Modulation of biliary lipid secretion by forskolin and cyclic AMP analogues

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Exposure of isolated perfused rat livers to either 100 μ M-forskolin, a potent activator of adenylate cyclase, or to 0.5 mM-concentrations of the cAMP analogues chlorophenylthio cAMP (CPTcAMP), dibutyryl cAMP (dbcAMP) and 8-bromo cAMP (8BrcAMP), to provoke increases in intracellular concentrations of cAMP, resulted in marked changes in bile volume and composition. Bile flow reached a peak after 10 min, before declining towards control levels, and an increase in several secretory parameters was also observed at this time. At 20 min, a substantial decrease in the output of both phospholipid and cholesterol was evident, and this suppression of secretion was maintained throughout the remainder of the experiment. The order of effectiveness of the cAMP-elevating agents at decreasing biliary lipid output was CPTcAMP > forskolin > dbcAMP > 8BrcAMP. Biliary output of bile acids was essentially unaltered compared with controls; similarly, no decrease in the secretion of protein and triacylglycerols into the perfusion medium was observed. This suggests that the elevation of intracellular levels of cAMP may cause a selective inhibition of biliary lipid output rather than a more general inhibition of hepatic secretion.

INTRODUCTION

Bile acid secretion is associated with increased bile flow and enhanced biliary lipid output; this indicates an important determining role for bile acids in phospholipid and cholesterol secretion (Wheeler, 1972; Sewell *et al.*, 1980; Gurantz & Hofmann, 1984; Coleman, 1987; Rahman & Coleman, 1987; Coleman *et al.*, 1988). A recent theory of biliary lipid output suggests that lipid vesicles fuse with the canalicular membrane to form more fluid microdomains; such domains are more susceptible to removal by the detergent action of bile acids and are replenished by lipid from within the cell, via microtubule-mediated vesicular transport (Lowe *et al.*, 1984; Coleman, 1987; Rahman & Coleman, 1987; Coleman *et al.*, 1988).

Biliary lipid output is linearly dependent on bile acid secretion at physiological rates (Rahman & Coleman, 1987). However, at high rates of bile acid secretion, a maximum secretory rate exists for biliary lipids, possibly reflecting the maximum rate of lipid synthesis, delivery and secretion (Barnwell *et al.*, 1986; Coleman, 1987; Coleman *et al.*, 1988).

This suggests that the hepatocyte is capable of sensing variations in the amount of bile acids passing through the cell and being secreted. By altering the control of lipid provision, of vesicle assembly and transport, and of membrane processing accordingly, delivery of the correct amount of lipid into bile is ensured (Coleman, 1987; Rahman & Coleman, 1987; Coleman *et al.*, 1988). The maximum rate of secretion possibly reflects the inability of the cell to respond to the bile acid signalling at a sufficient rate.

The exact point of interaction of bile acids in hepatocytes to provoke biliary lipid secretion is not fully understood, but bile acids themselves, or via some form of second messenger, may be acting as regulators of

biliary lipid secretion. Studies of this association between bile acid and biliary lipid secretions have been explored largely in a physiological or physicochemical manner, e.g. in micelle formation, but the involvement of second messengers has been little examined. It has been reported that cyclic AMP (cAMP), a known hormonal second messenger, decreases cholesterol concentration and output in dog liver (Kortz *et al.*, 1984), and appears to provoke choleresis in dogs and in the isolated perfused rat liver (Thomsen & Larsen, 1981; Schirmer *et al.*, 1986; Knodell *et al.*, 1987).

The present study further investigates the role of cAMP in the modulation of biliary lipid secretion in the isolated perfused rat liver. Intracellular levels of cAMP were raised by using analogues of cAMP, namely 8-(4-chlorophenylthio) cAMP (CPTcAMP), 8-bromo cAMP (8BrcAMP) and dibutyryl cAMP (dbcAMP), and by using forskolin, a unique and potent activator of adenylate cyclase in a wide variety of tissues (Metzger & Lindner, 1981; Seamon & Daly, 1981; Seamon *et al.*, 1981; Daly *et al.*, 1982; Birnbaumer *et al.*, 1983; Hersey *et al.*, 1983; Whetton *et al.*, 1983; Wong & Martin, 1983). A range of secretory parameters in bile and perfusion fluid were measured to establish whether cAMP caused a general inhibition of secretion or whether the effect was confined to biliary lipid secretion.

