Bile salts stimulate mucin secretion by cultured dog gallbladder epithelial cells independent of their detergent effect

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1. Bile salts stimulate mucin secretion by the gallbladder epithelium. We have investigated whether this stimulatory effect is due to a detergent effect of bile salts. 2. The bile salts taurocholic acid (TC) and tauroursodeoxycholic acid (TUDC) and the detergents Triton X-100 (12.5–400 μ M) and Tween-20 (0.1–3.2 mM) were applied to monolayers of cultured dog gallbladder epithelial cells. Mucin secretion was studied by measuring the secretion of [³H]*N*-acetyl-D-glucosamine-labelled glycoproteins. We also attempted to alter the fluidity of the apical membrane of the cells through extraction of cholesterol with β -cyclodextrin (2.5–15 mM). The effect on TUDC-induced mucin secretion was studied. Cell viability was assessed by measuring lactate dehydrogenase (LDH) leakage or ⁵¹Cr release. 3. In contrast with the bile salts, the detergents were not able to cause an increase in

mucin secretion without causing concomitant cell lysis. Concentrations of detergent that increased mucin release (> 100 μ M Triton X-100, > 0.8 mM Tween-20), caused increased LDH release. Incubation with β -cyclodextrin resulted in effective extraction of cholesterol without causing an increase in ⁵¹Cr release. However, no effect of the presumed altered membrane fluidity on TUDC (10 mM)-induced mucin secretion was observed. 4. The stimulatory effect of bile salts on mucin secretion by gallbladder epithelial cells is not affected by the fluidity of the apical membrane of the cells and also cannot be mimicked by other detergents. We conclude that the ability of bile salts to cause mucin secretion by the gallbladder epithelium is not determined by their detergent properties.

INTRODUCTION

Mucin is the major secretory product of the gallbladder epithelium. Mucin aggregates in aqueous solution to form an extended polymer that is responsible for the viscoelastic and gel-forming properties of mucus. The mucus gel may serve as a mechanism protecting the gallbladder epithelium against high concentrations of detergent bile acids. However, little is known about the mechanisms regulating its secretion by the gallbladder epithelium. We have reported the successful long-term culturing and passaging of normal, well-differentiated gallbladder epithelial cells from the dog [1]; these cells form electrically leakproof monolayers and synthesize protein and mucus glycoprotein. We have used these cultured DGBEC to study mucin secretion by the gallbladder epithelium. We demonstrated that mucin secretion by these gallbladder epithelial cells could be stimulated by several secretagogues that caused an increase in intracellular cAMP, such as prostaglandin E₂, prostaglandin E₁, vasoactive intestinal peptide, adrenaline (epinephrine) and isoproterenol [2]. In a more recent study we have demonstrated that bile salts can act as intraluminal secretagogues of mucin secretion. Bile salts were found to cause a dose-dependent increase in mucin secretion by the gallbladder epithelium. Hydrophobic bile salts were shown to be more potent stimulators of mucin secretion than hydrophilic bile salts. Moreover, bile salts caused mucin hypersecretion at concentrations below their critical micellar concentration [3]. Therefore, we hypothesized that it is not the detergent effect of bile salt micelles that are operative in bile-salt-cell-membrane interactions. We speculated that it may rather depend on the presence of bile salt monomers, or simple micelles, inserting themselves in the lipid bilayer. However, the exact mechanism by which bile salts interact with the gallbladder epithelium and are

able to stimulate mucin secretion is not yet clear. Subsequent studies showed that multiple intracellular second-messenger pathways are involved in the regulation of mucin secretion by the gallbladder epithelium. In addition to an increase in intracellular cAMP, activation of protein kinase C was also found to cause mucin hypersecretion by dog gallbladder epithelial cells. However, none of these intracellular signalling pathways were found to be involved in bile-salt-induced mucin secretion [4]. In the present study we have investigated whether the detergent properties of bile salts determine the secretory response of the gallbladder epithelium to bile acids.

Part of this research has been presented at the Annual Meeting of the American Gastroenterological Association held in Washington, DC, U.S.A. on May 11–14, 1997, and has been published in abstract form [4a].

