Inhibitory action of cyclobutyrol on the secretion of biliary cholesterol and phospholipids

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A number of organic anions are known to decrease biliary secretion of cholesterol and phospholipid without affecting bile acid secretion. Cyclobutyrol (CB) is a choleretic agent which also inhibits biliary lipid secretion. Using isolated perfused rat liver we have studied this inhibition in relation to possible mechanisms suggested for other anions. Shortly after its administration to the isolated perfused liver, CB decreases biliary outputs of cholesterol and phospholipid, without changes in bile acid secretion, at low (450 nmol/min), high (1350 nmol/min) and nil taurocholate infusion rates. The absolute inhibition does not appear to be decreased by elevated bile acid secretion. There is a differential effect on secretion of cholesterol and phospholipid, more marked at low bile acid secretion rates. Biliary outputs of the canalicular membrane enzymes 5'-nucleotidase and alkaline phosphodiesterase I are also depressed by CB administration, but the anion does not affect the biliary output of bovine serum albumin or the output of rat serum albumin into the perfusion fluid. Since CB does not inhibit intracellular vesicular transport or apparently inhibit intracanalicular events, its effect is different from the effect of several other anions. From these studies it appears that the most likely effect of CB is exerted at the level of the canalicular membrane.

INTRODUCTION

A progressively increasing number of organic anions have been reported to dissociate the biliary secretion of cholesterol and phospholipid from that of bile acids, e.g. lysine acetylsalicylate (Erlinger *et al.*, 1975), ioglycamide (Bell *et al.*, 1978), sulphobromophthalein (Shaffer & Preshaw, 1981), bilirubin (Apstein & Robins, 1982), iopamide (Apstein & Robins, 1982), ampicillin (Apstein & Russo, 1985; Bellringer *et al.*, 1988a), sulphated glycolithocholic acid (Kuipers *et al.*, 1987), cefoperazone (Pattinson *et al.*, 1987), valproic acid and octanoic acid (Bellringer *et al.*, 1988b), ceftriaxone (Arvidsson *et al.*, 1988). All these compounds, when presented to the liver, dramatically decrease biliary lipid secretion, with little or no parallel effect on bile acid secretion.

The mechanism(s) and site(s) of this uncoupling are little understood, but do not appear to be operating, in the few cases studied so far, at the level of lipid uptake (Apstein, 1984) or synthesis (Apstein & Robins, 1982). Since some of these agents are choleretic, it has been proposed that their effects on biliary secretion might be due to the diluting effect on canalicular bile acid concentration and its subsequent lipid-holding capacity (Erlinger *et al.*, 1975).

An intracellular action of some of these compounds has been also suggested: e.g. ampicillin, cefoperazone, sulphated glycolithocholic acid and valproic acid could have a inhibitory effect on the intracellular processes of lipid assembly or delivery, or on membrane fusion (Apstein & Russo, 1985; Pattinson *et al.*, 1987; Kuipers *et al.*, 1987; Bellringer *et al.*, 1988a,b). This theory has been supported by the findings that some of these agents decrease not only biliary lipid secretion, but also the secretion of other non-lipidic materials both into the bile and into the sinusoids (Bellringer *et al.*, 1988a,b).

Finally, an intracanalicular inhibitory effect has also

been claimed. A physical association between these agents and mixed lipid micelles (Scharschmidt & Schmid, 1978; Tazuma & Holzbach, 1987) or lipid vesicles (Arvidsson *et al.*, 1988) in bile, and the consequent decrease in the capacity of these aggregates to solubilize cholesterol and phospholipid, as explanation for the phenomenon has elicited some controversy, owing, at least in part, to the limitation of techniques and conditions used to assess such associations (Scharschmidt & Schmid, 1978; Pattinson *et al.*, 1987; Tazuma *et al.*, 1988).

