Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: possible role of p-glycoprotein

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Abstract Acylcoenzyme A:cholesterol acyltransferase (ACAT) and/or cholesteryl esters have been implicated as important factors in the normal assembly of apolipoprotein (apoB)-containing lipoproteins. The predominant substrate for ACAT is believed to originate from cholesterol contained within the plasma membrane. To investigate a possible role of intestinal plasma membrane cholesterol in triacylglycerol-rich lipoprotein synthesis and secretion, CaCo-2 cells were incubated with agents that are known to interfere with cholesterol transport from the plasma membrane to the ER. Progesterone, verapamil, and trifluoperazine significantly decreased the movement of cholesterol from plasma membrane to endoplasmic reticulum (ER) in CaCo-2 cells. Without altering the synthesis of apoB and independent of their effects on cellular cholesterol esterification, progesterone, verapamil, and trifluoperazine decreased the basolateral secretion of triacylglycerols, cholesteryl esters, and immunoreactive and newly synthesized apoB. The three agents also interfered with the esterification of cholesterol absorbed from taurocholate micelles. As progesterone, verapamil, and trifluoperazine are recognized inhibitors of p-glycoprotein, a variety of agents that have been shown to interfere with p-glycoprotein function were tested to investigate their effects on cholesterol transport and apoB secretion. All the agents significantly decreased in parallel both cholesterol transport and apoB secretion. In contrast, methotrexate, an antimetabolite that does not interact with p-glycoprotein, had no effect. Nigericin, a potassium ionophore, which causes alkalinization of intracellular vesicles, also caused a profound inhibition of cholesterol transport and apoB secretion. Preventing plasma membrane cholesterol from arriving at the ER, or inhibiting the esterification of plasma membrane cholesterol, does not alter apoB secretion. However, the results suggest a possible role for p-glycoprotein in normal cholesterol trafficking and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells. It is postulated that p-glycoprotein might function to maintain the acidic environment of transport vesicles, and therefore, could play a role in the transport of lipids by the intestine.—**Field, F. J., E. Born, H. Chen, S. Murthy, and S. N. Mathur.** Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: possible role of p-glycoprotein. *J. Lipid Res.* 1995. **36:** 1533–1543.

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Newly synthesized cholesterol is transported from its site of synthesis, the rough endoplasmic reticulum (ER), to the plasma membrane by an energy-dependent vesicular pathway that is independent of the secretory pathway (1, 2). Trafficking of cellular cholesterol from the plasma membrane back to the ER has also been demonstrated (3). It is believed that transport of internalized plasma membrane cholesterol to the ER is also mediated by a vesicular carrier that shuttles the sterol between the two membranes. A number of agents, including progesterone, trifluoperazine, colchicine, and nigericin, have been shown to interfere with this movement of cholesterol from plasma membrane to ER (3, 4). The mechanism for this interference is unknown. There is evidence to suggest that this incoming plasma membrane cholesterol is the major source of substrate for acylcoenzyme A:cholesterol acyltransferase (ACAT), an integral ER membrane protein that catalyzes the esterification of intracellular cholesterol (5). In cells incubated with agents that interfere with normal cholesterol trafficking, the substrate supply for ACAT becomes limiting and the rate of esterification of cellular cholesterol decreases (4, 5).

The importance of ACAT and/or the requirement of cholesteryl esters for the normal assembly and secretion of triacylglycerol-rich lipoproteins by liver and intestine has been debated (6, 7, reviews). In cultured human

Abbreviations: apoB, apolipoprotein B; ACAT, acylcoenzyme A:cholesterol acyltransferase.

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intestinal cells, prolonged inhibition of ACAT activity and the depletion of cellular cholesteryl esters caused the accumulation of cellular triacylglycerols and a decrease in their secretion (8). In HepG2 cells and cultured rabbit hepatocytes, the secretion of apoB was closely coupled to the amount of intracellular cholesteryl esters, suggesting a role for ACAT and/or cholesteryl esters in the transport of apoB-containing lipoproteins (9–11). Other studies, however, have disputed the relationship between cholesteryl esters and the secretion of apoB (12–14). As plasma membrane cholesterol is thought to be the primary substrate for ACAT, we postulated that the internalization and transport of plasma membrane cholesterol to the ER may be necessary for the normal assembly and secretion of triacylglycerol-rich lipoproteins by the intestine. The present study was done, therefore, to investigate mechanisms for cholesterol trafficking in intestinal cells and to determine the importance of plasma membrane cholesterol in triacylglycerol-rich lipoprotein synthesis and secretion.

The results of this study demonstrate that progesterone, trifluoperazine, and verapamil, agents that interfere with vesicular-mediated transport of cholesterol from the plasma membrane to the ER, independent of their inhibition of cholesterol esterification, decrease the secretion of apoB and lipids by CaCo-2 cells. These agents also interfere with the transport of absorbed cholesterol to the ER, indicating that the intracellular transport of cholesterol delivered to intestinal cells in bile salt micelles is vesicular-mediated. As progesterone, trifluoperazine, and verapamil are known to reverse the action of p-glycoprotein (15, review), a plasma membrane transport protein which is present in apical membranes of intestinal cells (16), p-glycoprotein or a related member of the p-glycoprotein ATP-binding cassette family, is implicated as a possible mediator of vesicular transport and thus, of cellular cholesterol trafficking and lipoprotein secretion in intestinal cells.