MATERIALS AND METHODS

Chemicals and reagents

Vitrogen was purchased from Celtrix Laboratories (Palo Alto, CA, U.S.A.). Tissue-culture plates were from Falcon (Lincoln Park, NJ, U.S.A.), and Transwell inserts (24.5 mm diameter, 3.0 μ m pore size) were obtained from Costar (Cambridge, MA, U.S.A.). [³H]*N*-Acetyl-D-glucosamine and Na₂⁵¹CrO₄ were from ICN Biomedicals (Irvine, CA, U.S.A.). [¹⁴C]Acetic acid was obtained from Amersham Corporation (Arlington Heights, IL, U.S.A.). Tauroursodeoxycholic acid (TUDC) and taurocholic acid (TC) were purchased from Calbiochem (La Jolla, CA, U.S.A.), and Tween-20 was from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Filters with a pore size of 0.45 μ m were

Abbreviations used: DGBEC, dog gallbladder epithelial cells; LDH, lactate dehydrogenase; PTA, phosphotungstic acid; TC, taurocholic acid; Cl₃Ac, trichloroacetic acid; TUDC, tauroursodeoxycholic acid; FCS, foetal-calf serum.

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obtained from Gelman Sciences (Ann Arbor, MI, U.S.A.). Triton X-100, β -cyclodextrin, and other chemicals and cell-culture media were purchased from Sigma (St. Louis, MO, U.S.A.), except where noted.

Isolation and culture of DGBEC

Gallbladder epithelial cells were isolated from dog gallbladder as previously described [1] by trypsin treatment. Stock cultures were grown on 60-mm-diameter Petri dishes coated with 1 ml of Vitrogen gel (1:1 mixture of Vitrogen and media) in Eagle's minimum essential medium, supplemented with 10% (v/v) foetal-calf serum (FCS), 2 mM L-glutamine, 20 mM Hepes, 100 i.u./ml penicillin, and 100 μ g/ml streptomycin. Medium was changed twice a week, and the cells were maintained in a 37 °C incubator with 5% CO₂. The cells were passaged when confluent (every 10–14 days), using trypsin (2.5 g/l) and EDTA (1 g/l) treatment.

Mucin secretion assay

Mucin assays were performed as described by Kuver et al. [2] with slight modifications. DGBEC were grown to confluence on Transwell inserts without Vitrogen in six-well tissue-culture plates. The cells were labelled overnight (16–24 h) with 2 μ Ci/well of [³H]N-acetyl-D-glucosamine in medium containing 10% FCS. The [³H]N-Acetyl-D-glucosamine was added to the lower compartment of the wells, exposing only the basolateral side of the cells to the label. After labelling, the cells were washed with sterile PBS, pH 7.4, for 30 min, followed by washing for another 30 min with serum-free medium to remove unincorporated label. Next, serum-free medium or solutions of different activators in serum-free medium were added to the apical side of the cells. Serum-free medium was added to the basolateral side of the wells and the plates were returned to the incubator. After a 4 h incubation, 1 ml of apical medium was harvested from each Transwell and spun at 500 g for 10 min to pellet released cells. A 0.5 ml portion of the supernatant was then mixed with 8 ml of 10% (w/v) trichloroacetic acid/1% (w/v) phosphotungstic acid (Cl₃Ac/PTA), vortex-mixed and incubated overnight at 4 °C. The cells in the Transwells were washed once with PBS and harvested with trypsin/EDTA. After collection they were again washed with PBS and spun down at 500 g for 10 min. Part of the resulting cell pellet was used for protein determination, using the bicinchoninic acid protein assay described by Smith et al. [5]. The remaining cells were treated with Cl₃Ac/PTA in a manner similar to the processing of the medium samples. After precipitation overnight the samples were spun at 1500 g for 15 min, and the resulting protein pellet was washed twice, first with 5 ml of Cl₃Ac/PTA, then with 2 ml of 90% (v/v) ethanol. Finally the pellets were dissolved in 0.5 ml of water and counted for radioactivity in 10 ml of scintillation fluid. Results were expressed as percentages of control (c.p.m. per mg of cell protein). Values are means $\pm$ S.E.M. for triplicate wells.