Recently, another choleretic organic anion, cyclobutyrol [α -(1-hydroxycyclohexyl)butyric acid; CB], shortly after its oral administration in the biliary-fistula rat, has been reported to decrease biliary secretion of cholesterol and phospholipid without inhibitory effect on bile acid secretion, and a possible effect of CB at the level of the canalicular membrane as the (or one of the) mechanism(s) involved in the inhibition was suggested (Monte *et al.*, 1989).

We decided to study the possible mechanism of the inhibitory action of this compound on a number of secretory parameters, using a different experimental model, the isolated perfused rat liver, in which extra-hepatic factors (such as gastrointestinal absorption) can be avoided. This preparation allows the secretion rate of bile acids to be selected and controlled, and hence the relationship of the inhibitory action of CB on bile-acid-stimulated lipid secretion at different bile acid secretion rates can be investigated.

MATERIALS AND METHODS

Materials

Antisera to rat and bovine serum albumins were purchased from Nordic Immunological Laboratories, Maidenhead, Berks., U.K. Sagatal was obtained from

May and Baker, Dagenham, Essex, U.K. Cannulation tubing PP10 was manufactured by Portex, Hythe, Kent, U.K., and heparin was made by Weddel Pharmaceuticals, London E.C.1., U.K. The sodium salt of taurocholic acid was purchased from Calbiochem/Behring Corp., Bishops Cleeve, Herts., U.K. CB was kindly given by Dr. R. Jimenez, Department of Physiology and Pharmacology, University of Salamanca, Spain. All other fine chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Methods

Male Wistar rats, weighing 250–280 g, were used throughout. These had been maintained on a standard laboratory diet under a constant light/dark cycle. The bile ducts of animals under pentobarbitone (Sagatal) anaesthesia were cannulated with PP10 tubing and their livers were then isolated *in situ* (Hems *et al.*, 1966) and perfused with 150 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), containing 1% (w/v) bovine serum albumin, 5 mM-glucose, 1.2 mM- CaCl_2 , a physiological amino acid mixture (see Barnwell *et al.*, 1983) and 20% (w/v) packed bovine red cells. This solution was recycled at a constant flow rate of 16 ml/min, gassed continuously with O_2/CO_2 (19:1) and maintained at $37 \pm 0.5^\circ\text{C}$ in a thermostatically controlled cabinet.

At 10 min after the start of the perfusion, a continuous portal infusion into the liver of sodium taurocholate (10 mM in 0.9% NaCl) was initiated, at a rate of 450 nmol/min (TC 450) or 1350 nmol/min (TC 1350). After the first 30 min, the perfusion fluid was changed to fresh medium to replace substrates used by the liver. Then 15 min later, CB (sodium cyclobutyrates, dissolved in 1 ml of sterile 0.9% NaCl) at a dose of 150 mg/kg body wt. (based on earlier studies; Monte *et al.*, 1989) was added, with complete mixing, to the perfusion fluid. Controls received an equal volume of saline only.

In another group of experiments, no bile acid infusion

was performed, and thus depletion of the endogenous bile acid pool occurred for 1 h (see Rahman & Coleman, 1986), before the addition of CB or saline.

Bile and perfusion-fluid samples were collected in pre-weighed tubes on ice. The volume of bile was determined gravimetrically, a density of 1 g/ml being assumed.

The health of the livers was monitored by analysing the extent of leakage into the perfusate of the cytosolic hepatocyte enzyme aspartate aminotransferase (EC 2.6.1.1). In no case was the leakage of this enzyme increased by any of the treatments performed.

Specific determinations

Aspartate aminotransferase was assayed using kits supplied by the Boehringer Corp., based on the method of Bergmeyer *et al.* (1978).

Bile acid concentrations were determined with hydroxysteroid dehydrogenase (EC 1.1.1.50) as described by Coleman *et al.* (1979).

Phospholipid present in bile was determined by the method of Bartlett (1959), after lipid extraction by the method of Bligh & Dyer (1959).

Rat serum albumin in the perfusion fluid and bovine serum albumin in bile were determined by quantitative radial immunodiffusion by the method of Mancini *et al.* (1965) with specific antisera. Authentic rat and bovine serum albumins were used for standardization.

