Regulation of apolipoprotein B secretion by biliary lipids in CaCo-2 cells

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Abstract The regulation of apoB synthesis and secretion by lipids present within bile was investigated in CaCo-2 cells grown on semipermeable filters. Bile acids decreased the basolateral secretion of immunoreactive apoB. Taurocholic acid decreased the secretion of newly synthesized apoB by increasing the rate of apoB degradation, but had no effect on the synthesis and secretion of apoA-I or trichloroacetic acid-precipitable proteins. The calcium ionophore, A23187, decreased apoB secretion similar to that observed for taurocholate. The addition of the ionophore and taurocholate together did not cause a further decrease in apoB secretion. Cholesterol or its hydroxylated derivative, 25-hydroxycholesterol, did not alter secretion of immunoreactive or newly synthesized apoB. Phosphatidylcholine increased apoB synthesis and secretion without affecting the synthesis or secretion of apoA-I. Phosphatidylcholine also reversed the effect of A23187 on apoB secretion. When phosphatidylcholine was added to the basolateral medium, apoB secretion was not altered. ApoB secretion was not increased by phospholipids of other classes. Dioleoylphosphatidylcholine increased apoB secretion, whereas dipalmitoylphosphatidylcholine did not. Fatty acid-labeled phosphatidylcholine was not hydrolyzed in the apical medium. Only 2% of the added phosphatidylcholine was cell-associated, and of this, 80% of the label remained as phosphatidylcholine with most of the remainder in triacylglycerols, fatty acids, and phosphatidylethanolamine. The results suggest that bile acids decrease apoB secretion by increasing its rate of degradation. This effect may be related to their ionophoric property. Cholesterol flux does not regulate apoB secretion. Phosphatidylcholine, independent of triacylglycerol flux and independent of its hydrolysis, increases the secretion of apoB by increasing apoB synthesis. Luminal phosphatidylcholine may play a role in apoB secretion in the intestine.—**Field, F. J., E. Born, H. Chen, S. Murthy, and S. N. Mathur.** Regulation of apolipoprotein B secretion by biliary lipids in CaCo-2 cells. *J. Lipid Res.* 1994. **35:** 749–762.

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Dietary lipids are transported by the intestine in large triacylglycerol-rich lipoproteins. An integral structural component of these lipoproteins is apolipoprotein (apo)B which is essential for the normal assembly, secretion, and catabolism of lipoproteins derived from the intestine. Through a novel post-transcriptional editing mechanism occurring in apoB mRNA of intestine, apoB is secreted as apoB-48, the amino-terminal half of apoB-100, an apolipoprotein synthesized and secreted exclusively by the liver (1, 2). Although the regulation of hepatic apoB synthesis and secretion has been extensively studied (3–8), there is a paucity of information on the regulation of apoB secretion by the intestine.

Studies designed to explore what factors might regulate the secretion of intestinal apoB could lead to further understanding of mechanisms involved in the absorption of luminal cholesterol and fat from the gut. Conflicting results were obtained from two earlier studies that investigated the effects of triacylglycerol flux on intestinal apoB secretion. In contrast to the results of Windmueller and Wu (9) who observed a direct relationship between apoB secretion and triacylglycerol flux, Davidson, Kollmer, and Glickman (10) found that the synthesis of intestinal apoB-48 was unrelated to the quantity and quality of triacylglycerol transported. However, in a chronic bile-diverted rat model, it was discovered that the reinfusion of either taurocholate or taurocholate and lysophosphatidylcholine into the duodenums of these animals caused a reexpression of apoB-48 synthesis (11). More recently, taking advantage of cell culture to investigate the regulation of apoB metabolism, different fatty acids were shown to regulate apoB secretion by CaCo-2 cells (12–15).

Because so little is known about the regulation of apoB synthesis and secretion by the intestine, the present investigation was performed in the human intestinal cell line, CaCo-2, to study the effects of biliary lipids on apoB secretion. Using cell culture, each lipid can be tested individually and its effect on apoB secretion can be determined without interference from the effects of other lipids. The results demonstrate that taurocholic acid and other bile acids decrease the secretion of newly synthesized and immunoreactive apoB. The influx of cholesterol

Abbreviations: apo, apolipoprotein; TCA, trichloroacetic acid; CMC, critical micellar concentration.

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does not alter apoB secretion. In contrast, incubation of cells with taurocholate and phosphatidylcholine significantly increases the synthesis and secretion of apoB. The results suggest that luminal phosphatidylcholine is important for the secretion of apoB by CaCo-2 cells.

METHODS AND MATERIALS

Trans-[^{35}S]methionine (1,100 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Phosphatidylcholine, L-dioleoyl(di-[1-^{14}C]oleoyl) (114 mCi/mmol) was from New England Nuclear (Boston, MA). Egg phosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, cholesterol, lysophosphatidylcholine, taurocholate, taurochenodeoxycholate, taurodeoxycholate, tauroursodeoxycholate, choline chloride, and fatty acid-poor bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). 25-Hydroxycholesterol was from Steraloids, (Wilton, NH). ApoB and apoB monoclonal antibody (clone No. 1607) (immunoglobulin G 26 fraction purified by column chromatography), and apoB sheep immunopurified polyclonal antibody conjugated to horseradish peroxidase were purchased from Biodesign International (Kennebunkport, ME). Rabbit polyclonal antibody (IgG fraction) specific for human apoB was from Calbiochem (San Diego, CA). Rabbit sera containing antibody to human apoA-I were graciously provided by Dr. Dennis Black, Department of Pediatrics, University of Chicago, Chicago, IL. Protein A bound to Sepharose was from Repli Gen (Cambridge, MA). TMB Microwell Peroxidase Substrate System was purchased from Kirkegaard and Perry Labs Inc. (Gaithersburg, MD). Ninety-six well Nunc-Immuno plates were from VWR Scientific (Batavia, IL). All other reagents were reagent grade.

Cell culture

CaCo-2 cells were grown in T-75 flasks as described previously (16). They were subcultured on polycarbonate micropore membranes (0.4 μm pore size) inserted in Transwells (Costar, Cambridge, MA). Unless otherwise stated, inserts of 6.5 mm diameter were used and cells were initially plated at a density of 0.2×10^5 cells per filter. Medium was changed every 2 days and the cells were used 14 days after plating.

Bile acid solutions

Bile acids were dissolved in a stock solution of 95% ethanol. Stock solutions of cholesterol, 25-hydroxycholesterol, fatty acids, and phospholipids were kept in chloroform under nitrogen. On the day of the experiment, appropriate amounts of the stock solutions were added together and dried under a stream of nitrogen. M199 (medium-199/Earle's Gibco, Grand Island, NY) containing 10 mM HEPES, pH 7.4, was added to make the correct concentrations of the lipids and vortexed until clear. The bile acid solutions were added to the apical medium unless otherwise specified. Multilamellar liposomes of phosphatidylcholine were prepared by drying appropriate amounts of phosphatidylcholine under a stream of nitrogen. M199 at 37°C was added and the mixture was vigorously vortexed.

Hydrolysis and/or uptake of [^{14}C]dioleoylphosphatidylcholine

Cells grown on semipermeable filters of 24.5 mm diameter were incubated for 18 h with 1 mM taurocholate and 500 μM phosphatidylcholine containing 0.2 μCi of dioleoylphosphatidylcholine/dish. In a total volume of 1.5 ml, the apical medium contained 750 nmol of phosphatidylcholine or 1500 nmol of fatty acid. To estimate the amount of nonspecific adsorption of the labeled phospholipid, some cells were pretreated for 1 h with a solution containing 37% formalin and M199 (1:1, vol/vol). These cells were washed twice with M199 to remove the formalin solution prior to adding the taurocholate and labeled phosphatidylcholine. After the overnight incubation, apical and basolateral media were collected and the cells were washed twice with 1 ml of 1 mM taurocholate. The washings were then combined with the apical medium. The cells were dissolved in 0.2 N NaOH and neutralized with 6 N HCl. The lipids from the media and cells were extracted in chloroform–methanol–water (final ratio of 1:1:0.9, vol/vol, pH < 3.0). Neutral lipids were separated by thin-layer chromatography using the solvent system of hexanes–diethylether–acetic acid 80:20:1 (vol/vol). Phospholipid separation was performed using the solvent system of chloroform–methanol–acetic acid–water–acetone 40:25:2:2:4 (vol/vol). Bands were

TABLE 1. Effect of bile acids on the secretion of apoB mass

Bile Acid	ApoB Mass
	ng/well
Control	256 ± 11
Taurocholate	
0.5 mM	252 ± 8
1.0 mM	167 ± 6[b]
5.0 mM	162 ± 10[b]
Taurochenodeoxycholate (1 mM)	159 ± 28[a]
Taurodeoxycholate (1 mM)	120 ± 16[b]
Tauroursodeoxycholate (1 mM)	194 ± 8[a]

Cells grown on semipermeable filters were incubated for 18 h with the respective bile acid added to the apical medium. The amount of apoB mass secreted into the basolateral medium was estimated by ELISA. The data represent the mean ± SE of at least three individual transwells assayed in triplicate.

[a] $P < 0.05$ vs. control.

[b] $P < 0.01$ vs. control.

localized by authentic standards and scraped from the plate and counted.