Effect of detergents on mucin secretion

The detergents Triton X-100 and Tween-20 were dissolved directly in serum-free media and were added to the DGBEC in concentrations ranging from 12.5 to 400 μ M and from 0.1 to 3.2 mM respectively. All solutions were sterile-filtered before use and were only added to the apical side of the cells. Cells were exposed to the detergents for 4 h, and mucin secretion was then measured as described above.

Effect of β -cyclodextrin on mucin secretion

β -Cyclodextrin was directly dissolved in serum-free medium in concentrations ranging from 2.5 to 15 mM. Solutions were sterile-filtered and added to the apical side of the DGBEC. After a 2 h preincubation, the cells were washed with PBS and serum-free medium for 30 min. Next the cells were incubated with either serum-free medium or solutions of 10 mM of TUDC in serum-free medium for 4 h. The bile salt was only added to the apical compartments of the Transwells. Mucin secretion was then measured as described above.

Measurement of cholesterol efflux

DGBEC were grown to confluence on Transwell inserts and incubated for 24 h with 0.5 μ Ci/well of [¹⁴C]acetic acid in media containing 10% FCS. Cells containing endogenously synthesized [¹⁴C]cholesterol were then washed with PBS and serum-free medium and subsequently incubated for 2 h with solutions of β -cyclodextrin (2.5–15 mM) in serum-free medium as described above. The efflux of radioactivity into the culture media, as well as the radioactivity in the cells, was then measured to demonstrate the extraction of [¹⁴C]cholesterol from the apical cell membrane by β -cyclodextrin. The [¹⁴C]cholesterol efflux per well (%) was calculated as follows:

$$\text{Efflux} = \left(\frac{^{14}\text{C in medium}}{^{14}\text{C in medium} + \text{cells}} \right) \times 100$$

Values are means $\pm$ S.E.M. for triplicate wells.

Measurement of cell viability

Cell viability was assessed by two different methods. In the experiments involving bile salts and detergents, the leakage of the endogenous enzyme lactate dehydrogenase (LDH) into the culture medium was measured, using the method of Amador et al. [6]. Results are expressed in units of LDH activity/mg of cell protein. Values are means $\pm$ S.E.M. for triplicate wells.

Addition of β -cyclodextrin to the culture medium of the cells interfered with the measurement of LDH activity in the medium. Therefore, in the experiments using β -cyclodextrin, cell viability was measured by means of a ⁵¹Cr-release assay. The cells were labelled overnight (16–24 h) with 2 μ Ci/well of Na₂⁵¹CrO₄ in medium containing 10% FCS. The label was added to the basolateral compartment of the wells. Subsequently, the ⁵¹Cr release into the culture media could then be measured, as well as the ⁵¹Cr content of the cells after harvesting. The ⁵¹Cr release (%) was calculated as follows:

$$^{51}\text{Cr release} = \left(\frac{^{51}\text{Cr in medium}}{^{51}\text{Cr in medium} + \text{cells}} \right) \times 100$$

The ⁵¹Cr release into the culture media was subsequently expressed as a percentage of the control. Values are means $\pm$ S.E.M. for triplicate wells.

RESULTS

Effect of TUDC and TC on mucin secretion by cultured DGBEC

We have previously demonstrated that bile salts regulate mucin secretion by cultured DGBEC [3]. We demonstrated that taurine-

Table 1 Effect of the bile acid TC on mucin secretion by cultured DGBEC

DGBEC were grown to confluence on Transwell inserts and incubated with either serum-free medium or solutions of TC in serum-free medium for 4 h. Mucin secretion and LDH release into the culture media were then measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for triplicate wells (* $P < 0.05$; ** $P < 0.01$ compared with control). The assay was repeated twice with similar results.