5'-Nucleotidase (EC 3.1.3.5) and alkaline phosphodiesterase I (EC 3.1.4.1) activities in bile were assayed as described by Godfrey *et al.* (1981).

Cholesterol was analysed as trimethylsilyl ether derivatives, in order to prevent loss of parent compound in the column, by a slight modification (Rahman & Coleman, 1986) of the method of Vanlerenberghe & Cassaigne (1968). This method was used (i) because of the small quantity of cholesterol present in rat bile, and (ii) because cholesterol at low concentrations cannot be detected satisfactorily by other methods, owing to interference by other molecules, e.g. bilirubin.

Table 1. Effect of CB on biliary secretion in the isolated perfused rat liver

After liver isolation, a continuous infusion of taurocholate, at a rate of 450 nmol/min (a, TC 450), 1350 nmol/min (b, TC 1350) or 0 nmol/min (c, No TC), was initiated. CB (150 mg/100 g body wt.) was added to the perfusion fluid after 45 min of taurocholate infusion (60 min after liver isolation in No TC experiments). Values are means $\pm$ S.E.M. for *n* observations; significant differences from values pre-treatment (or from controls in No TC) are indicated by * ($P < 0.05$), ** ($P < 0.01$). † Cholesterol concentration in bile was below the detection range (1 μM).

Parameter	(a) TC 450 (<i>n</i> = 5)		(b) TC 1350 (<i>n</i> = 4)		(c) No TC (<i>n</i> = 3)	
	Before CB Time (min)...	After CB Time (min)...	Before CB Time (min)...	After CB Time (min)...	Control Time (min)...	CB Time (min)...
Bile flow ($\mu\text{l}/\text{min}$)	18.9 $\pm$ 1.3	44.9 $\pm$ 4.9**	25.9 $\pm$ 0.8	48.0 $\pm$ 0.6**	12.3 $\pm$ 1.7	27.2 $\pm$ 2.2*
Bile acid output (nmol/min)	529 $\pm$ 28	473 $\pm$ 37	1257 $\pm$ 37	1270 $\pm$ 77	14.9 $\pm$ 3.8	12.4 $\pm$ 2.1
Phospholipid output (nmol/min)	64.4 $\pm$ 1.9	22.5 $\pm$ 3.9**	121 $\pm$ 9	85.4 $\pm$ 6.4*	1.36 $\pm$ 0.23	0.29 $\pm$ 0.08*
Cholesterol output (nmol/min)	6.93 $\pm$ 0.84	1.84 $\pm$ 0.16**	11.8 $\pm$ 0.99	7.13 $\pm$ 1.09*	0.26 $\pm$ 0.04	†
Cholesterol/phospholipid molar ratio	0.096 $\pm$ 0.006	0.071 $\pm$ 0.006*	0.098 $\pm$ 0.004	0.077 $\pm$ 0.006*	0.205 $\pm$ 0.018	†

Fig. 1. Effect of CB in the isolated perfused rat liver on (a) bile flow, and on biliary output of (b) bile acids, (c) phospholipid, (d) cholesterol, (e) 5'-nucleotidase, (f) alkaline phosphodiesterase I, (g) bovine serum albumin, and on (h) the output of rat serum albumin into the perfusion fluid

For details of animal preparation and assay procedures see the Materials and methods section. Infusion of taurocholate (450 nmol/min) was carried out throughout the experiment. At 45 min (denoted by the arrow) CB (150 mg/100 g body wt., dissolved in 1 ml of sterile 0.9% saline) was added to the recirculating perfusion. Controls received saline only. The values on the graphs are plotted to represent the end of each collection period, and are means $\pm$ S.E.M. Significant differences from controls are indicated by * ($P < 0.05$), ** ($P < 0.01$). Livers: $\circ$, control ($n = 4$); $\bullet$, treated with CB ($n = 5$).