MATERIALS AND METHODS

Materials

Sagatal (sodium pentobarbitone) was bought from May and Baker, Dagenham, Essex, U.K., and heparin was made by Weddel Pharmaceuticals, London E.C.1, U.K. The PP10 cannulation tubing was manufactured by Portex, Hythe, Kent, U.K., and taurocholate (sodium salt) was obtained from Calbiochem, Bishops Cleeve, Nottingham.

Abbreviations used: cAMP, cyclic AMP; CPTcAMP, 8-(4-chlorophenylthio) cAMP; 8BrcAMP, 8-bromo cAMP; dbcAMP, *N*⁶,*O*²-dibutyryl cAMP; RSA, rat serum albumin; BSA, bovine serum albumin.

Herts., U.K. Antisera to both rat serum albumin (RSA) and bovine serum albumin (BSA) were purchased from Nordic Immunological Laboratories, Maidenhead, Berks., U.K., and purified agar was obtained from Oxoid, Basingstoke, Hants., U.K.

dbcAMP, 8BrcAMP and CPTcAMP (all sodium salts), forskolin, hydroxysteroid dehydrogenase (EC 1.1.1.50; grade II), and all other fine chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Methods

Isolated perfused rat livers. Male Wistar rats weighing 245–280 g were used throughout; they were maintained on a standard laboratory diet with a constant 12 h-light/12 h-dark cycle.

Rats were anaesthetized with 1 ml of a 1+2 dilution of Sagatal. The bile duct was cannulated with 50 cm of PP10 tubing. Livers were isolated *in situ* and perfused with a recirculating medium consisting of supplemented Krebs–Ringer bicarbonate buffer (see Rahman & Coleman, 1986), into which was continuously infused 500 nmol of sodium taurocholate/min.

The perfusion fluid was replaced with fresh medium 60 min after isolation of the liver and, after a further 30 min, the liver was exposed to one of the following: CPTcAMP, 8BrcAMP or dbcAMP administered to the perfusion medium in 1 ml of sterile 0.9% NaCl to give a final perfusate concentration of 0.5 mM, or forskolin administered to the perfusion fluid in 1 ml of 95% (v/v) ethanol, giving a final concentration of 100 μ M. For controls, an equivalent amount of either sterile saline or 95% ethanol was added. Bile was collected throughout, in pre-weighed tubes stored on ice, and the volume was determined gravimetrically, assuming a density of 1 g/ml. All experiments were terminated 130 min after liver isolation.

Samples of perfusion fluid were collected every 10 min for glucose determination, and for RSA/BSA determinations. Aspartate aminotransferase (EC 2.6.1.1) was determined in samples of perfusion fluid at 130 min after isolation, to assess the extent of damage to the liver. Neither the cAMP analogues nor forskolin appeared to increase leakage of this enzyme from the liver (results not shown). Under the conditions used, all cAMP-elevating agents were observed to stimulate glucose concentration in perfusion fluid by 66–79% within 20 min compared with their respective controls.

Chemical determinations. Aspartate aminotransferase was measured in perfusion fluid with test kits based on the method of Bergmeyer *et al.* (1978), and supplied by Boehringer Mannheim.

Phospholipid content of bile was determined, after extraction of lipid by the method of Bligh & Dyer (1959), by using the method of Bartlett (1959). Bile salts were measured by using hydroxysteroid dehydrogenase (see Coleman, 1979). Cholesterol was determined by g.l.c., by the method of Vanlerenberghe & Cassaigne (1968), modified by Rahman & Coleman (1986).

Glucose levels were monitored with the Boehringer Mannheim test kit for glucose, based on glucose oxidase. Triacylglycerols were measured by the method of Wahlefeld (1974), with a Peridochrom test kit (Boehringer Mannheim).

BSA and RSA were measured in bile and perfusion

Fig. 1. Effect on bile flow of (a) 100 μ M-forskolin, (b) 0.5 mM-CPTcAMP, (c) 0.5 mM-dbcAMP and (d) 0.5 mM-8BrcAMP

cAMP-elevating agents were administered at zero time into the recirculating perfusion medium, as indicated by the arrow. Values are means $\pm$ S.E.M. of three controls (○) and four treated livers (●).

fluid respectively, by quantitative radial immunodiffusion, by the method of Mancini & Carbonara (1965).

Statistical analysis was performed by Student's *t* test.

RESULTS

Bile flow for control livers and those treated with forskolin and cAMP analogues is shown in Figs. 1(a)–(d). At 10–15 min, all compounds produced a significant peak in bile flow 25–95% above that at zero time ($P < 0.01$). Thereafter in all cases, bile flow declined, falling back to control levels by 30 min in the forskolin and 8BrcAMP series.