[TC] (mM)	Mucin secretion (% of control)	LDH release (units/mg of protein)
0	100 $\pm$ 8	1.2 $\pm$ 0.3
5	188 $\pm$ 13*	1.0 $\pm$ 0.1
10	218 $\pm$ 6**	0.9 $\pm$ 0
15	309 $\pm$ 22**	1.9 $\pm$ 0
20	646 $\pm$ 65**	8.2 $\pm$ 0.2**

conjugated bile salts cause a dose-dependent stimulation of mucin secretion by these cells. Hydrophobic bile salts were shown to be more potent stimulators of mucin secretion than hydrophilic bile salts. TUDC, a hydrophilic bile salt, was found to be the bile salt of choice to characterize the effects of bile salts on mucin secretion by gallbladder epithelial cells. When added to the DGBEC in concentrations ranging from 2.5 to 40 mM, a dose-dependent increase in mucin secretion was observed [3]. No toxicity, as indicated by increased LDH release from the cells, was found with any of the concentrations of TUDC tested. At a concentration of 40 mM of TUDC, a maximum stimulation of mucin secretion was obtained that was approx. 350% of control secretion after 4 h of incubation. An approx. 2-fold stimulation of mucin secretion could be obtained by the addition of 10 mM of TUDC (results not shown) [3]. TC, a more hydrophobic bile acid, was also found to be able to increase mucin secretion without causing concomitant LDH release from the cells. When added to the DGBEC at a concentration of 15 mM, mucin secretion increased to 309 $\pm$ 22% of control (100 $\pm$ 8%, $P < 0.01$), whereas LDH release remained unaltered. However, at a concentration of 20 mM the increase in mucin secretion became disproportionate (646 $\pm$ 65%) and was accompanied by an increase in LDH release from the cells, indicating a loss of structural integrity of the cells (Table 1) [3].

Effect of detergents on mucin secretion by cultured DGBEC

Next we investigated whether the stimulatory effect of bile salts on mucin secretion by DGBEC could be mimicked by other detergents. Therefore we studied the effect of two non-ionic detergents, Triton X-100 and Tween-20, on mucin secretion. DGBEC were incubated with either serum-free medium or solutions of Triton X-100 (12.5–400 μ M) or Tween-20 (0.1–3.2 mM) in serum-free medium for 4 h. Mucin secretion and LDH release into the culture medium were then measured as described in the Materials and methods section. The results are shown in Table 2. In contrast with bile salts, neither detergent was able to cause mucin release from the cells without causing a concomitant LDH release from the cells. As opposed to the experiments described above for TUDC and TC, increases in mucin secretion were always accompanied by increases in LDH release from the cells. At a concentration of 200 μ M, Triton X-100 increased mucin release by the cells to 291 $\pm$ 39% of control (100 $\pm$ 6%, $P < 0.05$). However, the LDH activity in the culture medium also increased from 20 $\pm$ 4 units/mg of protein for the control incubation, to 243 $\pm$ 32 units/mg of protein ($P < 0.01$) after incubation with 200 μ M Triton X-100. Similarly, 1.6 mM

Table 2 Effect of the detergents Triton X-100 (a) and Tween-20 (b) on mucin secretion by cultured DGBEC

DGBEC were grown to confluence on Transwell inserts and incubated with either serum-free medium or solutions of Triton X-100 or Tween-20 in serum-free medium for 4 h. Mucin secretion and LDH release into the culture media were then measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for triplicate wells (* $P < 0.05$; ** $P < 0.01$ compared with control). The assay was repeated twice with similar results.

(a)		
[Triton X-100] (μ M)	Mucin secretion (% of control)	LDH activity (units/mg of protein)
0	100 $\pm$ 6	20 $\pm$ 4
12.5	88 $\pm$ 6	8 $\pm$ 3*
25	136 $\pm$ 46	16 $\pm$ 1
50	130 $\pm$ 17	13 $\pm$ 2
100	113 $\pm$ 16	20 $\pm$ 3
200	291 $\pm$ 39*	243 $\pm$ 32**
400	284 $\pm$ 52*	823 $\pm$ 22**
(b)		
[Tween-20] (mM)	Mucin secretion (% of control)	LDH activity (units/mg of protein)
0	100 $\pm$ 21	9 $\pm$ 3
0.1	85 $\pm$ 19	13 $\pm$ 3
0.2	81 $\pm$ 5	22 $\pm$ 6*
0.4	92 $\pm$ 6	33 $\pm$ 12
0.8	139 $\pm$ 4	157 $\pm$ 30*
1.6	289 $\pm$ 27**	551 $\pm$ 43**
3.2	672 $\pm$ 61**	824 $\pm$ 61**

