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INHIBITION OF BILIARY LIPID AND PROTEIN SECRETION BY CYCLOSPORINE A IN THE RAT

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Abstract—We investigated the efffect of cyclosporine A (CyA) administered as a single i.v. dose of 20 and 40 mg/kg body wt, on biliary secretion of cholesterol, phospholipid, bile acid, and lysosomal marker and canalicular plasma membrane marker enzymes in anaesthetized Wistar rats. CyA reduced the concentration and biliary secretion of cholesterol, phospholipid and bile acid to a considerable extent; the inhibitory effect of CyA on the biliary secretion of phospholipid and bile acid was greater than that on cholesterol. The biliary outputs of acid phosphatase (AcP) and γ-glutamyltransferase (γ-GT) were also diminished by the drug, all these effects being dose-dependent. Maximum decreases in bile acid secretion were observed 10 min after administration, whereas those of cholesterol and phospholipid were delayed. Bile acid concentrations and secretion returned to pretest values at 30–50 min after CyA injection whereas those of cholesterol and phospholipid remained significantly reduced at this time point. The greater inhibitory effect of CyA on the biliary outputs of phospholipid and bile acid relative to cholesterol secretion together with the asynchronous fall and recovery of bile acid, cholesterol and phospholipid concentrations and secretion alter the cholesterol/bile acid, phospholipid/bile acid and cholesterol/phospholipid molar ratios as well as the lithogenic index, thus suggesting that CyA would uncouple biliary lipid secretion from bile acid secretion. Since under physiological conditions biliary lipid and γ-GT secretion is related to and dependent upon bile acid secretion, we propose that the CyA-induced inhibition on lipid and γ-GT secretion is, at least partly, secondary to the fall in bile acid output caused by the drug. However, since CyA inhibits secretory processes independent of the hepatobiliary flux of bile acid, such as the exocytic discharge of AcP, and because it also uncouples biliary lipid from bile acid secretion, other mechanisms and factors involved in lipid and protein secretion (such as intracellular transport, canalicular membrane fluidity and/or intracanalicular events) might also be altered by this drug.

Treatment with cyclosporine A (CyA‡) has contributed to enhancing allograft and patient survival after solid-organ transplantation and to reducing the manifestations of some autoimmune diseases [1, 2]. However, its therapeutic use is often limited by side effects including renal and hepatic dysfunctions. In both clinical and experimental studies, functional impairment of the liver is manifested by a syndrome of cholestasis that is accompanied by increased plasma levels of bilirubin and bile acid [1, 3–8]. Different authors have shown that CyA-induced cholestasis is due to an inhibition of the sinusoidal hepatic uptake of bile acid [7–12]; interference with the excretory step across the canalicular membrane of bile acid, bilirubin and cholephilic dyes have also been reported [5, 7, 13, 14], possibly related to changes in membrane fluidity [8, 11, 15].

Although the exact mechanism through which biliary lipids (cholesterol and phospholipid) are excreted into bile remain to be fully elucidated, it is well established that bile acid as well as generating bile flow also induces lipid secretion and in this sense a close association between bile acid and lipid secretion has been reported in all species studied (for a review see Refs. 16–19). Lipid secretion appears to involve vesicular transport (transcytosis) processes and exocytosis of vesicles whose major lipids are cholesterol and phospholipid. Different authors have indicated that vesicles containing biliary lipid precursors fuse with the canalicular membrane out of which cholesterol and phospholipid are continuously removed by the detergent action of bile acid and are solubilized in unilamellar vesicles and in bile acid mixed micelles [16, 19–26]. The presence of a number of canalicular membrane enzymes in bile, e.g. γ-glutamyltransferase (γ-GT), 5′-nucleotidase etc., seems to involve processes of microvesiculation, followed by the release and solubilization of these membrane proteins in response to bile acid secretion [16, 20, 21, 27–29]. By contrast, previously published data [16, 27, 28, 30–32] suggest that the exocytic discharge of lysosomal contents in bile is independent of the hepatobiliary flux of bile acid.

We have recently reported that CyA interferes with vesicle-mediated hepatic transport processes in

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‡ Abbreviations: AcP, acid phosphatase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BA, bile acid; CHE, cholinesterase; CHO, cholesterol; CPK, creatine phosphokinase; CyA, cyclosporine A; γ-GT, γ-glutamyltransferase; HBDH, hydroxybutyrate dehydrogenase; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; LI, lithogenic index of bile; PHO, phospholipid.

the rat; like phalloidin, an anti-cytoskeletal agent structurally similar to CyA [9], CyA induces cholestasis and reduces the biliary secretion of horseradish peroxidase (HRP)—a classic tracer of transcytosis processes—and that of bile acid and bilirubin [4–7].