ApoB and apoA-I measurements

The estimation of apolipoprotein mass by ELISA, estimation of apolipoprotein synthesis and degradation by pulse-chase, and immunoprecipitation of apolipoproteins were performed exactly as previously described (15).

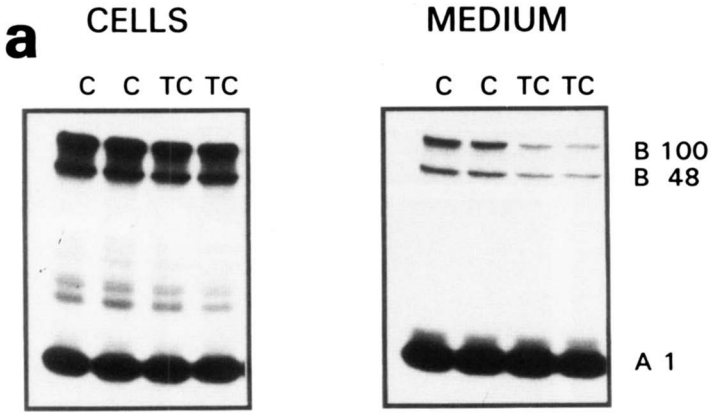
Cell viability and chemical analysis

The viability of cells was determined by measuring the release of cellular LDH as described (15) and by the release of formazan from tetrazolium salt (MTT assay) (17). No significant differences were observed among the treatments compared to controls. Protein was estimated by the method of Lowry et al. (18).

RESULTS

Bile acids

Because of the amphiphilic nature of bile acids and their recognized propensity to damage cells (19), taurocholic acid, the major bile acid of humans, was chosen to address the effects of bile acids on apoB secretion in this study. Except for tauroursodeoxycholic acid, taurocholic acid is the least toxic of the bile acids towards CaCo-2 cells (20). Cells were incubated for 18 h with 0.5, 1.0, or 5.0 mM taurocholate and the amount of apoB mass secreted into the basolateral medium was estimated (**Table 1**). The secretion of apoB mass was significantly decreased by 1 and 5 mM taurocholic acid. The inhibitory effect of 1 mM taurocholate on apoB mass secretion was very consistent but the degree of inhibition among



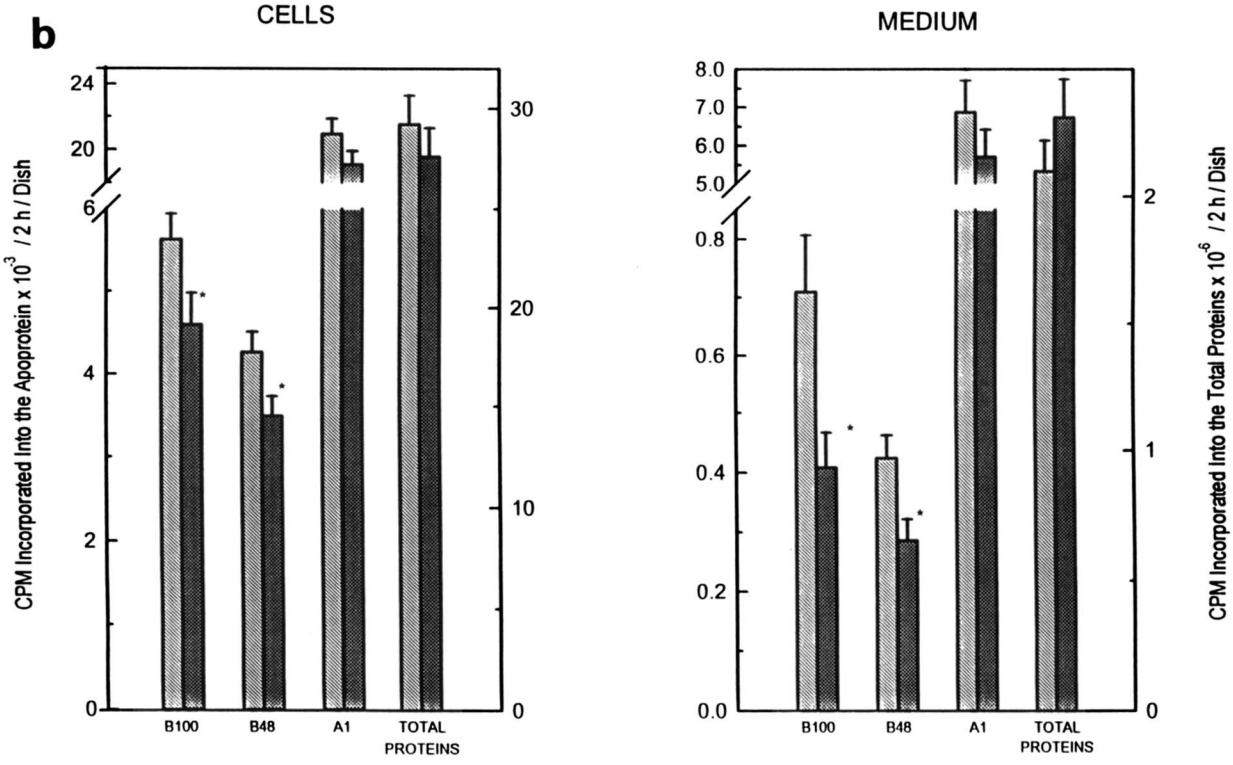


Fig. 1. Effect of taurocholate on the synthesis and secretion of apoB. Cells grown on semipermeable filters were incubated for 18 h with M199 (C) or M199 containing 1 mM taurocholate (TC) on the apical side. The basolateral medium contained M199 alone. After the incubation, the cells were pulsed for 2 h with 100 μCi of [^{35}S]methionine and the incorporation of label into apoB, apoA-I, and TCA-precipitable protein from cells and basolateral media was estimated after immunoprecipitation and polyacrylamide gel electrophoresis as described in Methods. a) This figure is a representative autoradiogram from one experiment. b) The data represent the mean ± SE of 22 transwells from four individual experiments; diagonal line, control; cross hatched, taurocholate; $^{*}P < 0.05$ vs. control.

many experiments varied from 20 to 50% (n > 50 dishes). The effect of taurocholate on apoB secretion was reversible. After an overnight incubation with 1 mM taurocholate, cells were allowed to recover by incubating them for 8 h in medium containing 20% fetal calf serum without the bile acid. The amount of apoB secreted after a second 18-h incubation with 1 mM oleic acid attached to albumin was similar to the amount secreted by control cells not initially exposed to taurocholate but treated in a similar manner (448 ng vs. 465 ng, respectively). At 1 mM concentrations, taurochenodeoxycholic, taurodeoxycholic, and tauroursodeoxycholic acids also decreased apoB mass secretion by 37%, 53%, and 25%, respectively.