Tween-20 significantly increased mucin secretion by the cells to 289 $\pm$ 27% of control (100 $\pm$ 21%, $P < 0.01$), whereas the LDH release increased from 9 $\pm$ 3 units/mg of protein to 551 $\pm$ 30 units/mg of protein ($P < 0.01$). At 0.8 mM, Tween-20 did not significantly increase mucin release by the cells, but LDH release was already found to have increased to 157 $\pm$ 30 units/mg of protein ($P < 0.05$).

Extraction of cholesterol from DGBEC with β -cyclodextrin

In a second set of experiments we investigated whether the fluidity of the apical membrane of the DGBEC determines the secretory response of the cells to bile salts. We tried to alter the fluidity of the apical membrane of the gallbladder cells by extraction of cholesterol. β -Cyclodextrin, which enhances the solubility of non-polar substances by incorporating them into its hydrophobic cavity and forming inclusion complexes, has previously been shown to selectively extract cholesterol, in preference to other membrane lipids, from the plasma membrane [7]. Cyclodextrins have also been used to extract cholesterol from fibroblasts and hepatoma cells [8], erythrocytes [7] as well as myometrial plasma membranes [9]. To demonstrate that indeed β -cyclodextrin is able to extract cholesterol from the membranes of the dog cells, we labelled the cells with endogenously synthesized [^{14} C]cholesterol. Experiments by Hayashi and Lee [10] showed that when these DGBEC were incubated with [^{14} C]acetic acid, they synthesized cholesterol *de novo* from this radioactive precursor. Moreover, the radioactive cholesterol was found to be incorporated into the membranes of the cells. Therefore DGBEC were labelled for 24 h with [^{14} C]acetic acid, washed, and subsequently incubated for 2 h with various doses of β -cyclodextrin (2.5–15 mM). [^{14} C]Cholesterol efflux into the culture media of

Table 3 β -Cyclodextrin-mediated [^{14}C]cholesterol efflux from cultured DGBEC

DGBEC were grown to confluence on Transwell inserts and incubated with either serum-free medium or solutions of β -cyclodextrin (2.5–15 mM) in serum-free media. After 2 h of incubation, [^{14}C]cholesterol efflux and ^{51}Cr release into the culture medium were then measured as described in the Materials and methods section (Table 2a). [^{14}C]cholesterol efflux was also measured after incubation with serum-free medium or 10 mM β -cyclodextrin for 1, 2, and 4 h (Table 2b). Values are means $\pm$ S.E.M. for triplicate wells (** $P < 0.01$; *** $P \leq 0.001$ compared with control; $^{n.s.}$, not significant compared with control). The assay was repeated twice with similar results.

(a)			
[β -Cyclodextrin] (mM)	[^{14}C]cholesterol efflux (% of total)	^{51}Cr release (% of control)	
0	0.9 $\pm$ 0.2	100 $\pm$ 20	
2.5	5.4 $\pm$ 0.4***	116 $\pm$ 8 $^{n.s.}$	
5	7.7 $\pm$ 0.4***	93 $\pm$ 27 $^{n.s.}$	
10	9.6 $\pm$ 0.6***	125 $\pm$ 10 $^{n.s.}$	
15	9.5 $\pm$ 0.5***	99 $\pm$ 1 $^{n.s.}$	