METHODS AND MATERIALS

Trans-[^{35}S]methionine (1,100 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). [7-^{3}H]cholesterol, [oleoyl-1-^{14}C]oleoyl CoA, [cholesterol-1,2,6,7-^{3}H]cholesteryl linoleate, [9,10-^{3}H]oleic acid, and [1,2,3-^{3}H]glycerol were purchased from New England Nuclear (Boston, MA). Oleoyl CoA, cholesterol, sodium taurocholate, oleic acid, verapamil, progesterone, nigericin, forskolin, amiodarone, reserpine, tamoxifen, colchicine, and methotrexate were from Sigma Chemical Co. (St. Louis, MO). Cyclosporine A was purchased from and 58-035 was a gift from Sandoz, Inc. (East Hanover, NJ). Trifluoperazine was purchased from SmithKline Beecham (Philadelphia, PA). 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 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, Dept. of Pediatrics, University of Arkansas. Protein A bound to Sepharose was purchased from RepliGen (Cambridge, MA). TMB Microwell Peroxidase Substrate System was 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 (17). They were subcultured on polycarbonate micropore membranes inserted in Transwells (Costar, Cambridge, MA). Cell were used 14 days after plating and medium was changed every 2 days.

Esterification of plasma membrane cholesterol

Plasma membrane cholesterol was labeled by incubating cells for 90 min at 4°C with 50 μCi [^{3}H]cholesterol in M199 (medium-199/Earle's, Gibco, Grand Island, NY) containing 1% delipidated fetal calf serum. The solution containing the labeled cholesterol was added to the top well (apical side) only. To remove unincorporated labeled cholesterol, cells were washed twice with cold M199. They were then incubated in M199 containing 0.1% fatty acid-poor bovine serum albumin at 37°C for the time depicted in the figure legends. Agents to be tested were added to both the apical and basolateral medium. Control cells were handled in the same fashion except the medium contained only the vehicle for the agent. After the incubation, cells were washed, scraped from the filter, and lipids were extracted with chloroform–methanol 2:1. Unlabeled cholesterol and cholesteryl oleate mass were added as carriers. The lipids were separated by thin-layer chromatography and cholesterol and cholesteryl ester bands were localized by authentic standards, scraped from the plate, and counted.

ApoB and apoA-I measurements

The estimation of apolipoprotein mass by ELISA, estimation of apolipoprotein synthesis and secretion by pulse-chase, and immunoprecipitation of apolipoproteins were performed exactly as previously described (18).

Lipid synthesis

The rates of lipid synthesis and secretion were estimated by the incorporation of labeled oleic acid or glycerol into the respective lipid within cells and that secreted into the basolateral medium as described (13).

Cholesterol uptake and esterification

Cells were incubated at 37°C with 100 μM [^{3}H]cholesterol solubilized in 1 mM taurocholate in M199 (specific activity, 70,000 dpm/nmol) added to the upper well. Micellar solutions were prepared as previously described (19). At the times specified, cells were rinsed twice with cold M199, scraped from the filter, and lipids were extracted with chloroform–methanol 2:1. Lipids were separated by thin-layer chromatography and the bands corresponding to cholesterol and cholesteryl esters were scraped from the plate and counted.

Enzyme and chemical analyses

Acylcoenzyme A:cholesterol acyltransferase activity and triacylglycerol mass were measured as described (20, 21).

RESULTS

Esterification of plasma membrane cholesterol

To estimate the movement of plasma membrane cholesterol to the ER in CaCo-2 cells, plasma membrane cholesterol was first labeled by incubating cells at 4°C with [^{3}H]cholesterol. After extensive washing, cells were placed in an incubator at 37°C and the amount of labeled cholesteryl esters formed from plasma membrane cholesterol was estimated. The rate of formation of cholesteryl esters was linear over 8 h (data not shown). **Figure 1** shows the effects of increasing concentrations of verapamil, trifluoperazine, progesterone, or methotrexate on the esterification of plasma membrane cholesterol. In control cells incubated in the absence of agents, between 4–6% of plasma membrane cholesterol was esterified by 6 h. Progesterone appeared to be the most potent inhibitor of cholesterol movement to the ER, inhibiting cholesterol esterification by 50% at a concentration of 5 μM and having a maximal inhibitory effect of 95% at 30 μM. Verapamil and trifluoperazine had similar effects on cholesterol transport causing a 50%