Since CyA inhibits transcytosis and bile acid secretion, two factors involved in lipid secretion, the major aims in the present study were (1) to evaluate the effect of CyA on the biliary secretion of cholesterol and phospholipid and on the changes that take place in the molar ratios between biliary lipid and bile acid and, (2) to attempt to discover whether mechanisms other than the inhibitory effect of CyA on bile acid secretion might modify biliary lipid and protein secretion following acute single-dose intravenous administration of CyA in anaesthetized rats.

MATERIALS AND METHODS

Chemicals. CyA, in powder form, was a gift from Sandoz A.G. (Basel, Switzerland). CyA was initially dissolved at 20° in 100 μL of anhydrous ethanol and diluted in 900 μL of Intralipid™ [4, 7]. Intralipid, a fat emulsion commonly used for parenteral nutrition and composed of soya bean oil, egg yolk, lecithin and glycerol, was purchased from Laboratorios Fides (Barcelona, Spain). Sodium pentobarbital was obtained from Claudio Barcia (Madrid, Spain). 3α-Hydroxysteroid dehydrogenase (EC 1.1.1.50), β-nicotinamide adenine dinucleotide, bile acid and the different kits and reagents for the determination of the enzymatic activities and substrate concentrations evaluated in bile and in liver homogenates were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and Sigma Chemical Co. (St Louis, MO, U.S.A.). All other reagents were of the highest quality available commercially.

Animals and experimental procedures. Male Wistar rats weighing 240–260 g were housed in a room maintained at 22° with humidity ranging from 45% to 55% and under a constant light cycle (12 hr, light/12 hr dark).They received standard laboratory diet (Panlab, Barcelona, Spain) and water *ad lib.* before the experiments. The protocols followed in the experimental procedures with the animals were in accordance with the indications of the *Guide to the Care and Use of Experimental Animals* (Canadian Council on Animal Care) which is routinely used at our laboratory.

The rats were anaesthetized with sodium pentobarbital (50 mg/kg) given intraperitoneally and a median laparotomy was performed. The bile duct and right jugular vein were cannulated with polyethylene tubing for collecting bile samples and administration of solutions, respectively. Losses in body temperature were prevented by a thermostatically controlled warming plate and the rectal temperature was maintained at 37°. A routine tracheotomy was performed on all animals. After an equilibration period of 25–30 min, bile was collected in pre-weighed tubes on melting ice and bile samples were immediately stored at −40° until required for analysis. At the end of the experiments the rats were killed by exsanguination. Livers were

Table 1. Effects of CyA on biliary secretion in the rat

	Control		CyA-20		CyA-40	
	Before	During	Before	During	Before	During
Bile flow						
μL/min/100 g	7.27 ± 0.42	7.62 ± 0.46	8.52 ± 0.29	6.34 ± 0.18*	7.61 ± 0.51	5.33 ± 0.47*
Bile acid						
mmol/L	30.8 ± 3.1	26.4 ± 1.7	26.2 ± 2.6	15.1 ± 2.2*	34.0 ± 1.9	18.8 ± 1.8*
nmol/min/100 g	230 ± 19	197 ± 20	218 ± 20	98 ± 15*	263 ± 18	106 ± 12*
Cholesterol						
mmol/L	0.395 ± 0.034	0.339 ± 0.021	0.368 ± 0.021	0.199 ± 0.021*	0.348 ± 0.012	0.144 ± 0.009*
nmol/min/100 g	3.05 ± 0.39	2.86 ± 0.42	3.10 ± 0.20	1.28 ± 0.13*	2.92 ± 0.21	0.93 ± 0.07*
Phospholipids						
mmol/L	3.71 ± 0.29	3.17 ± 0.18	3.20 ± 0.20	1.23 ± 0.10*	3.64 ± 0.25	1.15 ± 0.11*
nmol/min/100 g	29.5 ± 4.9	25.5 ± 3.7	27.2 ± 2.4	7.8 ± 0.6*	30.5 ± 2.2	7.2 ± 0.4*
Molar ratios						
CHO/BA	0.013 ± 0.001	0.013 ± 0.001	0.014 ± 0.001	0.014 ± 0.002	0.011 ± 0.001	0.009 ± 0.001
PHO/BA	0.128 ± 0.011	0.130 ± 0.011	0.110 ± 0.012	0.078 ± 0.011*	0.107 ± 0.007	0.063 ± 0.008*
CHO/PHO	0.104 ± 0.008	0.106 ± 0.005	0.120 ± 0.010	0.165 ± 0.012*	0.104 ± 0.008	0.157 ± 0.014*

Results for bile flow and bile acid, cholesterol and phospholipid biliary concentrations and secretion, and for the CHO/BA, PHO/BA and CHO/PHO molar ratios in controls and CyA-treated rats, *before* (min 0–30 of assays) and *during* the CyA-induced cholestasis period (min 30–70 of assays). Control rats received CyA vehicle; CyA was administered at doses of 20 mg/kg body wt (CyA-20) or 40 mg/kg body wt (CyA-40).