RESULTS

The effects of CB on a number of secretory parameters in the isolated livers were seen shortly after its administration and, in most cases, a maximal effect was observed within 20–30 min.

Effects of CB on secretory parameters at a low bile acid infusion rate (TC 450)

After 30–45 min of taurocholate infusion (450 nmol/min) a steady state in bile secretion was achieved (Table

1a). At that point CB was added into the perfusion medium. Bile flow increased 2.5-fold and subsequently returned towards control levels; 90 min after CB administration, flow values were not significantly different from controls (Fig. 1a). Bile acid output was unaffected by CB administration (Fig. 1b). Phospholipid and cholesterol secretion into bile (Figs. 1c and 1d) decreased by approx. 65% and 75% respectively, and stayed at the lower level throughout the remainder of the perfusion.

The activities in bile of the two canalicular membrane enzymes assayed, 5'-nucleotidase and alkaline phospho-

Fig. 2. Effect of CB in the isolated perfused rat liver on (a) bile acid/(phospholipid + cholesterol) molar ratio [BA/(PI + Ch)] and (b) cholesterol/phospholipid molar ratio (Ch/PI)

For details see the Materials and methods section and the legend to Fig. 1. Livers: □, control ($n = 4$); ■, treated with CB ($n = 5$).

diesterase I (Figs. 1e and 1f) were significantly decreased after CB administration and, as for the lipids, remained low even when the bile flow returned to control values (Fig. 1a).

The anion had no inhibitory effect either on the biliary output of bovine serum albumin (Fig. 1g) or on the secretion of rat serum albumin into the perfusion fluid (Fig. 1h).

Fig. 2 shows the calculated bile acid/(phospholipid + cholesterol) molar ratio (Fig. 2a) and cholesterol/phospholipid molar ratio (Fig. 2b) in control and CB experiments. The former molar ratio increased after CB administration, owing to the decrease in biliary outputs of phospholipid and cholesterol, without change in the bile acid output. The cholesterol/phospholipid molar ratio (Fig. 2b) decreased after CB administration, indicating a significantly greater effect of CB on the secretion of cholesterol relative to phospholipid.

Effects of CB on secretory parameters at a high bile acid infusion rate (TC 1350)

The effects of CB on biliary secretion in the presence of 1350 nmol of taurocholate/min are shown in Table 1(b). CB increased bile flow 2-fold; total bile acid secretion remained unaltered by the compound.

Phospholipid and cholesterol secretion rates at both low (TC 450) and high (TC 1350) taurocholate infusion rates are compared in Fig. 3. The administration of the same dose of CB decreased the biliary secretion of phospholipid and cholesterol in similar intensity in both series of experiments (Figs. 3a and 3b), the difference between values before and after CB administration being

Fig. 3. Effect of CB in the isolated perfused rat liver on the biliary output of (a, c) phospholipid and (b, d) cholesterol

For details see the Materials and methods section and the legend to Fig. 1. Infusion of taurocholate was carried out throughout the experiment at a rate of 450 nmol/min (TC 450; △; $n = 5$) or 1350 nmol/min (TC 1350; ▲; $n = 4$). CB was added in both groups at the time denoted by the arrow. (a, b) Means $\pm$ S.E.M. of the absolute values; (c, d) means $\pm$ S.E.M. of the percentage variation over pre-administration values.

41.9 ± 3.7 nmol/min of phospholipid and 5.09 ± 0.91 nmol/min of cholesterol in TC 450 and 35.9 ± 8.7 nmol/min of phospholipid and 4.66 ± 0.65 nmol/min of cholesterol in TC 1350. However, when calculated as percentage decreases from pre-administration values (Figs. 3c and 3d), the effect of the anion on these lipids therefore appeared less extensive at the high bile salt infusion rate: thus CB decreased phospholipid and cholesterol biliary outputs by 65 % and 75 %, respectively in TC 450, and only by 35 % and 50 % in TC 1350.