Fig. 2. Effect on biliary bile acid output of (a) $100\ \mu\text{M}$ -forskolin, (b) $0.5\ \text{mM}$ -CPTcAMP, (c) $0.5\ \text{mM}$ -dbcAMP and (d) $0.5\ \text{mM}$ -8BrcAMP

cAMP-elevating agents were administered at zero times into the recirculating perfusion medium, as indicated by the arrow. Values are means $\pm$ S.E.M. of three controls (○) and four treated livers (●).

Forskolin and the cAMP analogues produced a much smaller increase in bile acid output, a small peak being evident at 10 min, but the extent of this was only 0.2–17% higher than at zero time and did not achieve statistical significance (Figs. 2a–2d).

Fig. 3. Effect on biliary phospholipid output of (a) $100\ \mu\text{M}$ -forskolin, (b) $0.5\ \text{mM}$ -CPTcAMP, (c) $0.5\ \text{mM}$ -dbcAMP and (d) $0.5\ \text{mM}$ -8BrcAMP

cAMP-elevating agents were administered at zero time into the recirculating perfusion medium, as indicated by the arrow. Values are means $\pm$ S.E.M. of three controls (○) and four treated livers (●).

Fig. 4. Effect on biliary cholesterol output of (a) 100 μ M-forskolin, (b) 0.5 mM-CPTcAMP, (c) 0.5 mM-dbcAMP and (d) 0.5 mM-8BrcAMP

cAMP-elevating agents were administered at zero time into the recirculating perfusion medium, as indicated by the arrow. Values are means $\pm$ S.E.M. of three controls (○) and four treated livers (●).

After an initial small fall at 5 min, and a subsequent non-significant small rise at 10 min, forskolin caused a more substantial and significant ($P = 0.01$) fall in output of phospholipid and a fall in cholesterol ($P = 0.06$), at 20 min (Figs. 3a and 4a), to approx. 50% of the zero-time

values in each case. This decreased output was then maintained throughout the remainder of the experiment. The cAMP analogues showed essentially the same overall trends in lipid output, with a small increase at 10 min and a decrease at 20 min, although CPTcAMP and 8BrcAMP did not show initial small falls in lipid output at 5 min. All the analogues depressed biliary output at 20 min and onwards, although to varying degrees. CPTcAMP decreased phospholipid output by 50% (Fig. 3b), and cholesterol output by 60% (Fig. 4b) ($P < 0.01$), dbcAMP provoked a smaller decrease, 25% for phospholipid and 20% for cholesterol ($P = 0.05$) (Figs. 3c and 4c), and 8BrcAMP gave only a 10% decrease in phospholipid output while decreasing cholesterol output by 30% ($P = 0.06$) (Figs. 3d and 4d).

In all cases, the movement of BSA from perfusion fluid to bile reached a peak at 10 min before returning to control levels within 5 min, and then remained at control levels for the duration of the experiment (results not shown). RSA output into both bile and perfusion fluid, and the release of triacylglycerols into the perfusate, were unaffected throughout the experiments (results not shown).

DISCUSSION

Forskolin, a potent activator of adenylate cyclase, and the cAMP analogues CPTcAMP, 8BrcAMP and dbcAMP were used to raise intracellular levels of cAMP. The concentration of these analogues, 0.5 mM, was the same as that used for CPTcAMP by Pelech *et al.* (1981, 1982) and by Tijburg *et al.* (1989), allowing a direct comparison to be made. Several workers have reported maximal stimulation of adenylate cyclase activity in a variety of tissue preparations treated with 100 μ M-forskolin (Seamon *et al.*, 1981; Birnbaumer *et al.*, 1983; Schneyer *et al.*, 1983; Whetton *et al.*, 1983; Wong & Martin, 1983), and, on this basis, livers were therefore exposed to forskolin at 100 μ M. All cAMP-elevating compounds stimulated glucose output into the perfusion fluid by 66–79% above controls, indicating a stimulatory effect on hepatic glucose production within the time scale of the changes in biliary parameters.