The effect of taurocholate on the synthesis and secre-

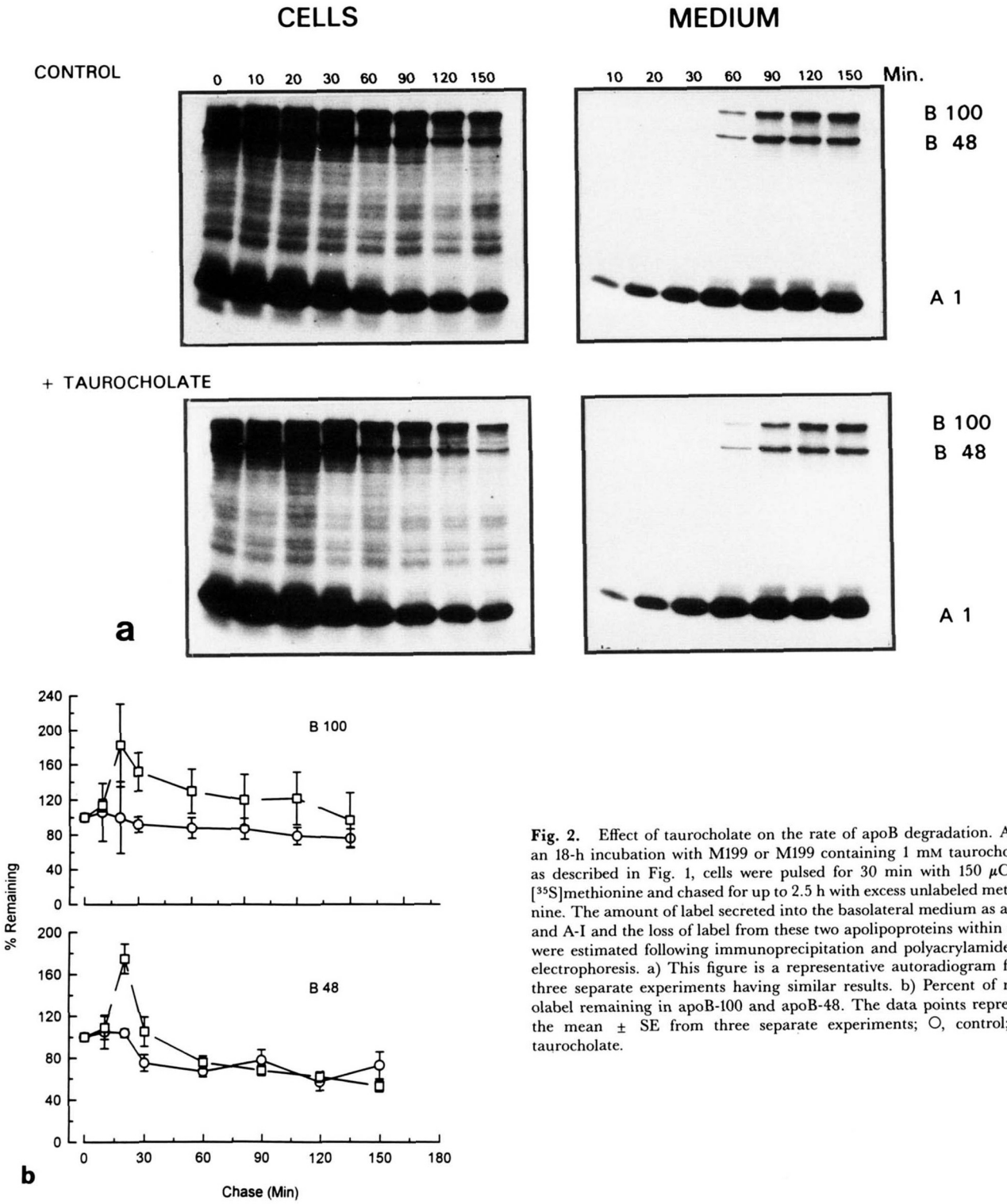


Fig. 2. Effect of taurocholate on the rate of apoB degradation. After an 18-h incubation with M199 or M199 containing 1 mM taurocholate as described in Fig. 1, cells were pulsed for 30 min with 150 μCi of [^{35}S]methionine and chased for up to 2.5 h with excess unlabeled methionine. The amount of label secreted into the basolateral medium as apoB and A-I and the loss of label from these two apolipoproteins within cells were estimated following immunoprecipitation and polyacrylamide gel electrophoresis. a) This figure is a representative autoradiogram from three separate experiments having similar results. b) Percent of radiolabel remaining in apoB-100 and apoB-48. The data points represent the mean ± SE from three separate experiments; ○, control; □, taurocholate.

tion of newly synthesized apoB was then addressed. Cells were incubated overnight with 1 mM taurocholate. The following day, the incorporation of [^{35}S]methionine into apoB within cells and that secreted into the basolateral medium was estimated. The incorporation of label into total TCA-precipitable protein and apoA-I was used as a control to study the specificity of the effect of the bile acid on apoB. **Fig. 1a** shows a representative autoradiogram after immunoprecipitation and separation of proteins by polyacrylamide gel electrophoresis after a 2-h pulse with [^{35}S]methionine. Fig. 1b shows the amount of radioactivity in the protein bands from four separate experiments. The incubation of cells with taurocholate caused a modest but significant decrease in the synthesis of apoB-100 and B-48. It had no effect on the incorporation of label into cellular apoA-I or TCA-precipitable proteins. Taurocholate did, however, cause a significant decrease in the secretion of newly synthesized apoB without altering the secretion of labeled apoA-I or total proteins.

To investigate the effect of the bile acid on apoB degradation, cells incubated for 18 h with taurocholic acid were pulsed for 30 min with [^{35}S]methionine and then chased for up to 3 h with excess unlabeled methionine. The loss of label in cellular apoB and the appearance of apoB in the basolateral medium were estimated after immunoprecipitation and separation of the proteins by polyacrylamide gel electrophoresis (**Fig. 2a and b**). It can again be appreciated from the gel (Fig. 2a) that the secretion of labeled apoB was significantly decreased in cells incubated with taurocholate. There was also a more rapid loss of label in cellular apoB, suggesting that apoB was turning over faster and less of the labeled apoB remained within the cell after the chase period. Fig. 2b shows the percent of radiolabel remaining in apoB-100 and B-48 during the chase from three individual experiments. The peak of incorporation of the label into apoB occurred at 15 min into the chase. The rate of disappearance of the label from apoB-100 and B-48 from this maximal point

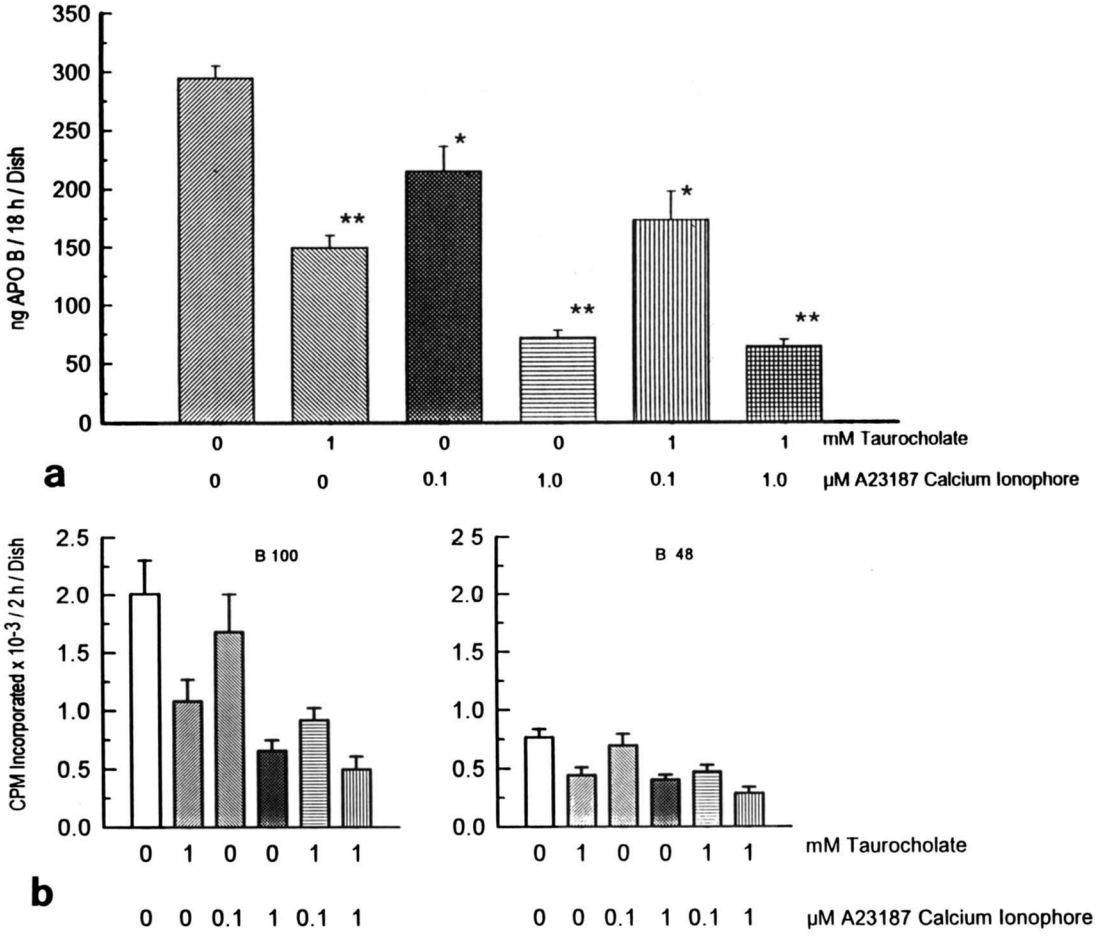


Fig. 3. Effect of A23187 with or without taurocholate on apoB secretion. Cells were incubated for 18 h with M199 containing 0.1 or 1.0 μM of the calcium ionophore A23187, 1 mM taurocholate, or A23187 plus taurocholate on the apical side. a) The amount of apoB mass secreted into the basolateral medium was estimated by ELISA. The data represent the mean ± SE of six individual transwells. b) The incorporation of [^{35}S]methionine into apoB secreted basolaterally was determined at 2 h. The values are the mean ± SE of three transwells; $^{*}P < 0.05$ vs. control; $^{**}P < 0.001$ vs. control.

was more rapid in cells incubated with taurocholate compared to what was observed in control cells. In contrast, taurocholate had no effect on the turnover or secretion of labeled apoA-I. These data would suggest that taurocholate causes a decrease in the secretion of apoB mass and newly synthesized apoB by enhancing apoB degradation within cells.

In hepatocytes, bile acids have been shown to release intracellular calcium (21, 22). To investigate whether calcium flux was playing a role in the inhibition of apoB secretion by taurocholate, cells were incubated with the calcium ionophore, A23187, with or without taurocholate. **Fig. 3** shows these results for immunoreactive and newly synthesized apoB. At both concentrations, the calcium ionophore decreased the secretion of apoB mass (Fig. 3a). When taurocholate and the calcium ionophore were added together, there was no additional decrease in apoB secretion. Similar results were obtained when the incorporation of [^{35}S]methionine into apoB secreted into the basolateral medium was determined (Fig. 3b). The results suggest that taurocholate and the calcium ionophore may be acting by a similar mechanism to decrease apoB secretion.