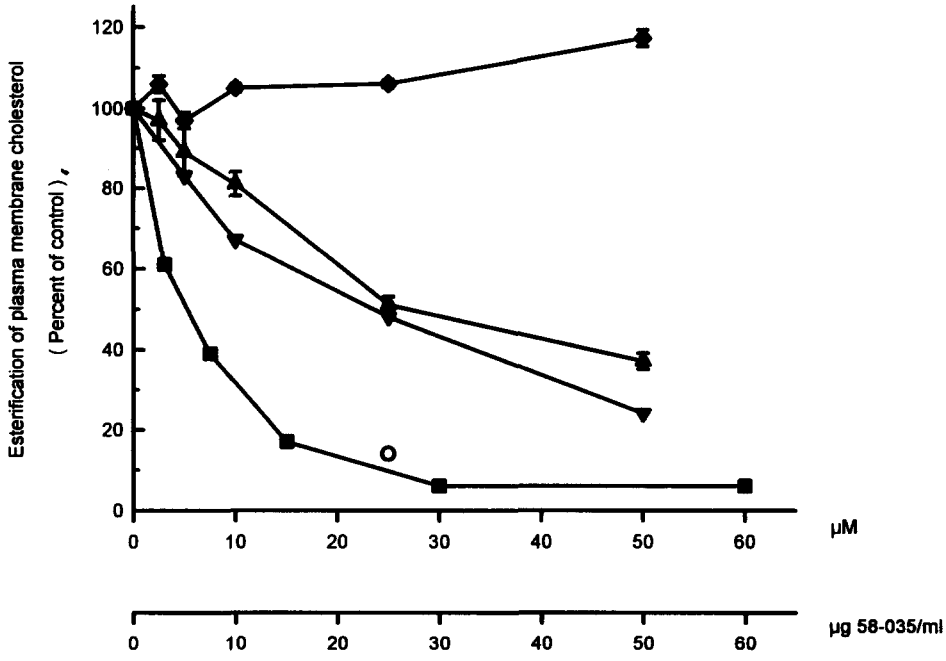

Fig. 1. Effect of progesterone, verapamil, trifluoperazine, methotrexate, or 58-035 on the esterification of plasma membrane cholesterol. Cells were washed twice with ice-cold M199. The basal well was washed once and filled with 2.6 ml of M199. The apical medium was then replaced with cold M199 containing 1% delipidated fetal calf serum and 50 μCi of [^{3}H]cholesterol. After 90 min at 4°C, the apical and basal medium were removed, the cells were washed twice with M199, and a solution containing M199 and 0.1% fatty acid-poor bovine serum albumin plus or minus the agents to be tested was added to the apical and basal wells. Control cells received only the vehicle. After 6 h at 37°C, the cells were washed, scraped from the filter, and the lipids were extracted and separated by thin-layer chromatography. Bands corresponding to unesterified and esterified cholesterol were scraped from the plate and counted. The data represent the mean ± SE of three dishes at each concentration. 58-035 was added at a concentration of 25 μg/ml. (▲) verapamil; (♦) methotrexate; (▼) trifluoperazine; (■) progesterone; (○) 58-035.

inhibition at a concentration of approximately 25 μM. At 50 μM, the highest concentration used, verapamil and trifluoperazine decreased cholesterol esterification by 65 and 75%, respectively. In contrast, methotrexate, an agent that does not interfere with p-glycoprotein function, had no effect on the movement of plasma membrane cholesterol to the ER as the percent of plasma membrane cholesterol esterified was similar to controls. As expected, incubating cells with 25 μg/ml of the ACAT inhibitor, 58-035, decreased the esterification of plasma membrane cholesterol by 86%.

Figure 2 shows data demonstrating the effect of temperature on the esterification of plasma membrane cholesterol. At 4°C, the amount of label found in cholesteryl esters was very small, representing 0.15% of labeled unesterified cholesterol. Moreover, the percent esterified was not altered by the presence of trifluoperazine. Compared to 4°C, 3- and 30-fold more labeled plasma membrane cholesterol was esterified at 23°C and 37°C, respectively. Trifluoperazine significantly inhibited the esterification of cholesterol at both 23°C and 37°C, suggesting that trifluoperazine was interfering with a temperature-sensitive transport process.

Estimating the inhibition of movement of plasma membrane cholesterol to the ER by measuring the decrease in the formation of cholesteryl esters from labeled plasma membrane cholesterol assumes that the agents themselves do not inhibit ACAT activity. To address whether these agents act directly on ACAT, total membranes were prepared from CaCo-2 cells. After adding the respective agent to the enzyme assay mixture, ACAT activity was estimated (**Table 1**). At 1 μg/ml, 58-035, used as a control for this experiment, decreased ACAT activity by 82%. In contrast, trifluoperazine, verapamil, and methotrexate did not inhibit ACAT. In dose-dependent fashion, however, progesterone decreased ACAT activity. At 30 μM, progesterone was as effective as 58-035 in inhibiting ACAT. Thus, it is clear that in cells incubated with progesterone, a decrease in the rate of esterification of plasma membrane cholesterol may not be a good indicator of progesterone's effect on cholesterol transport to the ER.

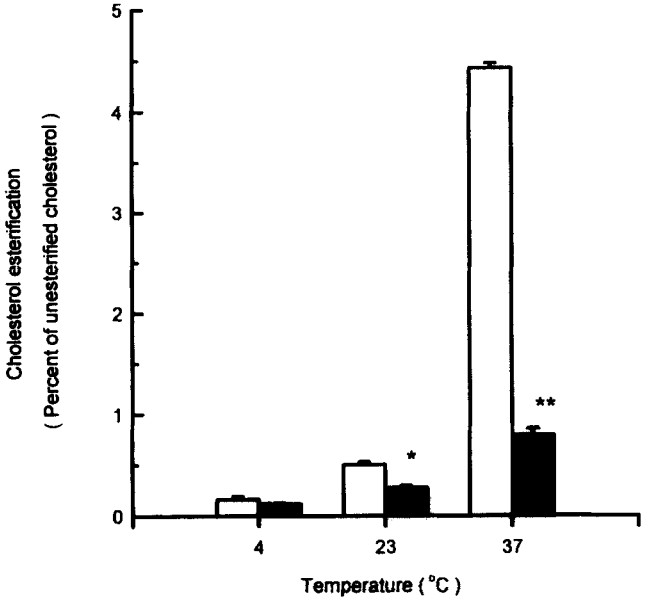

Fig. 2. Effect of temperature on the esterification of plasma membrane cholesterol. The experiment was performed essentially as described in Fig. 1 except that after labeling plasma membrane cholesterol, cells were maintained for 6 h at 4°C, 22°C, or 37°C in the presence or absence of 50 μM trifluoperazine. The data represent the mean ± SE of three to four dishes. (□) control; (■) trifluoperazine.