Effects of CB on secretory parameters in the absence of bile acid infusion (No TC)

In this series of experiments, the time of bile collection was modified in order to deplete the endogenous bile acid pool: liver perfusions in the absence of bile acid infusion were performed during 60 min, after which bile acid secretion stabilized, owing to depletion of the bile acid pool, and CB or saline was then added. The control for this series is the 60–90 min period in livers not treated with CB.

After 60 min of the liver perfusion, without infusion of bile acids, bile acid concentration in bile was 1.17 ± 0.14 mM; the levels of other biliary parameters are shown in Table 1(c). Administration of CB at that point increased bile flow and decreased phospholipid and cholesterol outputs, without affecting bile acid output. The effect on cholesterol secretion was such that its concentration in bile could be detected in only one animal, but in the other two animals the value was below $1 \mu\text{M}$ (the minimum range of detection of the technique).

DISCUSSION

In the isolated perfused rat liver, CB causes a transitory increase in bile volume and a simultaneous, but more permanent, depression in the biliary secretion of phospholipid and cholesterol, dissociating these from the secretion of bile acids at all bile acid secretion rates.

Possible intracellular effect

The maximal effect on biliary lipid secretion amounts to a depression of 70 % and this is achieved within 20 min of administration; such a rapid, major, effect makes inhibition of synthesis as a major determinant of the effect of CB, as for other anions (Pattinson *et al.*, 1987; Bellringer *et al.*, 1988a), unlikely. Moreover, in the rat, biliary cholesterol secretion is largely independent of new hepatic synthesis (Turley & Dietschy, 1981), and, in rats treated with CB for 10 days, no changes in the hepatic levels of cholesterol and phospholipids were detected (Monte, 1988).

Several other organic anions cause decreases in a number of materials transported by intracellular vesicle movement (Bellringer *et al.*, 1988a,b). Since the outputs of bovine serum albumin into bile and of rat serum albumin into the perfusion fluid are not affected by CB (Figs. 1g and 1h), inhibition of general vesicular transport is therefore unlikely to be present, though a more selective inhibitory effect of CB specifically on vesicular biliary lipid transport, or on the assembly of biliary lipids in the Golgi apparatus (see Crawford & Gollan, 1988), cannot be excluded.

In a preliminary series of experiments (results not shown), incubation of suspensions of isolated hepatocytes with CB at doses above those used here produced

Fig. 4. Relation between bile flow and biliary output of bile acids on control (□) and CB-treated (■) livers

The values correspond to 30 min and 45 min samples in all the experimental groups for the controls ($n = 32$) and to 65 min and 75 min samples (maximum of choleretic response) in the CB experiments ($n = 21$).

no changes in hepatocyte viability or in intracellular ATP concentration, suggesting that a decrease in cellular energy for the transport of biliary lipids was unlikely.

Possible intracanalicular effect

Choleretic organic anions, by their dilution of canalicular bile, may cause a decrease in the effective concentration of bile acids available for lipid solubilization (Erlinger *et al.*, 1975). We think that this is unlikely to be the principal explanation for the phenomenon, however, since, at the end of the experiments, when bile flow has returned to control values, biliary secretion of cholesterol and phospholipid, and of biliary enzymes, which also are dependent on bile acid concentration (Barnwell *et al.*, 1983), remained inhibited, i.e. did not return to control levels. Moreover, some of the anions uncoupling biliary lipid from bile acid secretion do not cause cholestasis (Apstein & Robins, 1982), and other choleretic compounds do not have such an effect on biliary lipids (Hardison & Apter, 1972).

Incorporation of CB into bile acid micelles could possibly interfere with the subsequent solubilization of biliary lipids. In preliminary experiments using the technique of Benzonana (1969) to detect critical micellar concentration, we have obtained no evidence for the influence of CB on that of taurocholate (results not shown), and other inhibitory anions (Pattinson *et al.*, 1987; Reuben *et al.*, 1982; Tazuma *et al.*, 1988) do not appear to aggregate into bile acid micelles; some association between a metabolite of CB with biliary micelles could, however, be possible.