Effect of cholesterol flux

To address the effect of sterol flux on apoB secretion, cholesterol or its hydroxylated derivative, 25-hydroxycholesterol, was solubilized with 1 mM taurocholic acid and added to cells for 18 h. The amount of apoB within cells and that secreted into the basolateral well was then determined (**Fig. 4**). ApoB mass within cells was not affected by any of the treatments. As shown above, cells incubated with taurocholate alone secreted less apoB into the lower well. The addition of cholesterol or 25-hydroxycholesterol did not alter the amount of apoB mass secreted. To study the effect of cholesterol flux on the secretion of newly synthesized apoB, cells were incubated for 18 h with taurocholic acid with or without the sterols. They were then incubated for up to 3 h with [^{35}S]methionine and the secretion of labeled apolipoproteins into the basolateral medium was assessed (**Fig. 5**). As expected, taurocholate decreased the secretion of labeled apoB. The addition of cholesterol or 25-hydroxycholesterol did not significantly alter the amount of newly synthesized apoB secreted compared to that secreted from cells incubated with taurocholate alone. The secretion of labeled apoA-I was also not altered by the sterols except at 3 h at which time less A-I was secreted. The combined data suggest that changes in cholesterol flux do not affect the secretion of immunoreactive or newly synthesized apoB.

Effect of phospholipid flux

As phosphatidylcholine is the predominant biliary phospholipid, cells were incubated for 18 h with 1 mM taurocholic acid plus increasing concentrations of phosphatidylcholine. ApoB mass was estimated within cells

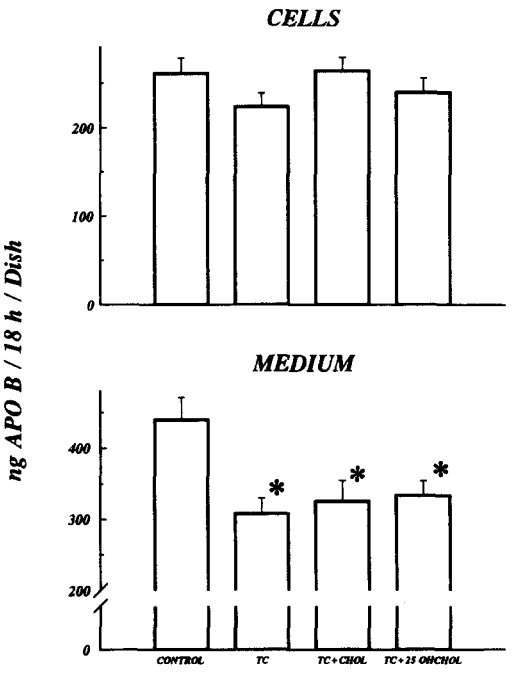


Fig. 4. Effect of cholesterol flux on apoB mass. Cells were incubated for 18 h with M199 (control), M199 containing 1 mM taurocholate (TC), M199 containing 1 mM taurocholate and 500 μM cholesterol (CHOL), or 25 μM 25-hydroxycholesterol (25 OHCHOL). The amount of apoB mass within cells and that secreted into the basolateral medium was estimated by ELISA. The data represent the means ± SE of six individual transwells assayed in triplicate; $^*P < 0.01$ vs. control.

and the basolateral medium (**Fig. 6**). Again, the amount of apoB secreted by cells incubated with taurocholic acid was significantly decreased. With the inclusion of 250 or 500 μM phosphatidylcholine, however, the amount of apoB secreted into the basolateral well was increased compared to cells incubated with taurocholate alone. In cells incubated with these concentrations of phosphatidylcholine, intracellular apoB mass was also significantly increased over controls. Phosphatidylcholine alone, added as multilamellar liposomes, increased apoB mass within cells and that secreted basolaterally compared to controls. Thus, phosphatidylcholine has an independent effect on apoB secretion and is not acting solely to prevent the inhibitory effect of taurocholate on apoB secretion. Moreover, phosphatidylcholine reversed the inhibitory effect of the calcium ionophore on apoB secretion. In the basolateral medium of cells incubated with medium alone, 1 μM A23187, or A23187 and 500 μM phosphatidylcholine, apoB mass was 201 ± 15, 69 ± 5, and 294 ± 18 ng apoB secreted/18 h per dish, respectively.

To investigate the specificity of the effect of phosphatidylcholine on apoB mass secretion, cells were incubated for 18 h with 1 mM taurocholate and 500 μM of phosphatidylethanolamine, sphingomyelin, or phosphatidylserine (**Fig. 7**). Only phosphatidylcholine in-

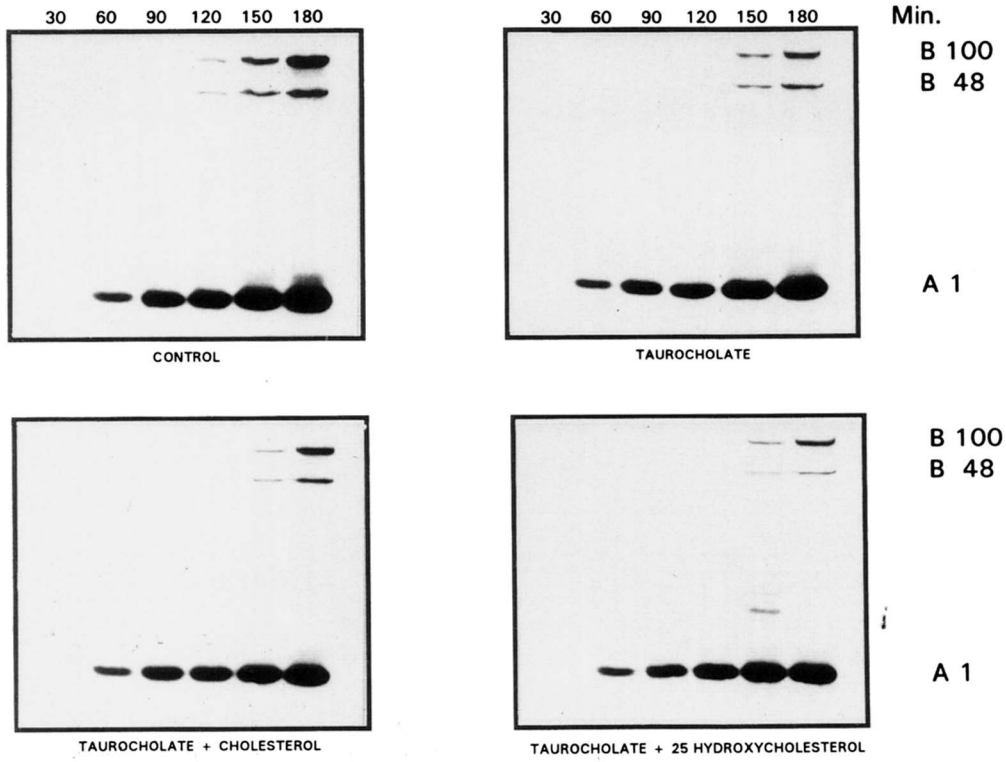


Fig. 5. Effect of cholesterol flux on the secretion of newly synthesized apoB. After an 18 h incubation with M199 (control), M199 and 1 mM taurocholate, 1 mM taurocholate plus 500 μM cholesterol or 25 μM 25-hydroxycholesterol, cells were pulsed for up to 3 h with 100 μCi of [^{35}S]methionine. The rate and the amount of labeled apoB and A-I secreted were estimated following immunoprecipitation and polyacrylamide gel electrophoresis as described in Methods. The figure is a representative autoradiogram from two experiments with similar results.

creased the amount of apoB found within cells and the basolateral medium.

Similar to biliary phosphatidylcholine, phosphatidylcholine derived from egg contains mostly palmitic (*sn*-1), oleic and linoleic acids (*sn*-2). To address whether the fatty acid composition of the phosphatidylcholine molecule was important in the regulation of apoB secretion, cells were incubated for 18 h with 1 mM taurocholate and 500 μM egg phosphatidylcholine, dioleoylphosphatidylcholine, or dipalmitoylphosphatidylcholine. After the incubation, the amount of apoB mass within cells and that found in the basolateral medium was estimated (**Fig. 8**). Cells incubated with egg phosphatidylcholine contained more apoB and secreted more apoB mass than cells incubated with the other two phosphatidylcholines. Dioleoylphosphatidylcholine was almost as potent as egg phosphatidylcholine in increasing the amount of apoB mass secreted into the basolateral well. In contrast, phosphatidylcholine containing two palmitic acid moieties had no effect on the amount of apoB found within cells or in the basolateral medium compared to cells incubated with taurocholate alone.

To assess whether hydrolysis or uptake of phosphatidylcholine was playing a role in this regulation, cells were incubated for 18 h with 1 mM taurocholate and fatty acid-labeled phosphatidylcholine. To estimate nonspecific adsorption of the phospholipid, some cells were treated with formalin prior to adding the label. After the incubation, the amount of fatty acid label in the apical and basolateral medium and that associated with cells was determined (**Fig. 9**). Fig. 9A shows that the amount of radioactivity found in the basolateral medium of viable cells was similar to formalin-treated cells. The amount of label remaining in the apical medium was also similar in viable cells versus formalin-treated cells. There was, however, approximately 30 nmol of labeled fatty acid, representing 2% of the total added, which was cell-associated and could not be attributable to nonspecific adsorption.