ApoB mass secretion

To address whether agents that inhibited trafficking of plasma membrane cholesterol to the ER also interfered with the secretion of apoB-containing lipoproteins, cells were incubated for 18 h with 1 mM oleic acid and increasing concentrations of verapamil, trifluoperazine, progesterone, methotrexate, or 58-035. The amount of apoB mass within cells and the amount secreted into the basolateral medium were then estimated. **Figure 3** shows these results. Control cells incubated with 1 mM oleic acid alone secreted approximately 200 ng/well of apoB into the basolateral medium and contained 60 ng/well of apoB intracellularly. Compared to the secretion of apoB by control cells, the secretion of apoB was significantly decreased in cells incubated with verapamil, trifluoperazine, or progesterone. In contrast, methotrexate did not inhibit the secretion of apoB, nor did the potent ACAT inhibitor, 58-035. These results suggest that progesterone (despite its inhibition of ACAT activity), trifluoperazine, and verapamil decreased apoB secretion independently of their interfer-

TABLE 1. Effect of trifluoperazine, verapamil, methotrexate, progesterone, and 58-035 on ACAT activity

	ACAT Activity	Percent of Control
	pmol/mg/30 min	%
Control	489 ± 5	100
Trifluoperazine	450 ± 8	92
Verapamil	434 ± 9	89
Methotrexate	537 ± 21	110
Progesterone		
3 μM	378 ± 9[a]	77
15 μM	212 ± 9[a]	43
30 μM	98 ± 10[a]	20
58-035	88 ± 15[a]	18

Total membranes (100 μg) prepared from CaCo-2 cells were preincubated for 10 min with 50 μM trifluoperazine, verapamil, or methotrexate, 1 μg/ml of 58-035, or increasing concentrations of progesterone. After the incubation, ACAT activity was determined. The data represent the mean ± SE of four determinations.

[a]$P < 0.001$ versus control.

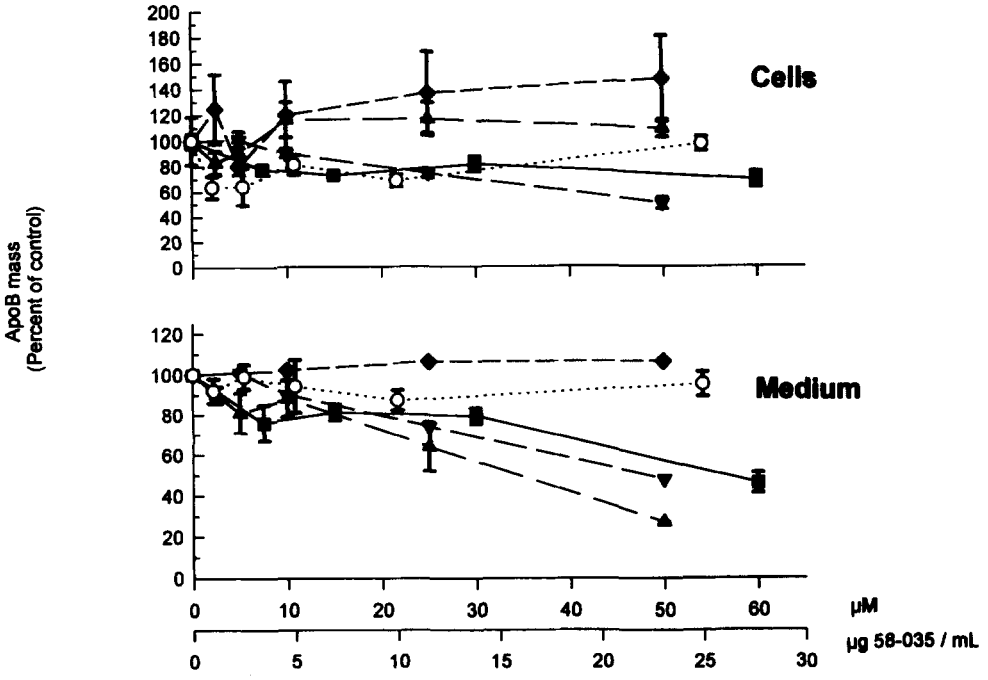

Fig. 3. Effect of progesterone, verapamil, trifluoperazine, methotrexate, or 58-035 on cellular apoB mass and apoB mass secretion. The cells and basolateral wells were washed twice with M199. The basal well was filled with 0.6 ml of M199. The top well was filled with 0.1 ml of M199 containing 1 mM oleic acid attached to 0.33 mM fatty acid-poor bovine serum albumin. The agent to be tested was added to both the basal and apical medium. Control cells received the vehicle only. After 16 h, apoB mass secreted into the basolateral medium was estimated by ELISA. The presence of the agents did not alter the detection of apoB by the assay. The data represent the mean ± SE of 3–10 wells at each concentration. (▲) verapamil; (♦) methotrexate; (▼) trifluoperazine; (■) progesterone; (○) 58-035.

ence with cholesterol esterification. Moreover, although progesterone, trifluoperazine, and verapamil interfere with the secretion of apoB, apoB mass does not accumulate in these cells.