Bile flow and bile acid output

The substantial peak in bile flow observed after treatment with both the cAMP analogues and forskolin is in agreement with the cAMP-induced choleresis reported in dogs and isolated perfused rat livers (Barnhart & Combes, 1975; Schirmer *et al.*, 1986; Knodell *et al.*, 1987). The exact mechanism of this substantial (25–95%) choleresis is unknown, but, since bile acid output is increased by only 0.2–17% after 10 min, it therefore implies that the choleric effect of cAMP is largely due to stimulation of a bile-acid-independent bile flow, as suggested by other workers (Barnhart & Combes, 1975; Thomsen & Larsen, 1981; Schirmer *et al.*, 1986). Although the mechanism of bile-acid-independent bile flow is unclear, it is thought to involve the excretion of Na^+ , Cl^- and HCO_3^- ions (Klaassen & Watkins, 1984). Several workers have suggested that in dogs cAMP might influence Na^+ transport and the Na^+/K^+ -ATPase system (Orloff & Handler, 1967; Meyers & Jones, 1979b; Thomsen & Larsen, 1981; Schirmer *et al.*, 1986), although others have doubted that accumulation of cAMP is physiological in bile formation (Baker & Kaplan, 1976; Poupon *et al.*, 1978).

Biliary lipid secretion

The increases in biliary lipid secretion at 10 min are coincident with, and probably reflect, the increase in bile acid secretion at this time. By 20 min onwards, however, both with the cAMP analogues and with forskolin, the observed decrease in output of both phospholipid and cholesterol is not related to bile acid secretion, and suggests that cAMP might play a more specific role in the partial inhibition of biliary lipid secretion. The order of effectiveness of the agents used is CPTcAMP > forskolin > dbcAMP > 8BrcAMP. This order may reflect the balance between penetration of the cell by each cAMP-elevating agent and its degradation within the cell, the requirement for metabolic transformation to an active species and its ability to activate target enzymes. In other studies CPTcAMP was reported to be 100 times more potent than dbcAMP at activating cAMP-dependent protein kinase, 20 times more resistant to degradation than dbcAMP, and does not require metabolic transformation for activity, which may explain its potency for inhibition of lipid secretion (Miller *et al.*, 1975).

A decrease in cholesterol concentration and output from dbcAMP-treated dog livers has been reported (Kortz *et al.*, 1984), and several workers have observed decreased biliary cholesterol secretion in dogs, using glucagon (Meyers & Jones, 1979a,b; Bickerstaff *et al.*, 1983; Schirmer *et al.*, 1986). The effects of cAMP on phospholipid output are not so widely reported. Meyers & Jones (1979b) observed a decrease in phospholipid secretion in biliary-fistula dogs treated with glucagon, and in cultured rat hepatocytes cAMP has been reported to decrease synthesis of phosphatidylcholine (Pelech *et al.*, 1981, 1982, 1984) and phosphatidylethanolamine (Tijburg *et al.*, 1989).

The relationship between decreased synthesis and decreased output of biliary lipids is, however, unclear. Although cAMP decreases the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (Meyers & Jones, 1979b; Kortz *et al.*, 1984), and Pelech *et al.* (1981, 1982, 1984) have reported a strong correlation between decreased activity of CTP:choline-phosphate cytidyltransferase and inhibition of phosphatidylcholine synthesis, the influence of this on biliary lipid secretion has been challenged by several other workers (Bickerstaff *et al.*, 1983; Kortz *et al.*, 1984; Schirmer *et al.*, 1986).

The reported reduction in the cytidyltransferase activity is apparent after approx. 2 h (Pelech *et al.*, 1981, 1982, 1984). However, in the present study, inhibition of secretion of biliary lipid was seen within 20 min; thus the reported time scale for inhibition of phospholipid synthesis observed by Pelech *et al.* (1984) does not appear to correlate with the inhibition of phospholipid output that we have observed in this study.

Butterwith *et al.* (1984) have shown that inactive phosphatidate phosphohydrolase becomes bound to membranes involved in glycerolipid synthesis, for the generation of diacylglycerol before phospholipid synthesis; cAMP was found to promote translocation of the phosphatidate phosphohydrolase back to the cytosol, thereby inactivating it and decreasing availability of diacylglycerol for phospholipid synthesis. Tijburg *et al.* (1989) suggested that phosphatidylethanolamine synthesis might be decreased by cAMP via an indirect mechanism involving the availability of diacylglycerols, and reported a 30% decrease in diacylglycerol levels in

hepatocytes 20 min after cAMP administration. However, if this was the mechanism by which phospholipid secretion was decreased, then a parallel decrease in triacylglycerol output might be expected in the present experiments, but this was not observed. Furthermore, it is unlikely that decreased synthesis via this pathway would have any appreciable effect on net lipid output within as short a time as 20 min.