Data are given as means ± SEM for 6–8 rats in each group. Significant differences from preadministration (before) values are indicated by * ($P < 0.05$). For details of assay procedures and CyA vehicle composition see Materials and Methods.

quickly removed, weighed and washed with an ice-cold isotonic saline solution. Small pieces weighing about 1 g were removed from the organs and stored at −40° until analysis.

The animals were divided into three groups receiving an equal volume (1 mL, i.v., given over 50–60 sec) of CyA vehicle (Intralipid plus absolute ethanol, 9:1, v/v; used as control experiments) or the solution containing 20 or 40 mg of CyA/kg. After collecting two baseline 15 min bile samples, the solutions were injected. Bile was collected for a further 70 min over 10-min intervals until the end of assays. In previous studies it has been ascertained that acute i.v. administration of this CyA vehicle does not lead to observable effects on biliary secretion in the anaesthetized rat when compared with animals injected with physiological saline [4–7]. The doses of CyA used in this study were higher than those usually employed in man; however, the dose most frequently used in acute and chronic treatment in this species ranges between 20 and 50 mg CyA/kg [7, 8, 11, 14, 33]. Additionally, the immunotherapeutic dose normally used in the rat is 25 mg/kg/day [33].

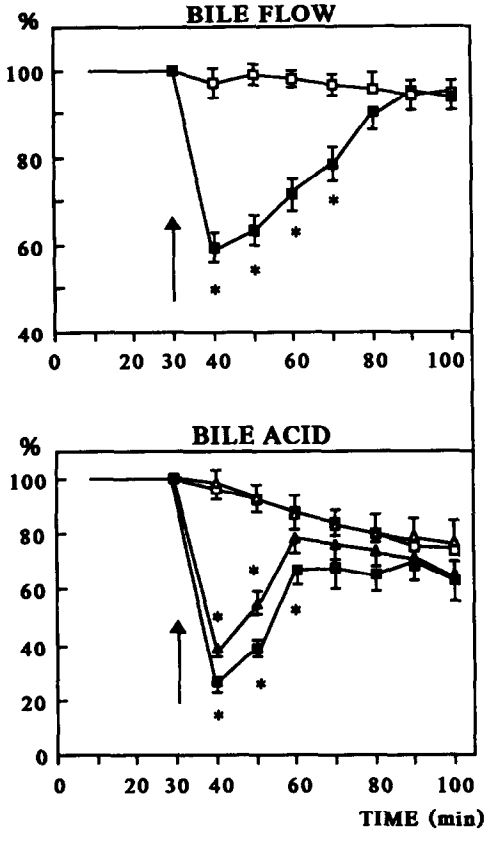

Fig. 1. Effects of CyA on bile flow and bile acid concentration and secretion in anaesthetized rats. Changes in bile flow and biliary bile acid concentration and secretion rates after i.v. administration (denoted by the arrow) of CyA vehicle (open symbols) or CyA (20 mg/kg, filled symbols). In the lower panel bile acid concentration are represented by triangles and bile acid secretion by squares. The changes relative to the means of the control period (min 0–30 of assays) are given as means ± SEM for 6–8 rats in each group. *P < 0.05 significantly different from control experiments.

Specific determinations. Bile flow was determined gravimetrically without correction for specific gravity, assuming a bile density of 1 g/mL. Total bile acid concentrations in bile were measured enzymatically with 3α-hydroxysteroid dehydrogenase [34]. Cholesterol concentrations in bile were determined by a modification of the method of Bolton *et al.* [35] using a commercial kit (Cholesterol enzymatique PAP, Biomerieux, Charboniers les Bains, France). Phospholipid concentrations in bile were measured by a commercial enzymatic method (Phospholipides enzymatique PAP, Biomerieux) based on that of Takayama *et al.* [36]. Acid phosphatase (AcP, EC 3.1.3.2), alkaline phosphatase (ALP, EC 3.1.3.1), alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1), cholinesterase (CHE, EC 3.1.1.8), creatine phosphokinase (CPK, EC 2.7.3.2), hydroxybutyrate dehydrogenase (HBDH) and lactate dehydrogenase (LDH, EC 1.1.1.27) activities, at 30°, and the concentrations of calcium, cholesterol, creatinine, glucose, phospholipids, phosphorus, total proteins, triglycerides, urea and uric acid in liver homogenates were measured by optimized methods routinely used in our laboratory on an automated analyser (Hitachi, model 717). The lithogenic index of bile was calculated by the method of Thomas and Hoffman [37]. γ-GT (EC 2.3.2.2) and acid phosphatase activities in bile were determined by the methods described by Persijn and Van der Slik [38] and Hillmann [39], respectively. Enzyme activities in bile were expressed as U/L and in liver homogenates as U/g liver.