ApoB synthesis and secretion

The effects of progesterone, trifluoperazine, and verapamil on apoB synthesis and secretion were examined by preincubating cells for 1 h with the respective agent. The apical medium was then replaced with medium containing 1 mM oleic acid and [^{35}S]methionine, and the agents were added back to the apical medium of those cells that had them initially. The incorporation of label into cellular and basolateral apoB and apoA-I was estimated after immunoprecipitation and polyacrylamide gel electrophoresis. Progesterone, trifluoperazine, and verapamil did not affect the rate of incorporation of [^{35}S]methionine into cellular apoB, apoA-I, or total proteins (data not shown). **Figure 4** shows representative autoradiograms of labeled apoB and apoA-I immunoprecipitated from the basolateral medium from these experiments. In two separate experiments with six transwells at each time point, the secretion of apoB-100 and apoB-48 into the basolateral medium was significantly decreased by all three agents. The effects of the agents on apoA-I secretion were not as pronounced. Verapamil and trifluoperazine decreased apoA-I secretion by 20 and 45%, respectively ($P < 0.05$) with progesterone having little effect (NS). Compared to controls, only trifluoperazine decreased the secretion of TCA-precipitable protein (30%, $P < 0.05$).

Lipid synthesis and secretion

To estimate the effects of progesterone, trifluoperazine, verapamil, and methotrexate on the rate of synthesis and secretion of lipids, cells were incubated for 18 h with 1 mM oleic acid in the presence or absence of the respective agent. After washing the cells, lipid synthesis and secretion were estimated by adding labeled oleic acid and measuring the incorporation of label into cholesteryl esters, triacylglycerols, and phospholipids within cells and that secreted into the basolateral medium. The results are shown in **Table 2.** To compare the effects of the four agents, the data are expressed as a percent of control cells incubated in the absence of the inhibitors. Incorporation of oleic acid into cellular total lipids was not altered by any of the agents (data not shown). Trifluoperazine decreased cholesteryl ester syn-

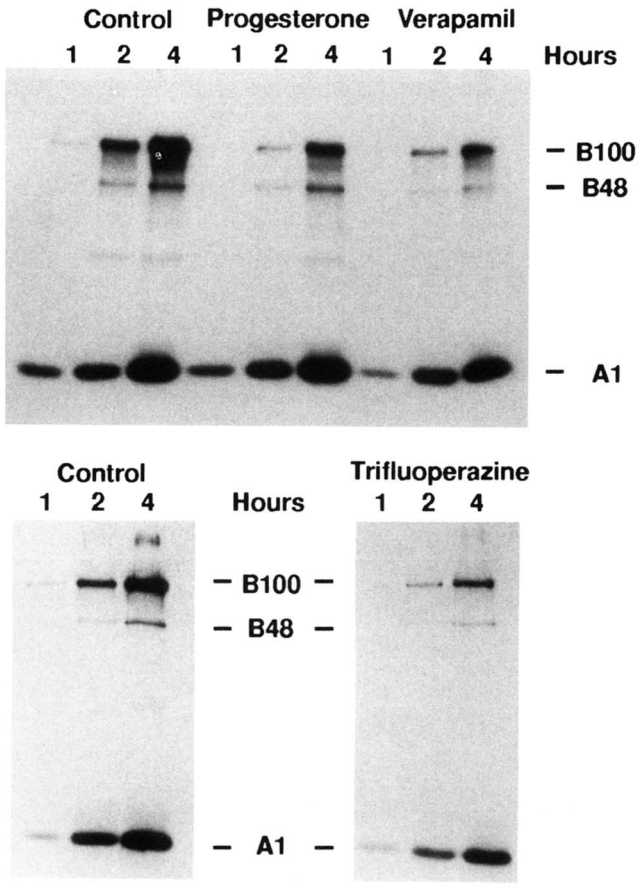

Fig. 4. Effect of progesterone, verapamil, or trifluoperazine on the synthesis and secretion of apoB. The cells and basolateral wells were washed twice with M199. The basal well was filled with 0.6 ml of M199. The cells were preincubated for 1 h in methionine-free medium containing 50 μM of the respective agent. The apical medium was then removed, the cells were washed and incubated with M199 containing 1 mM oleic acid attached to 0.33 mM albumin along with 150 μCi of [^{35}S]methionine and the agent to be tested. At the end of the incubation, apoB and apoA-I were immunoprecipitated from the cells and basolateral medium and the apolipoproteins were separated by polyacrylamide gel electrophoresis. The figure is a representative autoradiogram of apolipoproteins recovered from the basolateral medium from one of two separate experiments showing similar results.

thesis at the two higher concentrations and triacylglycerol synthesis at 50 μM. Phospholipid synthesis was unaffected. Verapamil also decreased the synthesis of cholesteryl esters without altering the synthesis of triacylglycerols or phospholipids. Progesterone decreased the incorporation of oleic acid into cellular cholesteryl esters and phospholipids without altering triacylglycerol synthesis. The secretion of labeled lipids, however, was significantly decreased by all three agents. In a concentration-dependent manner, progesterone decreased the secretion of cholesteryl esters, triacylglycerols, and phospholipids. Trifluoperazine did not alter phospholipid secretion, but cholesteryl ester and triacylglycerol secretion were significantly decreased. Verapamil, like progesterone, decreased cholesteryl ester and triacylglycerol secretion in a concentration-dependent manner. In contrast, methotrexate had no effect on lipid synthesis or secretion.