Cronholm *et al.* (1983) have shown that most of the phospholipid and cholesterol is secreted from pre-formed lipid pools within the liver and does not arise from synthesis *de novo*, and Schwartz *et al.* (1978) have estimated that only 16–20% of secreted biliary cholesterol has been newly synthesized. Rahman *et al.* (1986) reported that, in the isolated perfused rat liver, intrahepatic pools of lipid are capable of maintaining output for more than 2 h, despite the non-availability of lipoproteins. These observations further support the premise made above that decreased synthesis of lipid would not significantly decrease lipid output within 20 min. Thus, although inhibition of synthesis might play an important role in the longer-term inhibition of lipid secretion, it seems doubtful that it is responsible for the decreased lipid output observed in the shorter time-scale of the present study.

Since an increase in bile flow under conditions of constant bile acid output will decrease bile acid concentration, the decrease in biliary lipid output could reflect the decreased numbers of bile acid micelles, and thereby a lowered capacity to process the lipid from the canalicular membrane (Meyers & Jones, 1979a,b). However, the non-micellar analogue taurodehydrocholate is able to provoke an increase in bile flow and thereby to dilute the concentration of taurocholate without causing a decrease in biliary lipid outflow (Hardison & Apter, 1972; S. Hamlin, unpublished work). Moreover, and of more relevance to the present situation, the results of this investigation show that, although bile flow returns towards control levels after 30 min, biliary lipid output in all cases remains suppressed, suggesting that biliary bile acid dilution has little contribution to make to the effects of cAMP on biliary lipids (Figs. 1, 3 and 4).

It is therefore considered more probable in this case that the effects of cAMP have been to decrease lipid secretion in, or across, the hepatocyte, or lipid release at the canalicular membrane. The mechanism by which bile acids influence biliary lipid output is unknown, but it is possible that cytoskeletal or cytoskeletal-vesicle interaction may be a key regulatory site for bile-acid-stimulated biliary lipid secretion (Rahman & Coleman, 1987; Coleman *et al.*, 1988). cAMP might affect this site in some way, causing a dissociation of bile acid and biliary lipid output. The transcellular movement of vesicles destined for secretion of other materials is thought to require the disassembly of the actin filament network in the cell cortex (Cheek & Burgoyne, 1986). Elevation of intracellular cAMP is reported to inhibit disassembly of these actin filaments (Burgoyne & Cheek, 1987) and, in pancreatic acinar cells, also to inhibit nicotine-induced amylase secretion (see Cheek & Burgoyne, 1986), suggesting a possible mechanism for cAMP-induced inhibition of secretion. However, it is not clear whether such an effect on the disassembly of actin filaments would allow the specific inhibition only of the biliary lipids, in which case the disassembly would be localized, or whether a more general inhibition, as seen in

pancreatic acinar cells, would in fact be observed, i.e. an inhibition of protein secretion would also be apparent; there is, however, no interference with albumin secretion into the perfusion fluid.

Other secretory parameters

Most proteins are synthesized and secreted into the perfusion fluid rather than into bile (Kloppel *et al.*, 1986). However, some proteins, such as BSA and RSA, can be transported from the sinusoid into bile via two pathways (Kloppel *et al.*, 1986). Under normal circumstances, paracellular sieving makes a small contribution after 5 min (Lowe *et al.*, 1984; Kloppel *et al.*, 1986); the major route, however, is transcellular, involving microtubule-mediated transport, and a protein will appear approx. 20 min after it has been presented to the liver via the perfusion fluid. Triacylglycerols are also secreted by the liver via the sinusoidal pole, and this secretion is inhibited by microtubule-disrupting agents such as colchicine (Le Marchand *et al.*, 1973; Orci *et al.*, 1973).

Colchicine causes a general disruption of microtubule-mediated transport (Azhar *et al.*, 1985; Rahman & Coleman, 1987; Coleman *et al.*, 1988). If cAMP can cause a similar disruption, then secretion of RSA, BSA and triacylglycerols would be inhibited, whereas bile acid output, however, would not be expected to be affected, since bile acids are transported as discrete molecules rather than as vesicles (Rahman & Coleman, 1987). In the present series of experiments, a depression in plasma or biliary protein outputs was not observed after treatment with either forskolin or the cAMP analogues, suggesting that there is not a general inhibition of vesicular secretion. The inhibition thus appears to be localized to the secretion of biliary lipids. Such selective inhibition implies that cAMP is not simply disrupting the microtubular system, but has a more local effect along the pathway of lipid delivery into bile.

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