Statistics. Results are expressed as means ± SEM. The data were compared by the Mann–Whitney U test using a significance level of P = 0.05 to reject the null hypothesis. P values of 0.05 or less were considered statistically significant.

RESULTS

To allow easier comparison between the results obtained from control and CyA-treated rats, values are expressed as percentage variations with respect to the levels in basal conditions (before Intralipid or CyA administration). The amounts corresponding to 100% are shown in Table 1. Percent changes in bile flow and bile acid concentration and secretion are represented in Fig. 1 for the controls and CyA-20 experiments. Whereas Intralipid had no effects on biliary secretion, the administration of CyA resulted in a significant decrease in bile flow and bile acid secretion in all rats. Using values from the first 30 min of the assays as a baseline for comparison, the decreases in bile flow and bile acid concentration and secretion rates were significant within 10 min post-injection; later, there was a post-cholestatic recovery and at min 40–50 after CyA administration their values were similar to the baseline and control values (Fig. 1). This cholestatic effect of CyA was

very similar to that previously reported by us in the rat [4–7].

Basal biliary concentrations and secretion rates of cholesterol and phospholipid were similar in all three groups (Table 1). The changes in these parameters in the control and CyA-20 experiments are shown in Fig. 2. When CyA was administered, the biliary concentrations and outputs of cholesterol and phospholipid fell markedly. Maximum decreases in cholesterol and phospholipid concentrations and secretion were observed at min 20 post-injection (10 min later than in the case of those of bile acid). The recovery to baseline values occurred more slowly than was observed in the bile acid concentration and secretion rates; thus, at 30 min post-injection the bile acid concentration was again similar to control values, while those of cholesterol and phospholipid remained reduced (−50% and −70%, respectively, as compared to pretest values). Additionally, the reductions in phospholipid concentrations and secretion were more pronounced than those occurring in cholesterol and bile acid (Figs 1 and 2). For the 40 mg/kg dose of CyA the minimum concentrations of cholesterol and phospholipid were 73% and 86%, respectively, lower than the pretest values. In the case of the biliary secretion rates, the maximum decrease occurred at min 30 and their minimum values were 79% and 91%, respectively; again smaller than the baseline values.

Figure 3 shows the changes in the calculated cholesterol/bile acid (CHO/BA), phospholipid/bile acid (PHO/BA) and cholesterol/phospholipid (CHO/PHO) molar ratios and in the lithogenic index of bile (LI) in control and CyA-20 assays. Table 1 shows the values of these molar ratios in control and CyA-treated rats. All three molar ratios and the LI were increased immediately by the drug. The increases in the CHO/BA and CHO/PHO molar ratios are reflected in significant increases in the LI in the CyA-20 (+42%) and CyA-40 (+61%) experiments (data not shown). Later, the CHO/BA and PHO/BA molar ratios and the LI were reduced by the drug whereas the CHO/PHO molar ratio remained at a plateau until the end of the experiments.

The biliary outputs of the canalicular plasma membrane marker enzyme γ-GT and the lysosomal marker enzyme AcP were significantly reduced in the CyA-treated animals, while there were no changes with respect to the baseline values in the control rats (Fig. 4). Finally, Table 2 shows the activity/concentration data relating to the different enzymes and substrates analysed in the liver homogenates from control and CyA-treated rats; statistical comparison of the results obtained in both control and CyA assays did not reveal significant differences between either group, such that CyA cannot be said to affect the hepatic levels of the different parameters evaluated.