To further address and substantiate the effects of these agents on lipid synthesis and secretion, cells were incubated for 18 h with 1 mM oleic acid with or without increasing concentrations of progesterone or trifluoperazine. The next morning, labeled glycerol was added to the cells and the incorporation of label into cellular and basolateral triacylglycerols and phospholipids was estimated. In another experiment, the amount of triacylglycerol mass secreted into the basolateral medium was also estimated. The results are shown in **Table 3.** Progesterone decreased triacylglycerol and phospholipid synthesis by approximately 30 and 65%, respectively. In contrast, trifluoperazine did not alter the incorporation of glycerol into either cellular triacylglycerols or phospholipids. Both progesterone and trifluoperazine, however, markedly inhibited the secretion of glycerol-labeled lipids. The secretion of triacylglycerol mass was also decreased in a concentration-dependent manner by progesterone and trifluoperazine. Although differences exist between the results obtained by glycerol and oleic acid incorporation into cellular and medium lipids, these results, taken together with the effects of these agents on apoB secretion, strongly suggest that progesterone, trifluoperazine, and verapamil inhibit the secretion of triacylglycerol-rich lipoproteins by CaCo-2 cells.

Cholesterol uptake and esterification

In the intestine, dietary and biliary cholesterol are absorbed from bile salt micelles. To address whether cholesterol taken up from micelles enters and mixes with the pool of cholesterol in the plasma membrane, which is then internalized and shuttled to the ER, cells were preincubated for 1 h in the presence or absence of progesterone, trifluoperazine, or verapamil. Labeled cholesterol solubilized in 1 mM taurocholate was then added to the apical medium and the uptake of cholesterol and its esterification were estimated. The results shown in **Fig. 5** suggest that the amount of labeled cholesterol taken up by cells from micelles is not significantly affected by the agents. Trifluoperazine and verapamil, which significantly inhibited the transport of plasma membrane cholesterol to the ER (and thus its esterification), also decreased the esterification of the absorbed cholesterol. As expected, progesterone, which also inhibits ACAT directly, significantly decreased the esterification of the cholesterol taken up from micelles.

TABLE 2. Effect of trifluoperazine, verapamil, progesterone, or methotrexate on [^{3}H]oleic acid incorporation into cellular lipids and lipids secreted into the basolateral medium

	Cells			Medium		
	CE	TG	PL	CE	TG	PL
	% of control			*% of control*		
Trifluoperazine						
5 μM	103 ± 4	99 ± 2	103 ± 2	87 ± 6	86 ± 4	91 ± 2
10 μM	108 ± 3	106 ± 1	109 ± 1	85 ± 3	81 ± 3	97 ± 7
25 μM	64 ± 3[b]	109 ± 2	106 ± 3	22 ± 2[b]	42 ± 2[b]	77 ± 4[a]
50 μM	40 ± 1[b]	68 ± 3[b]	145 ± 3	14 ± 3[b]	3 ± 1[b]	107 ± 10
Verapamil						
5 μM	69 ± 6[a]	83 ± 4	85 ± 6	74 ± 5[a]	79 ± 3[a]	93 ± 8
10 μM	66 ± 2[a]	87 ± 5	82 ± 5	61 ± 4[a]	73 ± 3[a]	77 ± 3[a]
25 μM	46 ± 2[b]	87 ± 1	84 ± 2	18 ± 3[b]	40 ± 7[b]	115 ± 19
50 μM	40 ± 2[b]	82 ± 2	94 ± 3	6 ± 2[b]	5 ± 1[b]	87 ± 7
Progesterone						
7.5 μM	93 ± 7	110 ± 4	96 ± 4	76 ± 4[a]	84 ± 3	81 ± 3
15 μM	61 ± 3[a]	107 ± 4	88 ± 3	32 ± 3[b]	61 ± 2[b]	58 ± 4[b]
30 μM	25 ± 1[b]	109 ± 1	85 ± 2	9 ± 1[b]	39 ± 2[b]	40 ± 1[b]
60 μM	10 ± 2[b]	97 ± 1	64 ± 1[a]	nd[b]	10 ± 1[b]	29 ± 1[b]
Methotrexate						
5 μM	100 ± 8	101 ± 6	98 ± 3	96 ± 8	102 ± 4	106 ± 1
10 μM	106 ± 8	101 ± 6	97 ± 6	101 ± 8	101 ± 3	98 ± 2
25 μM	117 ± 6	113 ± 1	105 ± 2	107 ± 19	105 ± 5	98 ± 1
50 μM	104 ± 5	108 ± 5	113 ± 3	90 ± 2	108 ± 3	100 ± 3

CaCo-2 cells were incubated for 18 h with 1 mM oleic acid attached to 0.33 mM albumin and increasing concentrations of the respective agent. The cells were washed and [^{3}H]oleic acid (25 μM, sp act 5,000 dpm/nmol) was added for 4 h and the incorporation of label into cellular and basolateral medium lipids was determined. The data represent the mean ± SE of four transwells at each concentration. The average values for oleate incorporation into cellular CE, TG, and PL were approximately 14, 500, and 170 pmol incorporated/filter per 4 h, respectively, and for medium CE, TG, and PL were 1, 35, and 3 pmol incorporated/filter per 4 h, respectively. nd, not detected.