If cyclobutyrol (or if one of its metabolites) is incorporated into the bile acid mixed micelles, then a change in its own choleretic activity (during variation in bile acid secretion rates), or in the choleretic activity of bile acids (when the anion is present), would be expected. However, when the regression line relating bile flow and bile acid secretion was calculated, both before and during the maximum choleretic response to CB (Fig. 4), administration of the anion does not modify the choleretic

activity of bile acids, since the gradient is similar both with and without CB.

In the absence of bile acid infusion, the biliary concentration of bile acids is very low, probably below (or very close to) the critical micellar concentration. Therefore, the small amount of lipids present in bile are not likely to occur principally as mixed bile acid-lipid micelles, but as other aggregates (mainly vesicles) of cholesterol and phospholipids (see, e.g., Somjen & Gilat, 1983; Carey & Cohen, 1987). When CB was then added to such livers it caused a virtual elimination in the secretion of lipids (Table 1c). Thus a competition of CB with the lipids for the association with the bile acid molecules into the micelles cannot possibly be the only mechanism involved in the inhibition, since the number of micelles present will be vanishingly low.

When CB was administered at two different bile acid infusion rates (450 and 1350 nmol/min) (Fig. 3), it caused a decrease of almost similar amounts of lipids at bile acid perfusion rates varying over a 3-fold range. Inhibition by CB is therefore unlikely to be related to the total number of bile acid micelles present.

Action on the canalicular membrane

A number of enzymes, e.g. 5'-nucleotidase, alkaline phosphodiesterase I, leucine aminopeptidase etc., are derived mainly from the canalicular membrane and are released into bile in response to bile acid secretion (Godfrey *et al.*, 1981; Barnwell *et al.*, 1983). Their presence in bile may involve an initial microvesiculation of a region of the canalicular membrane, followed by 'solubilization' by the bile acids (Barnwell *et al.*, 1983; Coleman, 1987). Monte *et al.* (1989) have reported a decrease in the biliary output of plasma-membrane enzymes during the initial choleretic response to CB in the fistula rat. In the present experiments, 5'-nucleotidase and alkaline phosphodiesterase I output into bile was dramatically decreased (Figs. 1e and 1f), and remained low even though bile acid output is maintained and biliary volume returns to normal values (i.e. is not due to a dilution effect on bile acid potency).

The biliary secretion of cholesterol and phospholipids is also believed to be a process possibly involving vesiculation of more fluid, 'biliary-like', lipid microdomains in the canalicular membrane, followed by their transformation into mixed micelles by subsequent action of bile acids (Barnwell *et al.*, 1984; Coleman, 1987). Since the secretion of both biliary lipids and enzymes of the plasma membrane are depressed in a prolonged fashion by CB, or its metabolites, this points to an effect of CB (i) on the canalicular membrane directly or (ii) on the interaction of bile acids with the membrane.

Differential action on cholesterol and phospholipid secretion

In the biliary response to CB, cholesterol secretion is affected to a greater extent than phospholipid secretion (Fig. 2b). A proportionally greater decrease in cholesterol secretion has also been reported for several other anions (Shaffer & Preshaw, 1981; Apstein & Russo, 1985; Pattinson *et al.*, 1987; Arvidsson *et al.*, 1988).

In the present paper, the cholesterol/phospholipid molar ratio in samples before CB administration was constant at both high (TC 1350) and low (TC 450) bile acid infusion rates, but was increased when no taurocholate infusion was performed ('No TC', Table 1).

Administration of CB at this very low bile acid secretion rate resulted in a complete inhibition of cholesterol secretion (Table 1c). The more powerful inhibition of a second, bile-acid-independent, pathway of cholesterol secretion (see Hardison & Apter, 1972; Rahman & Coleman, 1986; Coleman *et al.*, 1989) by CB could explain this result [see also Bell *et al.* (1978) for ioglycamide], and also the consistent decrease in cholesterol/phospholipid molar ratio after CB administration (Table 1, Fig. 2b).

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