(b)			
[β -Cyclodextrin] (mM)	Time (h)	[^{14}C]cholesterol efflux (% of total)	^{51}Cr release (% of control)
0	1	1.6 $\pm$ 0.1	100 $\pm$ 5
0	2	1.6 $\pm$ 0.2	103 $\pm$ 19
0	4	1.8 $\pm$ 0.1	94 $\pm$ 11
10	1	6.6 $\pm$ 0.7**	118 $\pm$ 22 $^{n.s.}$
10	2	8.5 $\pm$ 0.7**	103 $\pm$ 7 $^{n.s.}$
10	4	10.4 $\pm$ 0.8**	116 $\pm$ 18 $^{n.s.}$

the cells was then measured as described in the Materials and methods section. As can be concluded from Table 3(a), incubation of the cells in serum-free medium caused almost no discernible [^{14}C]cholesterol efflux from the cells into the culture media. Only 0.9 $\pm$ 0.2 % of the total radioactivity was found in the culture media after the 2 h incubation. In contrast, incubation with increasing doses of β -cyclodextrin resulted in a dose-dependent efflux of [^{14}C]cholesterol from the cells. With 10 mM cyclodextrin a maximum [^{14}C]cholesterol efflux was observed of 9.6 $\pm$ 0.5 % of the total radioactivity. Exposing the cells to 15 mM of β -cyclodextrin did not result in a further increase in [^{14}C]cholesterol efflux from the cells (9.5 $\pm$ 0.5 % of total radioactivity). The [^{14}C]cholesterol efflux was also found to be time-dependent (Table 3b). Cells were incubated with serum-free medium or 10 mM of β -cyclodextrin for 1, 2 and 4 h and [^{14}C]cholesterol efflux was again measured. In the control incubation the [^{14}C]cholesterol efflux increased from 1.6 $\pm$ 0.1 % (1 h) to 1.8 $\pm$ 0.1 % (4 h) of the total radioactivity, whereas 10 mM cyclodextrin caused the [^{14}C]cholesterol efflux to increase from 6.6 $\pm$ 0.7 % (1 h) to 10.4 $\pm$ 0.8 % (4 h) of the total radioactivity. These experiments therefore demonstrate that concentrations of β -cyclodextrin up to 15 mM are indeed effective in extracting cholesterol from the apical membrane of the epithelial cells. Extraction of cholesterol from the epithelial cells by incubation with β -cyclodextrin apparently had no effect on the viability of the cells. Membrane integrity of the cells was assessed by means of a ^{51}Cr -release assay. Cells were labelled with $\text{Na}_2^{51}\text{CrO}_4$ and subsequently incubated with 2.5–15 mM β -cyclodextrin for 2 h or with 10 mM β -cyclodextrin for 1, 2 and 4 h. ^{51}Cr release into the culture media was then measured as described in the Materials and methods section. No significant changes ($P > 0.05$) in ^{51}Cr

Table 4 Effect of β -cyclodextrin on constitutive (a) and bile-salt-induced (b) mucin secretion by cultured DGBEC

DGBEC were grown to confluence on Transwell inserts and incubated with either serum-free medium or solutions of β -cyclodextrin in serum-free medium for 2 h. Cells were then washed and subsequently incubated with either serum-free medium or solutions of 10 mM of TUDC in serum-free medium for 4 h. Mucin secretion and ^{51}Cr release into the culture media were then measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for triplicate wells (* $P < 0.05$; ** $P < 0.01$ compared with control; $^{n.s.}$, not significant when compared with 10 mM TUDC). The assay was repeated twice with similar results.

(a)			
[β -Cyclodextrin] (mM)	Constitutive mucin secretion (% of control)	^{51}Cr release (% of control)	
0	100 $\pm$ 5	100 $\pm$ 7	
2.5	99 $\pm$ 3	87 $\pm$ 12	
5	94 $\pm$ 27	79 $\pm$ 7*	
10	71 $\pm$ 3*	84 $\pm$ 6*	
15	103 $\pm$ 6	114 $\pm$ 12	

(b)			
[β -Cyclodextrin] (mM)	+ [TUDC] (mM)	Bile-salt-induced mucin secretion (% of control)	^{51}Cr release (% of control)
0	0	100 $\pm$ 5	100 $\pm$ 7
0	10	187 $\pm$ 20*	69 $\pm$ 1*
2.5	10	174 $\pm$ 12** $^{n.s.}$	59 $\pm$ 9**
5	10	159 $\pm$ 16** $^{n.s.}$	96 $\pm$ 8
10	10	159 $\pm$ 23** $^{n.s.}$	90 $\pm$ 12
15	10	186 $\pm$ 17** $^{n.s.}$	120 $\pm$ 18

release were observed after the incubations with β -cyclodextrin when compared with the control incubations (Table 3).