[a]$P < 0.02$ versus control.

[b]$P < 0.001$ versus control.

P-glycoprotein, cholesterol trafficking, and lipoprotein secretion

A common denominator among progesterone, trifluoperazine, and verapamil is that they all have been shown to interact with p-glycoprotein in reversing the multidrug resistance phenotype. In the present study, these three agents interfered with the transport of cholesterol and the secretion of apoB, apoA-I, and lipids. In contrast, methotrexate, an agent that does not interact with p-glycoprotein, did not alter cholesterol trafficking or lipoprotein secretion. It was postulated, therefore, that p-glycoprotein or a member of the p-glycoprotein family may be involved in cholesterol trafficking and lipoprotein secretion in intestinal cells.

To investigate this, several other agents known to interact with p-glycoprotein were tested to examine their effects on cholesterol transport and apoB mass secretion. The results are shown in **Table 4.** Compared to cells incubated with vehicle alone, amiodarone, colchicine, cyclosporine A, forskolin, reserpine, and tamoxifen significantly decreased the transport of cholesterol and the secretion of apoB.

As it is believed that p-glycoprotein may function to maintain the acidification of endosomes by acting as a proton pump and chloride channel, nigericin, a potent potassium ionophore that causes the exchange of potassium ions for hydrogen ions, was used to address whether alkalinization of endosomes might be playing a role in the regulation of cholesterol transport and apoB secretion. In a concentration an order of magnitude less than p-glycoprotein inhibitors, nigericin caused a marked decrease in cholesterol transport and apoB secretion (Table 4), suggesting that maintenance of endosomal pH may be important in cholesterol trafficking and apoB secretion.

DISCUSSION

The results of this study clearly demonstrate that drugs which interfere with cholesterol trafficking from the plasma membrane to the ER also cause the disruption of triacylglycerol-rich lipoprotein secretion in CaCo-2 cells. Although it was initially postulated that esterified cholesterol synthesized from the plasma mem-

TABLE 3. Effect of progesterone and trifluoperazine on [^{3}H]glycerol incorporation into triacylglycerols and phospholipids within cells and those secreted into the basolateral medium and the secretion of triacylglycerol mass

	[^{3}H]Glycerol Incorporation				Triacylglycerol Mass
	Cells		Medium		Medium
	TG	PL	TG	PL	TG
	% of control				*% of control*
Progesterone					
7.5 μM	80 ± 2[a]	61 ± 3[b]	39 ± 2[b]	42 ± 2[b]	76
15 μM	77 ± 4[a]	42 ± 2[b]	22 ± 2[b]	26 ± 3[b]	63
30 μM	66 ± 2[b]	32 ± 1[b]	9 ± 1[b]	20 ± 2[b]	43
60 μM	74 ± 1[a]	35 ± 1[b]	8 ± 1[b]	27 ± 2[b]	15
Trifluoperazine					
5 μM	98 ± 6	107 ± 4	94 ± 4	86 ± 5	100
10 μM	96 ± 3	104 ± 2	65 ± 5[b]	63 ± 10[a]	96
25 μM	112 ± 5	125 ± 6	47 ± 5[b]	50 ± 6[b]	76
50 μM	88 ± 3	145 ± 5	5 ± 2[b]	8 ± 1[b]	51

The experiment was performed as described in Table 2. After the incubation, cells were washed and labeled glycerol (10 μCi, sp act 450 dpm/pmol) was added for 4 h and the incorporation of label into cellular and medium triacylglycerols and phospholipids was determined. For determining the amount of triacylglycerol mass secreted, after the 18-h incubation, the lipids were extracted from the basolateral medium and triacylglycerol mass was estimated. The data represent the mean ± SE from four transwells. Triacylglycerol mass estimates were performed in duplicate. The average values for glycerol incorporation into cellular TG and PL were 460 and 160 pmol incorporated/filter per 4 h, respectively; and for medium TG and PL were 20 and 5 pmol incorporated/filter per 4 h, respectively.

[a]$P < 0.02$ versus control.

[b]$P < 0.001$ versus control.