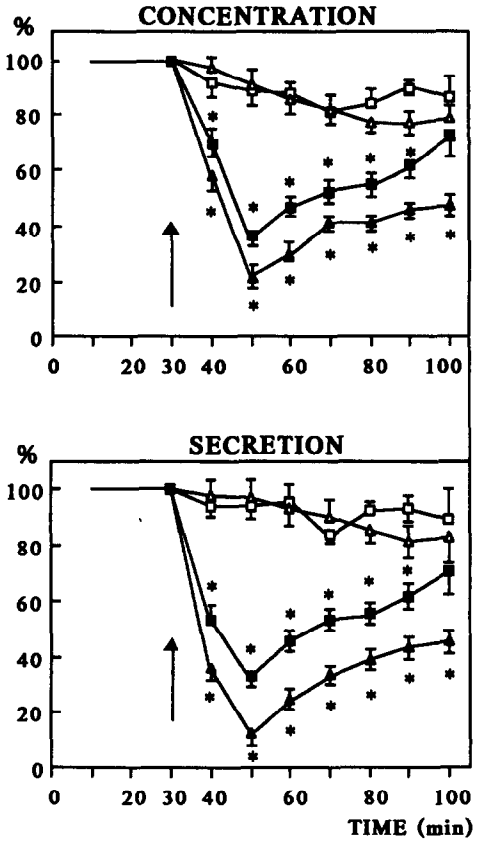

Fig. 2. Effects of CyA on the biliary lipid secretion in anaesthetized rats. Changes in biliary concentration and secretion rates of cholesterol (squares) and phospholipid (triangles) after i.v. administration (denoted by the arrow) of CyA vehicle (open symbols) or CyA (20 mg/kg, filled symbols). The changes relative to the means of the control period (min 0–30 of assays) are given as means ± SEM for 6–8 rats in each group. *$P < 0.05$ significantly different from control experiments.

DISCUSSION

We have observed that CyA reduces the biliary secretion of bile acid and that of bile constituents that depend on the hepatobiliary flux of bile acid, such as water, cholesterol, phospholipid or canalicular membrane enzymes, such as γ-GT. The drug also alters the calculated values of the molar ratios between bile acid and lipid, uncouples biliary lipid from bile acid secretion, and modifies the lithogenic index of bile. Finally, CyA inhibits secretory processes independent of bile acid flux, such as the exocytic discharge into bile of a lysosomal marker enzyme; namely, AcP.

It has been reported that CyA inhibits bile acid synthesis in cultured rat hepatocytes and in the rat *in vivo* [40]. However, in agreement with previous studies [4–7], the maximum decrease in bile acid secretion was achieved within 10 min of CyA administration and was also associated with rapid decreases in cholesterol, phospholipid and protein secretion. Such rapid effects on compounds so different means that it is unlikely that the inhibition of their synthesis would be a major determinant of the action of CyA. Moreover, in the rat, biliary cholesterol and phospholipid secretion is largely