Effect of β -cyclodextrin on mucin secretion by DGBEC

Having demonstrated that incubation of the DGBEC with β -cyclodextrin results in a depletion of cholesterol from the apical membrane of the cells, we next investigated the effect of β -cyclodextrin on mucin secretion by the cells. We studied whether incubation with β -cyclodextrin caused any changes in either constitutive or bile-salt-induced mucin secretion by the cells or affected cell viability. DGBEC were grown to confluence and labelled overnight with both [^3H]N-acetyl-D-glucosamine and $\text{Na}_2^{51}\text{CrO}_4$. Subsequently the cells were incubated for 2 h in either serum-free medium or solutions of β -cyclodextrin (2.5–15 mM) in serum-free medium. Next the cells were washed and incubated for another 4 h in either serum-free medium or 10 mM TUDC in serum-free medium, and mucin secretion was then measured as described in the Materials and methods section. Cell viability was assessed by means of the ^{51}Cr -release assay after the 4 h incubation with serum-free medium or bile-salt solution. The results are shown in Table 4. Constitutive mucin secretion by the epithelial cells appeared not to be affected by the treatment with β -cyclodextrin; only with 10 mM β -cyclodextrin was a small decrease in mucin secretion (71 $\pm$ 3 % of control 100 $\pm$ 5 %, $P < 0.05$) observed. Incubation of the epithelial cells with 10 mM TUDC for 4 h increased mucin secretion to 187 $\pm$ 20 % of control (100 $\pm$ 5 %). After preincubation for 2 h with different concentrations of β -cyclodextrin and subsequent treatment with 10 mM TUDC, mucin secretion remained elevated when compared with control ($P < 0.05$), but was not significantly different ($P > 0.05$) from mucin secretion after treatment with TUDC alone. Therefore, bile-salt-induced mucin secretion also seemed not to

be affected by alteration of the fluidity of the apical membrane. However, some decrease in ^{51}Cr release was observed after treatment with β -cyclodextrin (Table 4).

DISCUSSION

In the present study we used normal cultured DGBEC to investigate further the stimulatory effect of bile salts on mucin secretion by the gallbladder. By studying the interaction of detergents with the apical membrane of the DGBEC we have demonstrated that the stimulatory effect of bile acids on mucin secretion cannot be mimicked by other detergents. Non-ionic detergents like Triton X-100 and Tween-20 were not able to cause an increase in mucin secretion without causing concomitant cell lysis. By contrast, bile acids were found to be able to increase mucin secretion without exerting a cytotoxic effect on the cells. Also, bile salts were found to be potent stimulators of mucin secretion at concentrations well below their critical micellar concentration [3]. These findings therefore seem to point to a specific effect of the bile salts on the epithelial cells, rather than a simple detergent effect. The possibility that bile salts exert their stimulatory effect on mucin secretion by means of their membrane-solubilizing capacity, also appeared to be contradicted by other results. We used the cholesterol-solubilizing compound β -cyclodextrin to effectively extract cholesterol from the apical membrane of the DGBEC, which should alter the fluidity of a lipid bilayer structure such as the cell membrane. However, incubating the gallbladder cells with β -cyclodextrin did not result in an enhanced, or decreased, secretory response of the cells. Neither constitutive nor bile-salt-induced mucin secretion by DGBEC seemed to be affected by the lipid composition of the apical membrane of the cells. Therefore we conclude that the ability of bile acids to solubilize membrane lipids, and thereby interact with biological membranes, does not determine their effect on mucin secretion by the gallbladder epithelium. The stimulatory effect of bile acids on mucin secretion appears to be a specific physiological effect rather than a non-specific physical effect.