Of the label remaining in the apical medium, 98% was in phosphatidylcholine (Fig. 9B). The amount of label in apical free fatty acids, triacylglycerols, and lysophosphatidylcholine was not above background. Approximately 80% of the label which was cell-associated was in phosphatidylcholine (Fig. 9C). The remaining 20% was found mainly in triacylglycerols, free fatty acids, and phosphatidylethanolamine with smaller amounts

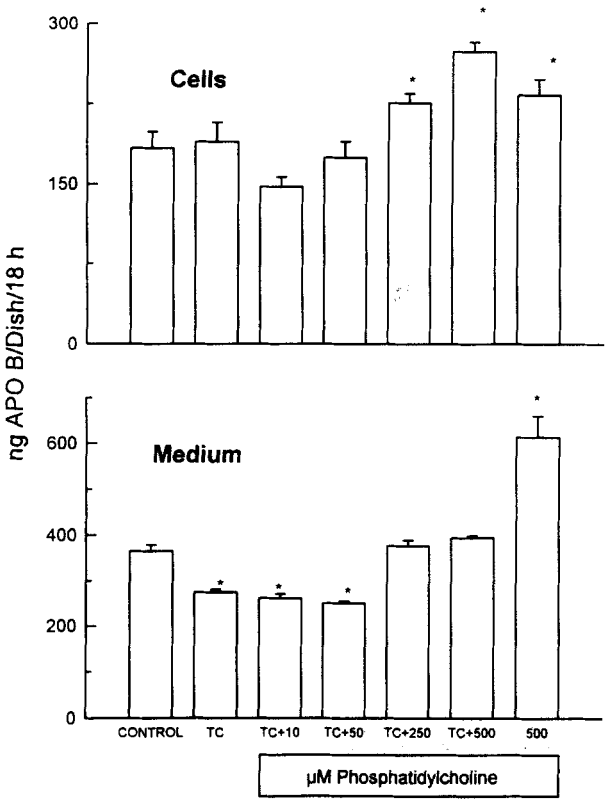


Fig. 6. Effect of phosphatidylcholine on apoB mass. Cells were incubated for 18 h with M199 (control), M199 containing 1 mM taurocholate (TC), TC and increasing concentrations of phosphatidylcholine, or 500 μM phosphatidylcholine multilamellar liposomes. The amount of apoB mass within cells and that secreted into the basolateral medium was estimated by ELISA (mean ± SE, n = 6 separate transwells assayed in triplicate); $^*P < 0.01$ vs. control.

found in other lipids. Thus, 2% of labeled phosphatidylcholine was cell-associated after an 18-h incubation. Most of the label remained as phosphatidylcholine. Small amounts of label in other lipids suggested hydrolysis and reincorporation of the phosphatidylcholine fatty acids.

Because only a small amount of the phosphatidylcholine was found to be cell-associated, the data suggested that the regulation of apoB secretion by taurocholate and taurocholate plus phosphatidylcholine involved the interaction of the lipid with the apical membrane. To determine the importance of this interaction, taurocholate or taurocholate and phosphatidylcholine was added instead to the basolateral side of the cells and the amount of apoB mass secreted was estimated (**Fig. 10**). Similar to what was observed when taurocholate was added apically, apoB mass secretion was significantly decreased by the bile acid. In contrast, however, taurocholate plus phosphatidylcholine did not increase apoB secretion when added to the basolateral medium. The data suggest that taurocholate regulates apoB secretion by interacting with either the apical or basolateral membrane, but interaction

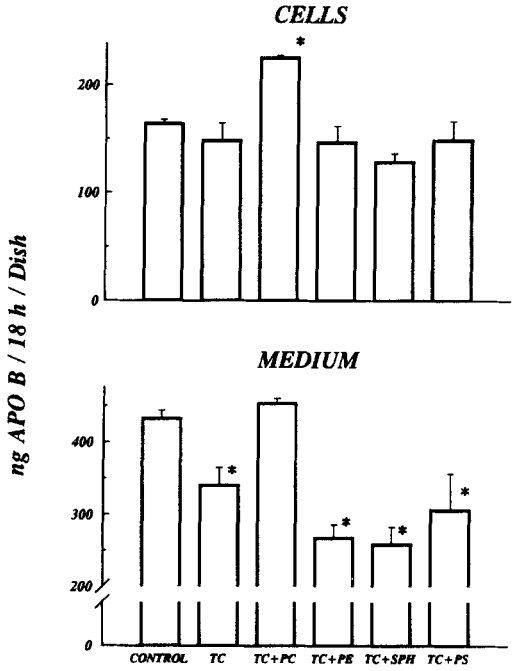


Fig. 7. Effect of phospholipids on apoB mass. The experimental design was exactly as described for Fig. 6 except that 500 μM of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SPH), or phosphatidylserine (PS), was added with 1 mM taurocholate (TC). The data represent the mean ± SE of six individual transwells assayed in triplicate; $^*P < 0.01$ vs. control.

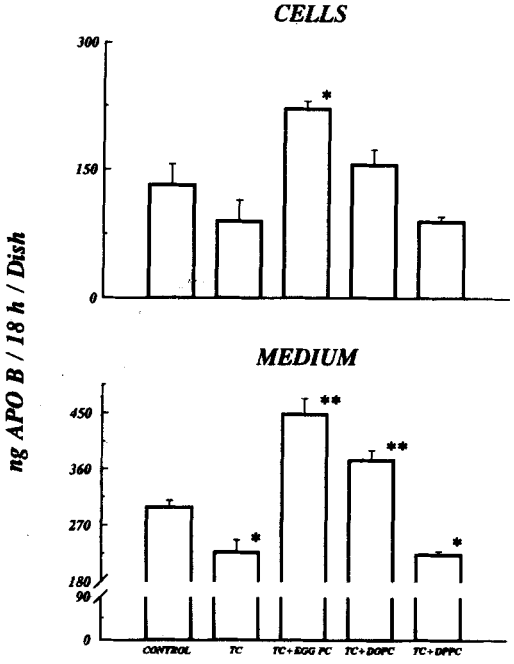


Fig. 8. Effect of the fatty acid composition of phosphatidylcholine on apoB mass. The experimental design was that described in Fig. 6: M199 (control); 1 mM taurocholate (TC); TC + 500 μM egg phosphatidylcholine (PC), dioleoylphosphatidylcholine (DOPC), or dipalmitoylphosphatidylcholine (DPPC). The data represent the mean ± SE of six separate transwells assayed in triplicate; $^*P < 0.05$ vs. control; $^{**}P < 0.02$ vs. control.

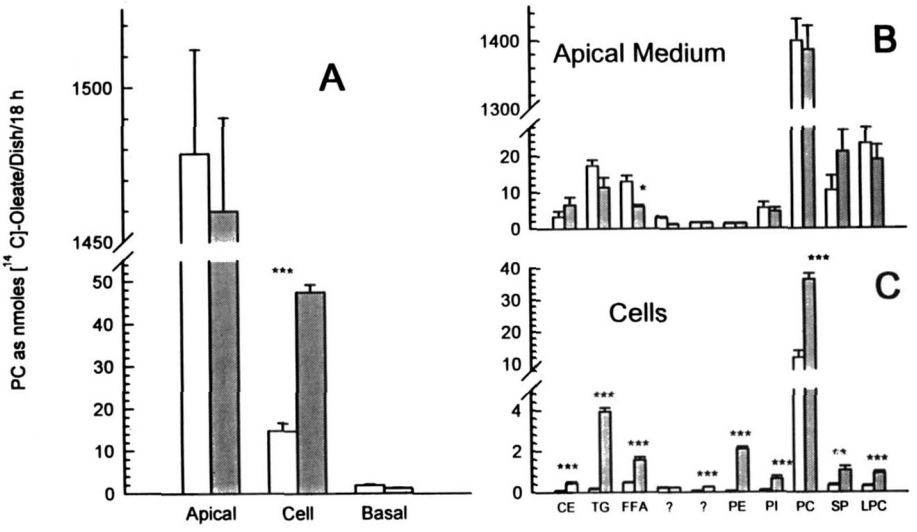


Fig. 9. Hydrolysis and/or uptake of [^{14}C]dioleoylphosphatidylcholine. Cells were grown on semipermeable filters and incubated for 18 h with 1 mM taurocholate and 500 μM phosphatidylcholine containing 0.2 μCi of [^{14}C]dioleoylphosphatidylcholine per filter. Nonspecific adsorption was estimated by pretreating some cells for 1 h with a 37% formalin solution prior to the addition of the labeled phosphatidylcholine. After the 18-h incubation, lipids were extracted from the cells and the apical and basolateral media and separated by thin-layer chromatography (see Methods). A) Total labeled fatty acid found in the apical and basolateral media and cell-associated. B) Labeled lipids in the apical medium. C) Labeled lipids within cells; n = 6 transwells for each treatment; results are expressed as nmol of fatty acid found in each lipid fraction; shaded column viable cells; open column formalin-treated cells; $^{*}P < 0.05$ vs. formalin-treated cells; $^{**}P < 0.01$ vs. formalin-treated cells; $^{***}P < 0.001$ vs. formalin-treated cells.

