCs with either shorter or longer acyl chains do not have this effect. These data would appear to be in agreement with the results presented in this work, i.e. DDPC and DLaPC appeared to be haemolytic since STDC-induced haemolysis was always less than the haemolysis induced by STDC and DDPC or DLaPC mixtures. PCs with longer acyl chains of 14 or more carbon atoms or shorter acyl chains of 6 carbon atoms had no apparent inherent haemolytic action since the percentage haemolysis was approximately the same or less with such PC–STDC mixtures than STDC alone.

Of the PCs studied, only DMPC, DPPC and DSPC had phase transition temperatures above 20°C. At a preparation of 60°C, all the PCs examined were above their phase transition temperatures. When the PC–bile salt mixtures were prepared above their phase transition temperatures and incubated at 20°C, only DPPC appeared to show any great change (i.e. an increase in excess of 100%) in the degree of protection afforded. DMPC has a phase transition temperature (24°C) very close to 20°C and a pre-transition temperature of 14°C. Hence, lack of change in protection by this compound is not unexpected. DPPC showed an increase in the degree of protection (Fig. 4) which might be due to a change in the formation and stability of the mixed micelles above the phase transition temperature of the phospholipid. When mixtures prepared at 60°C were reacted at 37°C and compared with those reacted at 20°C, the only sample to show any marked change in the percentage haemolysis attained was the DLPC–STDC mixture (Fig. 5). The mitigating effect of DLPC appears to be greatly reduced when the mixture is incubated at the higher temperature and it is probable that the increased temperature affects micelle formation and stability.

Short chain fatty acids (up to C9) have been shown to break the gastric mucosal barrier (Davenport 1964) whereas longer chain fatty acids do not appear to have a disruptive effect on the gastric mucosa (Duane et al 1980). Mixtures containing 33% palmitic acid, 11% stearic acid, 45% oleic acid and 11% linoleic acid, in proportions simulating those found in human gastric aspirates, have been shown not to disrupt the canine gastric mucosal barrier despite total concentrations of above 7 mM (Duane et al 1980). Similarly, in these experiments, the fatty acid salts were all found to be innocuous to membranes, but unlike the long chain PCs, they did not induce any mitigation of haemolysis due to bile salt or LPC.

To summarize, the presence of PC is important in determining the extent of damage induced by LPC and bile salts to a membrane. The mitigating effect appears to be related to a number of factors, including acyl chain length, degree of acyl chain saturation, bile salt/LPC to PC ratio and temperature. The most protective phosphatidylcholines are those with acyl chain lengths of C14 and above, i.e. those normally found in mammals. The fatty acids studied showed no protection against bile salt-induced damage and this suggests that the acyl chains of the PCs alone do not afford the protection attained. The synthetic double acyl chain compound, DHDP (Fig. 3) did not mitigate bile salt-induced membrane damage, suggesting that the presence of two long acyl chains on a molecule is not a prerequisite for protection.

These findings have implications for a number of fields of study, including those associated with understanding the aetiology of a number of gastrointestinal diseases and those designed to examine drug absorption both in the gastrointestinal tract and by other mucosal epithelia, where mixed micelles are employed to promote drug uptake.

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