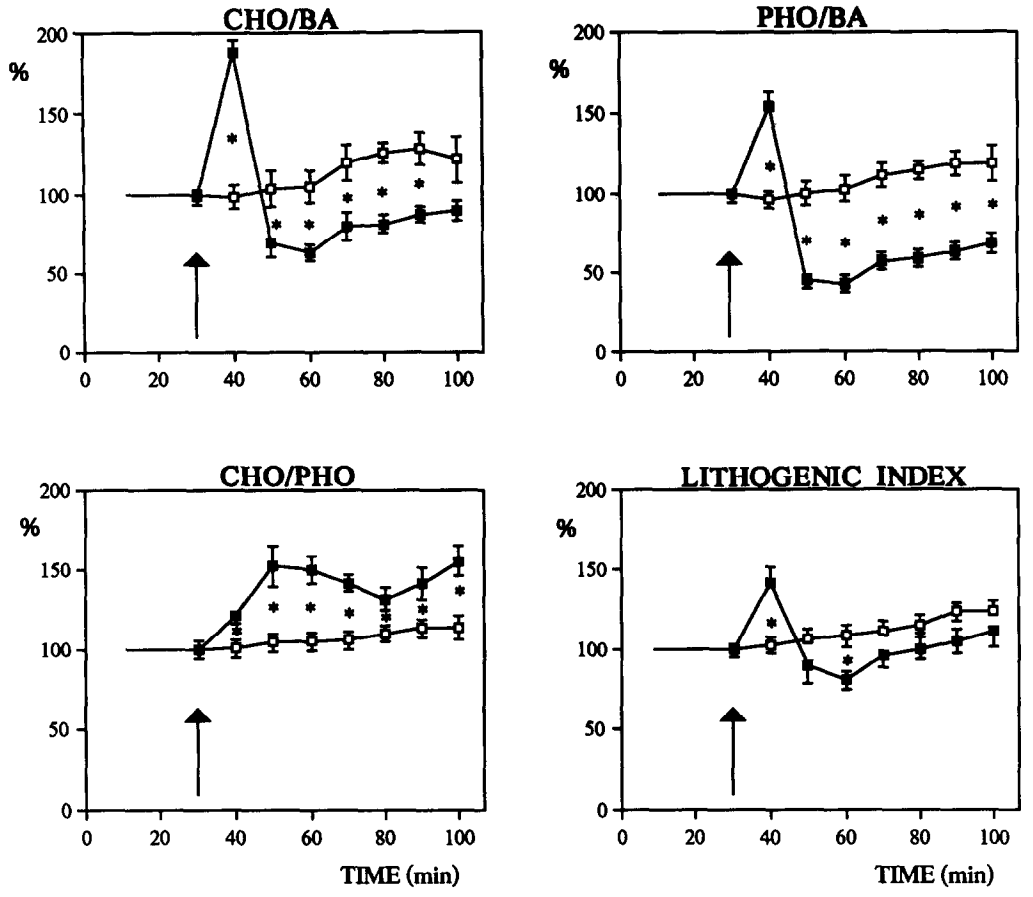

Fig. 3. Effects of CyA on the CHO/BA, PHO/BA and CHO/PHO molar ratio, and on lithogenic index of bile in anaesthetized rats. Changes in CHO/BA, PHO/BA and CHO/PHO molar ratios after i.v. administration (denoted by the arrow) of CyA vehicle (open symbols) or CyA (20 mg/kg, filled symbols). The changes in percentages relative to the means of the control period (min 0–30 of assays) are given as means ± SEM for 6–8 rats in each group. *$P < 0.05$ significantly different from control experiments.

independent of *de novo* hepatic synthesis [22, 23]. Additionally, Table 2 shows that CyA has no effects on the activities of the different enzymes evaluated in liver homogenates, or on the hepatic concentration of total proteins or of the other compounds, suggesting that the synthesis and elimination of these enzymes and substrates are unaffected by the drug.

Numerous investigations have shown that biliary secretion of lipid is related to and dependent upon bile acid secretion, there being a close association between bile acid and lipid secretion in all animal species studied [16–19, 21–23, 30], irrespective of the methodology used. Accordingly, the stimulation of bile acid secretion, e.g. by bile acid administration, increases lipid secretion; by contrast, when the transhepatic flux of the bile acid is low, as reported in man and animals with an interrupted enterohepatic circulation [41, 42], there is an associated decrease in the biliary secretion of lipid. A number of canalicular membrane enzymes are released into bile in response to bile acid secretion, whereas the discharge of lysosomal content into bile is an exocytic process that does not depend on the transhepatic flux of bile acid (see the introduction). Thus, a possible explanation for the CyA-induced inhibition of lipid and γ-GT secretion is that this is, at least in part, secondary to the fall in bile acid output caused by the drug.

Despite this, CyA induces a series of modifications in bile secretion which suggest that the drug (or its metabolites) might also inhibit biliary lipid and protein secretion directly and not only secondarily to the fall in bile acid secretion. Firstly, the intensity and duration of the inhibition exerted by the drug on the biliary secretion of bile acid and lipid, and the time course of these are very different. As shown in Figs 1 and 2, bile acid secretion first decreases and then returns rapidly to basal values; by contrast, cholesterol and phospholipid secretion decreases and recoveries to baseline values occur more slowly. Also, lipid and γ-GT secretion remains significantly reduced, even though the bile acid secretion rates return to values similar to those of the pretest period. Secondly, CyA induces significant alterations in the