There are numerous reports on the detergent properties of bile salts and their interactions with biological membranes [11–13], as well as their toxic effects on cells [14]. However, more recently there have been an increasing number of studies showing that bile salts, besides being harmful to cells, can also play important physiological roles. For example, bile salts have been shown to affect protein kinase C activity. TUDC activates protein kinase C in isolated rat hepatocytes [15]. Bile acids have been reported to repress cholesterol 7 α -hydroxylase transcription, and this effect is mediated through protein kinase C [16]. Moreover, bile acids were found to be potent and selective activators of protein kinase C isoenzymes from colorectal mucosa [17]. Also through an activation of protein kinase C, bile acids were found to inhibit hormone-induced cAMP production [18,19]. Our previous studies on the mechanism of bile-salt-induced mucin secretion have not yielded evidence to support the theory that bile acids exert their effect on DGBEC through an activation of protein kinase C or by causing an increase in either intracellular cAMP or Ca^{2+} in these cells. However, activation of protein kinase C did cause an increase in mucin secretion in these cells [4]. Recently, a study by Dray-Charier et al. [20] showed that, in cultured human gallbladder epithelial cells, mucin secretion is predominantly regulated by Ca^{2+} -dependent pathways implicating Ca^{2+} /calmodulin kinase II and protein kinase C. Ionomycin and PMA have been shown to stimulate mucin secretion, and the effects of these two agents were mediated by a Ca^{2+} -dependent pathway. Mucin secretion was also found to be

stimulated by extracellular ATP via P_{2u} receptors, cytosolic Ca^{2+} increase and protein kinase C. Moreover, the bile salt taurochenodeoxycholic acid was also shown to cause an increase in mucin secretion by increasing cytosolic Ca^{2+} and through activation of Ca^{2+} /calmodulin-dependent protein kinase II. Bile-salt-induced mucin secretion was shown to be inhibited by intracellular Ca^{2+} chelators. This is the first evidence that bile acids can stimulate mucin secretion via a direct activation of an intracellular signalling pathway.

Bile salts might act as Ca^{2+} ionophores by inserting themselves into the cell membrane or by actually entering the cells and causing a release of Ca^{2+} from intracellular stores, thereby bypassing the regulatory systems that maintain cellular Ca^{2+} homeostasis [21,22]. Studies by Zimniak et al. [21] demonstrated that taurine-conjugated bile acids were able to translocate Ca^{2+} from one aqueous phase, across a phospholipid bilayer, into a second aqueous phase. Moreover, the bile acids were able to do so at micromolar concentrations without causing membrane lysis. Hydrophobic bile acids were found to be more effective than hydrophilic bile acids. Thus, they concluded, bile acids can be regarded as true Ca^{2+} ionophores. This would imply that bile salts, if present at sufficient concentrations in body fluids, could exert their effects on a variety of cells by increasing intracellular Ca^{2+} concentrations. TUDC has been reported to deplete intracellular Ca^{2+} stores and mobilize extracellular Ca^{2+} [23,24]. Tauro lithocholic acid and taurodeoxycholic acid have also been demonstrated to cause an increase in free cytosolic Ca^{2+} concentration by mobilizing Ca^{2+} from the endoplasmic reticulum [25–27]. Another potential mechanism by which bile acids might interact with cell membranes is via the ileal bile-acid transporter. Recently it was demonstrated that the ileal Na^{+} -dependent bile-acid transporter is also present on the apical membrane of rat cholangiocytes and is functional in promoting apical uptake and transcellular transport of bile salt [28,29]. Perhaps the 'ileal' bile-acid transporter is present on the apical membrane of all biliary epithelial cells, including those of the gallbladder, allowing entry of non-micellar bile salts into the cell, thereby triggering a second-messenger response.

Bile salts might play an important physiological role throughout the body, by acting as signalling molecules. In this way, bile salts might be actively involved in the regulation of mucin secretion by gallbladder epithelial cells. The gallbladder epithelium would then be able to respond to a changing bile salt composition of bile and thus maintain an adequate mucus layer to protect the gallbladder against the detergent action of bile.

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