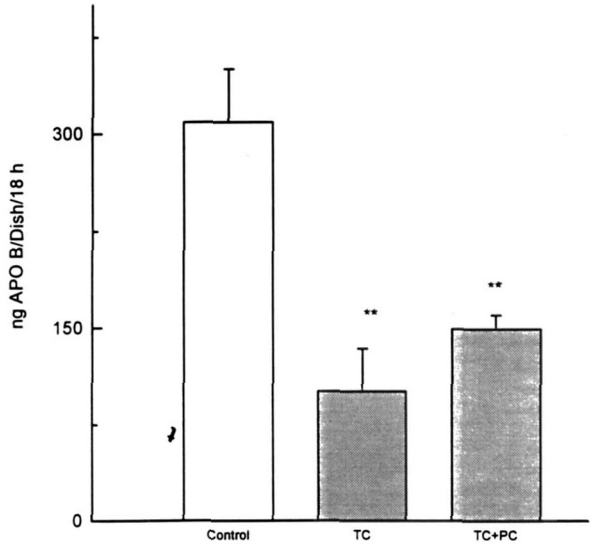


Fig. 10. ApoB secretion by cells incubated with taurocholate or taurocholate and phosphatidylcholine added to the basolateral medium. Cells grown on semipermeable filters were incubated for 18 h with M199, M199 containing 1 mM taurocholate or taurocholate plus 500 μM phosphatidylcholine added to the basolateral medium. After the incubation, the amount of apoB mass secreted into the basolateral medium was estimated. Taurocholate or phosphatidylcholine did not interfere with the estimation by ELISA of apoB mass secreted basolaterally. The data represent the mean ± SE of five transwells with the assays done in duplicate; $^{**}P < 0.01$ vs. control.

with the apical membrane is necessary for the regulation of apoB secretion by phosphatidylcholine.

To investigate the effect of phosphatidylcholine on the synthesis and secretion of newly synthesized apoB, cells were again incubated 18 h with 1 mM taurocholate and 500 μM phosphatidylcholine. The next morning the incorporation of [^{35}S]methionine into apoB within cells and that secreted into the basolateral medium was estimated. **Fig. 11** shows these results. The rate of incorporation of labeled methionine into cellular apoB was significantly increased in cells incubated with phosphatidylcholine. Moreover, the secretion of labeled apoB into the basolateral medium was markedly increased as well. Thus, phosphatidylcholine increases the synthesis of apoB and the secretion of newly labeled and immunoreactive apoB by CaCo-2 cells.

To address the effect of phosphatidylcholine on apoB degradation, cells were pulsed for 30 min with [^{35}S]methionine and chased for 3 h with unlabeled methionine after an overnight incubation with taurocholate or taurocholate plus phosphatidylcholine. The amount of label remaining in apoB and that secreted into the basolateral medium was estimated following immunoprecipitation and polyacrylamide gel electrophoresis. **Fig. 12a and b** show a representative autoradiogram (a) and the percent decrease of radioactivity within apoB-100, apoB-48, apoA-I from cells and basolateral media combined (b, three individual experiments). From the

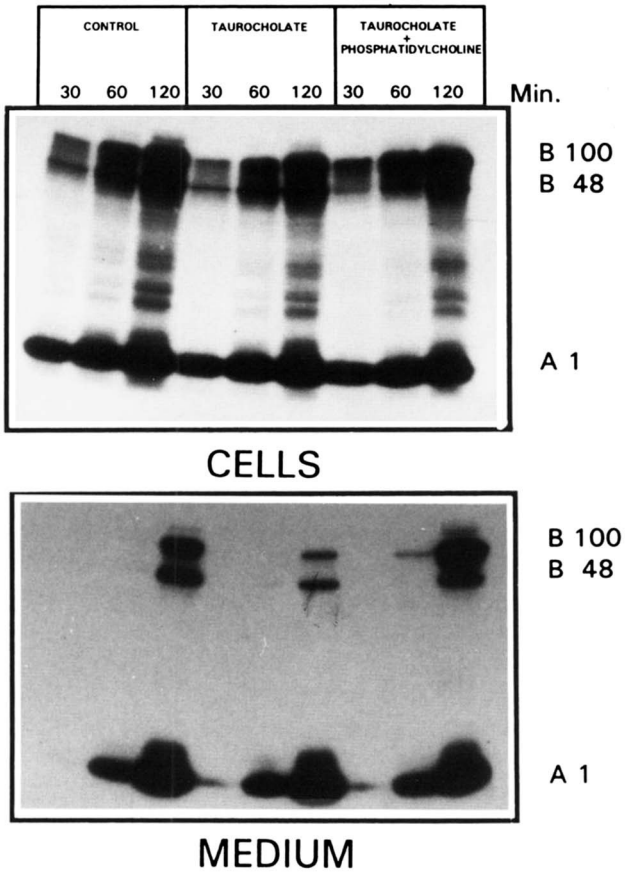


Fig. 11. Effect of phosphatidylcholine on the synthesis and secretion of apoB. After an 18-h incubation with M199 (control), 1 mM taurocholate, or taurocholate and 500 μM phosphatidylcholine, cells were pulsed for up to 2 h with 100 μCi of [^{35}S]methionine. The incorporation of label into cellular apoB and apoA-I and that secreted into the basolateral medium was estimated after immunoprecipitation and polyacrylamide gel electrophoresis. The figure is a representative autoradiogram from more than six separate experiments, all demonstrating similar results.

gel, it can be appreciated that after a 30-min pulse of [^{35}S]methionine, more apoB was synthesized in cells incubated with taurocholate plus phosphatidylcholine than in cells incubated with the bile acid alone (0 time point). More radiolabeled apoB was also secreted into the basolateral medium by cells incubated with phosphatidylcholine. The rate of decrease, or loss, of labeled apoB (cell plus medium), however, was not significantly altered by the presence of phosphatidylcholine (b).

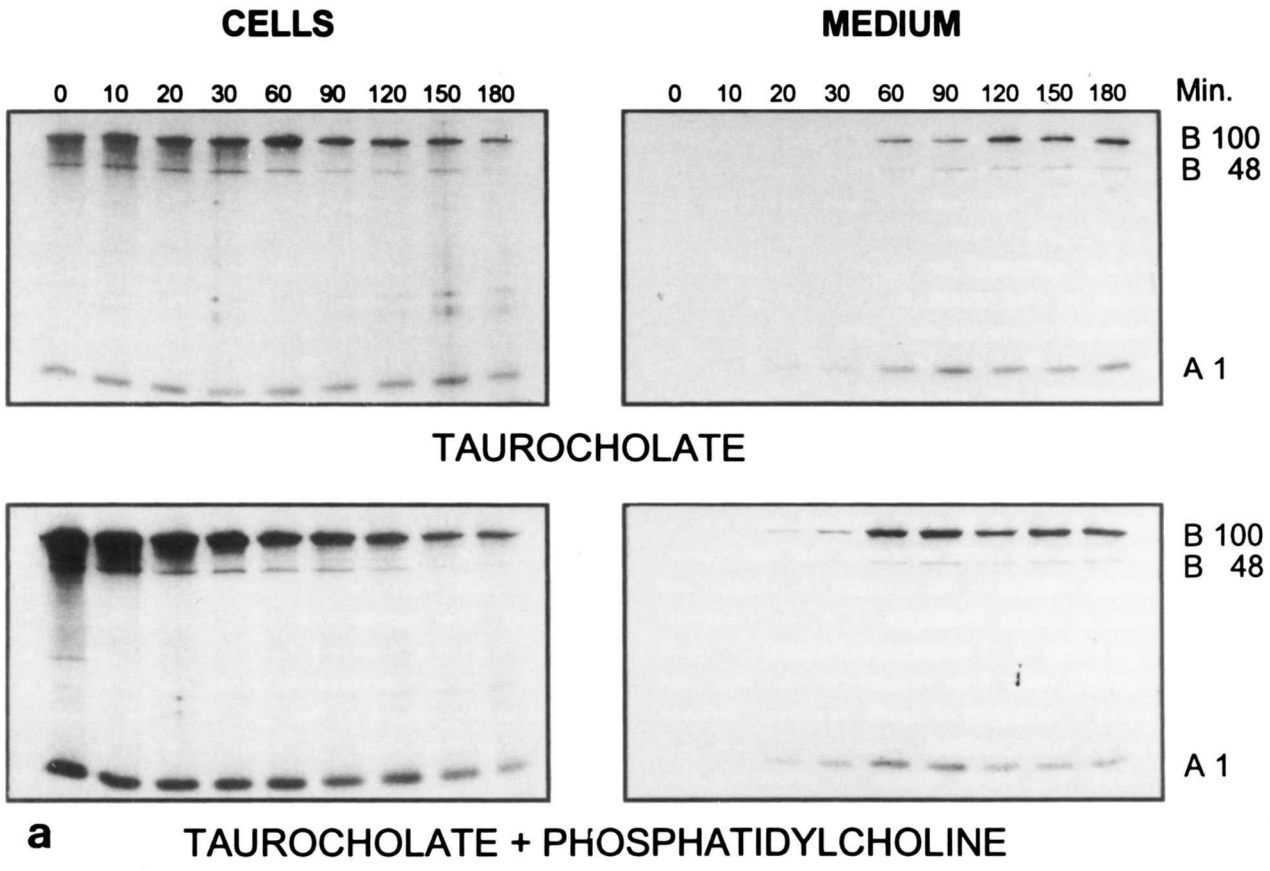
DISCUSSION

The results of this study clearly show that bile acids alone, independent of luminal lipids, inhibit the secretion of apoB by CaCo-2 cells. Taurocholate decreased the secretion of both immunoreactive and newly synthesized apoB without affecting apoA-I or total protein secretion. The predominant effect of taurocholate appeared to be on the degradation of cellular apoB as the bile acid only modestly decreased apoB synthesis. Intracellular degradation of this apolipoprotein is thought to be one mechanism by which the cell regulates the amount of apoB secreted (3, 23–25). How taurocholate earmarks more apoB for degradation rather than secretion is unknown. ApoB mass within the cell remained constant, suggesting that the amount of intracellular apoB was being maintained despite rather marked changes occurring in its rate of secretion. This conservation of cellular apoB despite changes in apoB secretion has been observed by others (13, 26, 27). It is recognized that bile acids facilitate the transport of lipids by the intestine (28). In light of the present results, they likely do so by facilitating lipid uptake from the lumen, not by enhancing lipoprotein secretion.