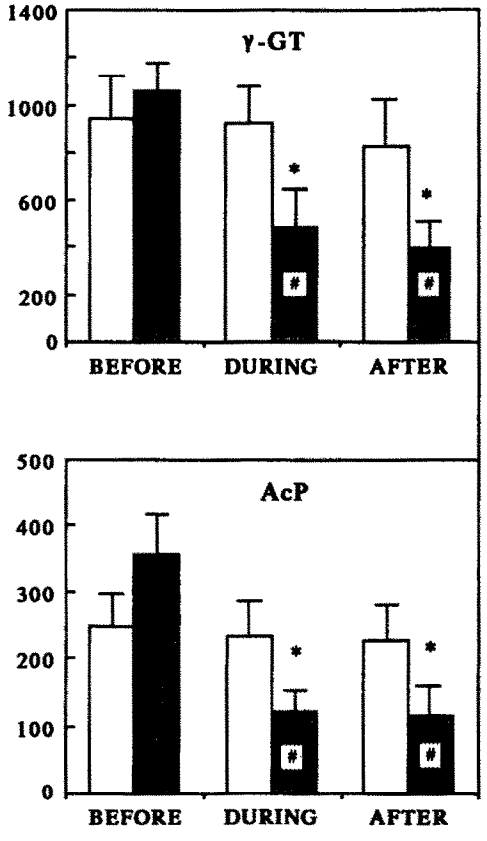

Fig. 4. Effects of CyA on the biliary secretion of canalicular plasma membrane (γ-GT) and lysosomal (AcP) marker enzymes in anaesthetized rats. Mean values ± SEM of γ-GT and AcP *before* i.v. administration (min 0–30 of assays) of CyA vehicle (open bars) or 20 mg/kg of CyA (filled bars), *during* the CyA-induced cholestasis period (min 30–70 of assays) and *after* the colestatic period (min 70–100 of assays). Biliary secretion rates are given as μU/min/100 g body wt, N = 6–8 rats in each group; *P < 0.05 significantly different from pretest values; #P < 0.05 significantly different from controls (CyA vehicle-treated rats).

Table 2. Mean values ± SEM of different enzyme activities and substrate concentrations in hepatic homogenates of control and CyA-treated rats

	Control	CyA
Enzymes activities (U/g liver)		
AcP	3.72 ± 0.22	4.05 ± 0.36
ALP	0.317 ± 0.037	0.320 ± 0.037
ALT	43.30 ± 4.37	52.55 ± 6.83
AST	114.7 ± 10.2	113.8 ± 15.9
CHE	0.748 ± 0.063	0.882 ± 0.094
CPK	4.42 ± 0.67	4.92 ± 0.88
HBDH	68.00 ± 9.01	87.20 ± 11.83
LDH	313.2 ± 44.3	388.3 ± 51.8
Substrate concentrations (mg/g liver)		
Calcium	0.024 ± 0.002	0.023 ± 0.002
Cholesterol	0.55 ± 0.07	0.55 ± 0.11
Creatinine	0.005 ± 0.001	0.007 ± 0.001
Glucose	6.77 ± 1.32	6.86 ± 1.33
Phosphorus	0.91 ± 0.14	0.91 ± 0.09
Phospholipids	5.88 ± 1.46	7.08 ± 1.52
Total proteins	87 ± 25	100 ± 9
Triglycerides	5.96 ± 0.66	5.81 ± 0.68
Urea	1.09 ± 0.11	1.13 ± 0.07
Uric acid	0.60 ± 0.10	0.64 ± 0.06

Rats received CyA vehicle (1 mL i.v., Intralipid plus ethanol, 9:1 v/v, used as control) or 20 mg/kg wt of CyA 80 min before liver homogenization.
N = 8–12 hepatic homogenates in each group.

CHO/BA and PHO/BA molar ratios; both molar ratios increase briefly (as a result of the greater and faster reduction in the concentration of bile acid) and thereafter decrease to values lower than those of the pretest period (due to the sustained reduction in the secretion of cholesterol and phospholipid). Thirdly, although the secretion patterns of cholesterol and phospholipid are in parallel, CyA increases the CHO/PHO molar ratio in a sustained fashion because the inhibitory effect of the drug on phospholipid secretion is higher than on cholesterol secretion. Finally, the biliary output of AcP, which does not depend on bile acid, is also reduced. Taken together, all these findings clearly suggest that CyA uncouples lipid secretion from bile acid secretion and also favour the hypothesis that CyA decreases biliary lipid and protein secretion directly and not only secondarily to the fall in bile acid output.

The uncoupling of biliary lipid secretion from bile acid has been described for a variety of substances in different experimental situations and species. However, the mechanisms responsible for this phenomenon remain to be fully elucidated and are still under debate. The uncoupling mechanisms proposed are (i) disturbances in intracellular transport of cholesterol and phospholipid, (ii) changes in the structure and function of the canalicular membrane that hinder the microvesiculation and removal of lipid from the membrane by bile acid, and (iii) a reduction in the membrane-solubilizing capacity of bile acid inside the bile canalicular lumen caused by an interaction between the uncoupling agent with bile acid [20, 24–26, 43–46].

Regarding possible alterations in transcellular transport, a number of workers have reported that the precursors of biliary lipid are transported by some vesicle-mediated process to the canalicular membrane to which they fuse until bile acid causes the release of membrane components. It is therefore possible that CyA might exert its effect by impairing this intracellular movement of cholesterol and phospholipid-containing vesicles and/or their fusion to the canalicular membrane, as reported for phalloidin—an anticytosketal compound structurally similar to CyA [9]—and other anticytoskeletal agents [16, 19, 20]. We have no direct evidence to confirm or disprove this hypothesis, although data previously published by us [4, 7] clearly show that CyA blocks trancytosis in the rat since the biliary secretion of HRP was impaired in anaesthetized rats receiving an i.v. dose of 10 or 20 mg of CyA per kg. Additionally, in our study the biliary excretion of

AcP was reduced by CyA and it is well known that this lysosomal (vesicular) transport system of proteins plays an important role in their secretion into bile [16, 27, 30–32].

Regarding the second mechanism, CyA could elicit changes in the structure and function of the canalicular membrane, either indirectly (by blocking the transcellular transport and supply of lipid vesicles—repair vesicles—involved in canalicular membrane recycling and repair, as reported above) or directly, as a result of the interaction of CyA with the components of the canalicular membrane. CyA is a highly lipophilic drug extensively metabolized in the liver by the cytochrome P450 system; all its metabolites have the cyclic undecapeptide structure of the parent compound and in the rat more than 60% is eliminated through the biliary route [1, 47]. Due both to its lipophilic property and high biliary excretion, CyA (or its metabolites) presumably induce physicochemical changes and/or changes in the fluidity of the canalicular membrane during translocation into the bile canaliculus. If such an alteration does in fact occur, one would expect other properties of the canalicular membrane, such as the activities of the bilirubin or sulfobromophthalein transport systems, to be affected; in this sense, an inhibition of the canalicular transport of bilirubin [5, 14] and sulfobromophthalein [13] has been demonstrated in rats treated with CyA. Finally, a striking finding reported by Whitington *et al.* [15] is that CyA alters hepatocyte plasma membrane lipid composition and markedly reduces its fluidity in the rat. Accordingly, via a reduction in membrane fluidity, CyA could cause a subsequent reduction in the removal of lipid and protein from the membrane by bile acid.

Additionally, it is possible that CyA may act inside the canalicular lumen. Current findings indicate that under physiological conditions, cholesterol and phospholipid are found in bile as mixed micelles with bile acid and as cholesterol–phospholipid vesicles. Intracanalicular physical interactions between CyA (or its metabolites) and the bile acid micelles and/or lipid vesicles could interfere with the subsequent removal from the membrane and solubilization of lipid and ectoenzymes, such as γ-GT, as has been proposed for other uncoupling agents that are extensively eliminated in bile [26, 43–46, 48]. Although we have not assessed the relative affinity of CyA for bile acids, indirect evidence of such an association has been reported. Mehta *et al.* [49] and other workers [50] have observed an extreme dependence of CyA intestinal absorption on bile acid. These authors have suggested that the stronger degree of absorption of CyA occurring when there is bile acid in the intestinal lumen would be mediated by a bile acid increase of CyA solubility. Therefore, since CyA and its metabolites are insoluble in water, once inside the canaliculus it is probable that they become incorporated into bile acid micelles. Moreover, Le Grue *et al.* [51] have indicated that CyA binds to phospholipid; if CyA–phospholipid binding does occur in bile, this could prevent the formation of mixed micelles, thus reducing the solubilizing capacity of bile acid. Finally, if CyA affects the formation of bile acid micelles or mixed micelles, then a change in the choleretic activity of bile acid would be expected. In this sense, we have previously reported an increase in the choleretic activity of bile acid in rats receiving an i.v. dose of CyA [7]. Accordingly, in the presence of high biliary concentrations of CyA, competition between the compound and the lipid for association with micelles could reduce the membrane-solubilizing capacity of bile acid and hence the secretion of lipid and protein into bile.

Finally, we observed that during the initial period of cholestasis, bile contained more cholesterol, as indicated by the transient increase in the lithogenic index. This new effect of the drug could be explained by the marked increases in the CHO/BA and CHO/PHO molar ratios during this period, in turn due to the asynchronic fall and recovery of the concentration and secretion of the three biliary compounds.

In summary, as well as inhibiting bile flow and bile acid secretion, CyA also inhibits the secretion of cholesterol and phospholipid in the rat. This latter effect is, at least in part, secondary to the inhibition of the hepatobiliary transport of bile acid. However, since CyA uncouples biliary lipid from bile acid secretion, and lipid and γ-GT biliary outputs remained low when bile acid output had returned to normal values, we hypothesize that other mechanisms and factors involved in lipid and protein secretion (at intracellular level, in the canalicular membrane and/or inside the bile canaliculus) might also be altered by the drug. To confirm these hypotheses and to evaluate whether this new hepatotoxic effect of the drug persists after prolonged treatment with CyA is a matter of further investigation.

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