Bile acids have been shown to cause cell injury at concentrations above their critical micellar concentration (CMC) (19, 20). In a recent study using CaCo-2 cells, cell injury did not occur at concentrations of bile acids below their CMC (20). In the present study, the concentrations of bile acids were purposefully kept below these concentrations. Although it could be argued that this may not mimic the in vivo situation, the potential cytotoxic effects of bile acids would likely have caused significant untoward effects on apoB secretion, or for that matter, the secretion of other proteins as well. Cell viability, as assessed by the MTT assay, the amount of protein per filter, and the secretion of apoA-I and total proteins were similar in control cells and cells incubated with taurocholic acid. Moreover, cells incubated with 1 mM taurocholate and then allowed to recover secreted similar amounts of apoB as did controls. Thus, possible cytotoxic effects of taurocholate cannot explain these results.

At 1 mM concentrations, bile salts exist within lipid bilayers with their long axis parallel to the membrane, a so-called reversed micelle (19). The formation of reversed micelles within a membrane has been postulated to cause the recognized ionophoric behavior of bile acids at low concentrations (29). As changes in calcium flux alter certain secretory processes, it was postulated that taurocholate may be altering apoB secretion by increasing intracellular calcium flux. The calcium ionophore, A23187, caused a similar decrease in the secretion of immunoreactive and newly synthesized apoB as did bile acids. The addition of the ionophore and taurocholate together did not cause a further decrease in apoB secretion, suggesting that both agents were acting through a common mechanism.

Results of previous studies investigating a possible role for calcium in the secretion of apoB-containing lipoproteins have been conflicting (8, 30). In a study by Hughes, Ordovas, and Schaefer (30), apoB mass secretion was increased in CaCo-2 cells incubated with 1 μM A23187. In that study, however, cells were cultured on plastic. Thus, apoB released from the apical membrane



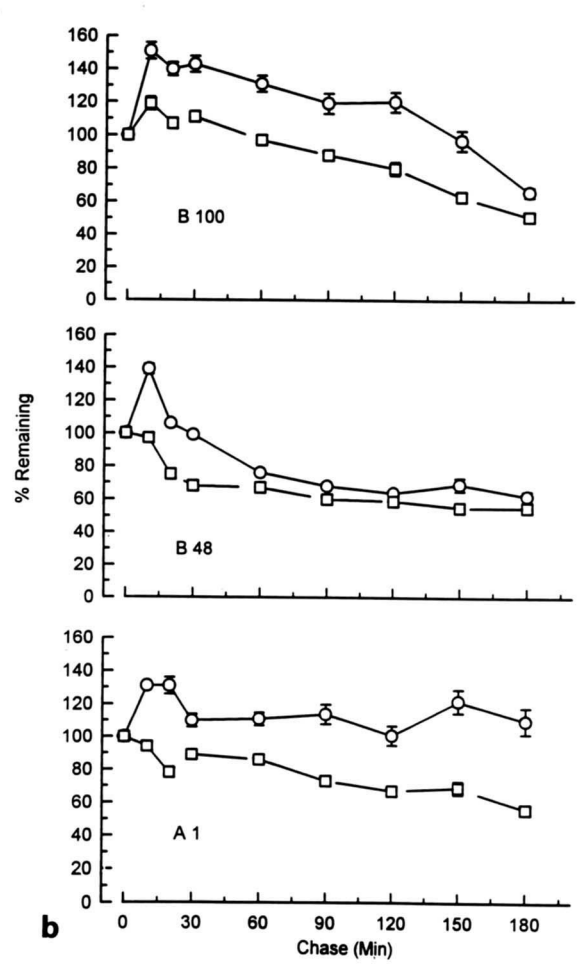


Fig. 12. Effect of phosphatidylcholine on apoB degradation. After an 18-h incubation with M199 containing 1 mM taurocholate or taurocholate plus 500 μM phosphatidylcholine, cells were pulsed for 30 min with 150 μCi of [^{35}S]methionine and chased for up to 3.0 h with excess unlabeled methionine. a) Representative autoradiogram after immunoprecipitation and polyacrylamide gel electrophoresis to separate the apoproteins. b) Percent decrease of radioactivity within apoB-100, B-48, and A-I from combined cell plus medium. The data represent the means ± SE of three individual experiments.

could not be distinguished from apoB released basolaterally. In addition, cells grown on filters, rather than plastic, act more like mature intestinal epithelium (13). The present results support the findings of Pullinger et al. (8) who observed a 35% decrease in apoB secretion by HepG2 cells with 1 μM A23187.

Few studies have addressed the regulation of apoB synthesis or secretion by cholesterol. In rat intestine, an increase in cholesterol absorption decreased the synthesis of apoB-48; whereas, apoB-48 synthesis was increased when cholesterol absorption was prevented (31). Davis and Malone-McNeal (32) observed that hepatocytes from control rats synthesized and secreted apoB at similar rates as hepatocytes from cholesterol-fed rats. In HepG2 cells, Dashti (33) found that 25-hydroxycholesterol, but not native cholesterol, increased apoB secretion and that this increase was directly related to the amount of cellular and medium cholesteryl esters. Pullinger et al. (8), however, failed to find an effect of 25-hydroxycholesterol on apoB secretion in HepG2 cells. Our results do not implicate cholesterol flux as a regulator of apoB secretion. Neither cholesterol nor its hydroxylated analogue, 25-hydroxycholesterol, which has been shown to down-regulate cholesterol synthesis and LDL receptor activity in CaCo-2 cells (34, 35), altered the secretion of apoB mass or the synthesis and secretion of newly synthesized apoB.

A somewhat unexpected observation was the marked increase in the synthesis and secretion of apoB by cells incubated with taurocholate plus phosphatidylcholine. A stimulatory effect of phosphatidylcholine on triacylglycerol transport in the intestine has been observed by others, although the effects have largely been attributed to the intraluminal hydrolysis of the phospholipid and the cellular uptake of lysophosphatidylcholine and fatty acids (36–40). The present results suggest an alternative mechanism. Although 2% of the added phosphatidylcholine was found to be cell-associated, the amount of fatty acid or lysophosphatidylcholine that could have been generated by hydrolysis is far below the amount that is observed to promote apoB secretion (12–15, 25). Furthermore, other classes of phospholipids that would have supplied similar amounts of fatty acids or lysophosphatidylcholine did not significantly alter apoB secretion.

The reasons why dioleoylphosphatidylcholine enhanced apoB secretion and dipalmitoylphosphatidylcholine did not are not entirely clear. It would seem, however, that the positioning of the unsaturated fatty acid on the *sn*-2 carbon atom of the phospholipid is important for its effect on apoB secretion. Replacing palmitate with oleate on the *sn*-1 position caused little effect on apoB secretion, whereas replacing oleate with palmitate at *sn*-2 nullified the stimulatory effect of the phospholipid. Tso, Lam, and Simmonds (36) also found that the nature of the fatty acid at the *sn*-1 position of phosphatidylcholine was not crucial for triacylglycerol transport by the gut. These investigators, however, found that dipalmitoylphosphatidylcholine was as effective (albeit somewhat less) as dioleoylphosphatidylcholine in enhancing lymphatic triacylglycerol transport in a biliary fistula rat model (39). Bennett Clark (37) also found that the effects of dipalmitoylphosphatidylcholine were less than those observed with vegetable phosphatidylcholine in perfused intestines of bile-intact and bile-diverted rats. Aside from the obvious differences between cell culture and intestinal perfusion, the reasons for these differences remain unclear. The present study was done in the absence of triacylglycerol flux, i.e., apoB secretion was increased by phosphatidylcholine in the absence of fat transport. In studies using bile-fistula rats, triacylglycerol flux was increased by the perfusion of fatty acids or triacylglycerols (36–39). Our results would suggest that the effect of phosphatidylcholine on apoB secretion is independent of triacylglycerol transport. This agrees with the results of Bennett Clark (37) in which she observed that the effect of phosphatidylcholine on triacylglycerol secretion into lymph was unrelated to the uptake of the lipid from the lumen.

Phosphatidylcholine increased apoB secretion in CaCo-2 cells by increasing the synthesis of apoB without altering its rate of degradation. As the release of intracellular fatty acids through the hydrolysis of phosphatidylcholine cannot explain the present results, the data suggest that the interaction of phosphatidylcholine with the apical membrane triggers some type of signal, which may involve calcium, which then leads to the increased secretion of apoB. With the concentration of phosphatidylcholine in bile being approximately 10 mM (40) and with a normal bile flow of 600 ml/24 h, it is likely that some luminal phosphatidylcholine will escape hydrolysis. Interaction of this unhydrolyzed phosphatidylcholine with the apical membrane of the enterocyte could play a role in regulating apoB secretion by the intestine.

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REFERENCES

1. Chen, S. H., G. Habib, C. Y. Yang, Z. W. Gu, B. R. Lee, S. A. Weng, S. R. Silberman, S-J. Cai, J. P. Deslypere, M. Rosseneu, A. M. Gotto, Jr., W-H. Li, and L. Chan. 1987. Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science.* **238:** 363–366.
2. Powell, L. M., S. C. Wallis, R. J. Pease, Y. H. Edwards, T. J. Knott, and J. Scott. 1987. A novel form of tissue-specific RNA processing produces apolipoprotein B-48 in intestine. *Cell.* **50:** 831–840.
3. Sparks, J. D., and C. E. Sparks. 1990. Insulin modulation

of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J. Biol. Chem.* **265:** 8854–8862.
4. Davidson, N. O., R. C. Carlos, M. J. Drewek, and T. G. Parmer. 1988. Apolipoprotein gene expression in the rat is regulated in a tissue-specific manner by thyroid hormone. *J. Lipid Res.* **29:** 1511–1522.
5. Sparks, J. D., C. E. Sparks, M. Bolognino, A. M. Roncone, T. K. Jackson, and J. M. Amatruda. 1988. Effects of nonketotic streptozotocin diabetes on apolipoprotein B synthesis and secretion by primary cultures of rat hepatocytes. *J. Clin. Invest.* **82:** 37–43.
6. Sorci-Thomas, M., M. D. Wilson, F. L. Johnson, D. L. Williams, and L. L. Rudel. 1989. Studies on the expression of genes encoding apolipoproteins B-100 and B-48 and the low density lipoprotein receptor in nonhuman primates. Comparison of dietary fat and cholesterol. *J. Biol. Chem.* **264:** 9039–9045.
7. Kroon, P. A., J. A. DeMartino, G. M. Thompson, and Y-S. Chao. 1986. Molecular cloning of partial cDNAs for rabbit liver apolipoprotein B and the regulation of its mRNA levels by dietary cholesterol. *Proc. Natl. Acad. Sci. USA.* **83:** 5071–5075.
8. Pullinger, C. R., J. D. North, B-B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* **30:** 1065–1077.
9. Windmueller, H. G., and A-L. Wu. 1981. Biosynthesis of plasma apolipoproteins by rat small intestine without dietary or biliary fat. *J. Biol. Chem.* **256:** 3012–3016.
10. Davidson, N. O., M. E. Kollmer, and R. M. Glickman. 1986. Apolipoprotein B synthesis in rat small intestine: regulation by dietary triglyceride and biliary lipid. *J. Lipid Res.* **27:** 30–39.
11. Davidson, N. O., M. J. Drewek, J. I. Gordon, and J. Elovson. 1988. Rat intestinal apolipoprotein B gene expression. *J. Clin. Invest.* **82:** 300–308.
12. Traber, M. G., H. J. Kayden, and M. J. Rindler. 1987. Polarized secretion of newly synthesized lipoproteins by the Caco-2 human intestinal cell line. *J. Lipid Res.* **28:** 1350–1363.
13. Moberly, J. B., T. G. Cole, D. H. Alpers, and G. Schonfeld. 1990. Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occurs post-transcriptionally. *Biochim. Biophys. Acta.* **1042:** 70–80.
14. Dashti, N., E. A. Smith, and P. Alaupovic. 1990. Increased production of apolipoprotein B and its lipoproteins by oleic acid in Caco-2 cells. *J. Lipid Res.* **31:** 113–123.
15. Murthy, S., E. Albright, S. N. Mathur, N. O. Davidson, and F. J. Field. 1992. Apolipoprotein B mRNA abundance is decreased by eicosapentaenoic acid in CaCo-2 cells. Effect on the synthesis and secretion of apolipoprotein B. *Arterioscler. Thromb.* **12:** 691–700.
16. Field, F. J., E. Albright, and S. N. Mathur. 1987. Regulation of cholesterol esterification by micellar cholesterol in CaCo-2 cells. *J. Lipid Res.* **28:** 1057–1066.
17. Hansen, M. B., S. E. Nielsen, and K. Berg. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods.* **119:** 203–210.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Coleman, R. 1987. Bile salts and biliary lipids. *Biochem. Soc. Trans.* **15:** 68S–80S.
20. Velardi, A. L. M., A. K. Groen, R. P. J. Oude Elferink, R. Van Der Meer, G. Palasciano, and G. N. J. Tytgat. 1991. Cell type-dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology.* **101:** 457–464.
21. Combettes, L., M. Dumont, B. Berthon, S. Erlinger, and M. Claret. 1988. Release of calcium from the endoplasmic reticulum by bile acids in rat liver cells. *J. Biol. Chem.* **263:** 2299–2303.
22. Beuers, U., M. H. Nathanson, and J. L. Boyer. 1993. Effects of tauroursodeoxycholic acid on cytosolic Ca^{2+} signals in isolated rat hepatocytes. *Gastroenterology.* **104:** 604–612.
23. Borchardt, R. A., and R. A. Davis. 1987. Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. *J. Biol. Chem.* **262:** 16394–16402.
24. Sato, R., T. Imanaka, A. Takatsuki, and T. Takano. 1990. Degradation of newly synthesized apolipoprotein B-100 in a pre-Golgi compartment. *J. Biol. Chem.* **265:** 11880–11884.
25. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266:** 5080–5086.
26. White, A. L., D. L. Graham, J. LeGros, R. J. Pease, and J. Scott. 1992. Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. *J. Biol. Chem.* **267:** 15657–15664.
27. Jackson, T. K., A. I. Salhanick, J. D. Sparks, C. E. Sparks, M. Bolognino, and J. M. Amatruda. 1988. Insulin-mimetic effects of vanadate in primary cultures of rat hepatocytes. *Diabetes.* **37:** 1234–1240.
28. Hofmann, A. F., and D. M. Small. 1967. Detergent properties of bile salts: correlation with physiological function. *Annu. Rev. Med.* **18:** 333–378.
29. Abramson, J. J., and A. E. Shamoo. 1979. Anionic detergents as divalent cation ionophores across black lipid membranes. *J. Membr. Biol.* **50:** 241–255.
30. Hughes, T. E., J. M. Ordovas, and E. J. Schaefer. 1988. Regulation of intestinal apolipoprotein B synthesis and secretion by Caco-2 cells. Lack of fatty acid effects and control by intracellular calcium ion. *J. Biol. Chem.* **263:** 3425–3431.
31. Davidson, N. O., A. M. Magun, T. A. Brasitus, and R. M. Glickman. 1987. Intestinal apolipoprotein A-I and B-48 metabolism: effects of sustained alterations in dietary triglyceride and mucosal cholesterol flux. *J. Lipid Res.* **28:** 388–402.
32. Davis, R. A., and M. Malone-McNeal. 1985. Dietary cholesterol does not affect the synthesis of apolipoproteins B and E by rat hepatocytes. *Biochem. J.* **227:** 29–35.
33. Dashti, N. 1992. The effect of low density lipoproteins, cholesterol, and 25-hydroxycholesterol on apolipoprotein B gene expression in HepG2 cells. *J. Biol. Chem.* **267:** 7160–7169.
34. Field, F. J., T. Shreves, D. Fujiwara, S. Murthy, E. Albright, and S. N. Mathur. 1991. Regulation of gene expression and synthesis and degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by micellar cholesterol in CaCo-2 cells. *J. Lipid Res.* **32:** 1811–1821.
35. Field, F. J., D. Fujiwara, E. Born, D. A. Chappell, and S. N. Mathur. 1993. Regulation of LDL receptor expression by luminal sterol flux in CaCo-2 cells. *Arterioscler. Thromb.* **13:** 729–737.
36. Tso, P., J. Lam, and W. J. Simmonds. 1978. The impor-

tance of the lysophosphatidylcholine and choline moiety of bile phosphatidylcholine in lymphatic transport of fat. *Biochim. Biophys. Acta.* **528:** 364–372.
37. Bennett Clark, S. 1978. Chylomicron composition during duodenal triglyceride and lecithin infusion. *Am. J. Physiol.* **235:** E183–E190.
38. Mansbach, C. M., II, A. Arnold, and M. A. Cox. 1985. Factors influencing triacylglycerol delivery into mesenteric lymph. *Am. J. Physiol.* **249:** G642–G648.
39. Tso, P., H. Kendrick, J. A. Balint, and W. J. Simmonds. 1981. Role of biliary phosphatidylcholine in the absorption and transport of dietary triolein in the rat. *Gastroenterology.* **80:** 60–65.
40. Balint, J. A., E. C. Kyriakides, H. L. Spitzer, and E. S. Morrison. 1965. Lecithin fatty acid composition in bile and plasma of man, dogs, rats, and oxen. *J. Lipid Res.* **6:** 96–99.