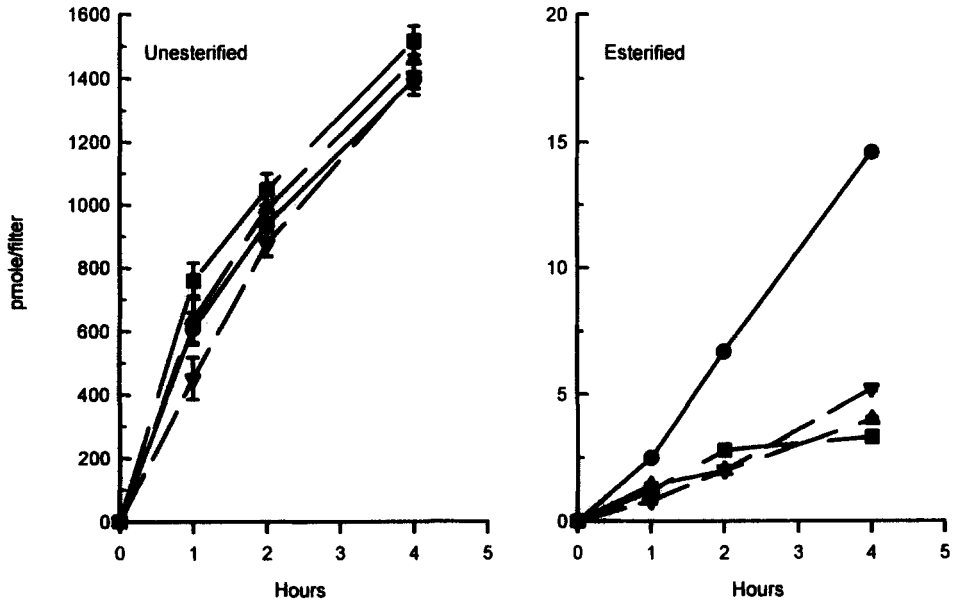

Fig. 5. Effect of progesterone, verapamil, or trifluoperazine on the uptake and esterification of micellar cholesterol. The cells and basolateral wells were washed twice with M199. The basal well was filled with 2.6 ml of M199 containing 50 μM of the agent to be tested. The cells were preincubated for 1 h with M199 containing 50 μM of the agent. After 1 h, the cells were washed twice with M199 and incubated with M199 containing 1 mM taurocholate and 100 μM of [^{3}H]cholesterol. The agent to be tested was added back to the apical medium. At 1, 2, and 4 h, the cells were washed with M199, scraped from the filter, and the lipids were extracted and separated by thin-layer chromatography. Bands corresponding to unesterified and esterified cholesterol were scraped from the plate and counted. The data represent the mean ± SE of four dishes at each time point from two different experiments. (●) control; (■) progesterone; (▲) verapamil; (▼) trifluoperazine.

TABLE 4. Effect of agents known to interfere with p-glycoprotein function on plasma membrane cholesterol esterification and apoB mass secretion

	Plasma Membrane	
	Cholesterol Esterification	ApoB Mass Secretion
	% of control	
Control	100	100
Amiodarone	34 ± 3[a]	72 ± 3[a]
Colchicine	44 ± 1[a]	33 ± 1[a]
Cyclosporine A	20 ± 3[a]	24 ± 1[a]
Forskolin	48 ± 2[a]	38 ± 2[a]
Reserpine	80 ± 6[b]	92 ± 1[b]
Tamoxifen	27 ± 1[a]	58 ± 3[a]
Nigericin	42 ± 2[a]	27 ± 3[a]

The experimental protocols were exactly as described for Figs. 1 and 3, respectively. The concentration of the agents was 50 μM except for colchicine and nigericin which were 500 μM and 1 μM, respectively. The data represent the mean ± SE of four transwells for plasma membrane cholesterol esterification and nine transwells for apoB mass. The average percentage of plasma membrane cholesterol esterified in 6 h was 5% for control cells. The average apoB mass secreted for control cells was 200 ng/filter.

[a]$P < 0.001$ versus control.

[b]$P < 0.05$ versus control.

brane cholesterol pool was necessary for normal lipoprotein assembly and secretion to occur, this was not the case. 58-035, a potent ACAT inhibitor that decreased the esterification of plasma membrane cholesterol by 86%, had no significant effect on apoB secretion. There is ongoing debate within the literature regarding the importance of cholesteryl esters, triacylglycerols, and phospholipids in hepatic lipoprotein secretion (6, 7). Our combined data generated in CaCo-2 cells would argue that there is little evidence for a relationship between newly synthesized cholesteryl esters and normal lipoprotein synthesis and secretion. In an early study, we found that the secretion of newly synthesized triacylglycerols was unaltered by inhibiting ACAT activity and cholesteryl ester synthesis by a potent ACAT inhibitor, PD128,042 (22). In a more recent study, CaCo-2 cells incubated with sphingomyelinase secreted significantly less apoB despite an increase in the rate of cholesteryl ester synthesis and cholesteryl ester mass within these cells (12). We would conclude, therefore, that the assembly and secretion of apoB-containing lipoproteins by CaCo-2 cells are independent of changes occurring in rates of cholesterol ester synthesis or cholesteryl ester mass.

It is possible that interfering with the arrival of unesterified cholesterol to the ER could disrupt lipoprotein secretion by decreasing the amount of sterol required for the normal assembly of surface components. In hepatocytes, for example, there is evidence to suggest that triacylglycerol-rich lipoprotein secretion can be decreased under conditions in which unesterified cholesterol is limiting (23). From recent studies, however, we believe this can be ruled out as well. In CaCo-2 cells incubated with phosphatidylcholine or lysophosphatidylcholine, the esterification of cholesterol was markedly decreased because of an efflux of plasma membrane cholesterol into the medium (13, 24). Despite a marked decrease in the influx of plasma membrane cholesterol and a decrease in the synthesis of cholesterol esters, both phosphatidylcholine and lysophosphatidylcholine significantly increased the secretion of apoB. Thus, the results would suggest that progesterone, trifluoperazine, and verapamil decrease apoB secretion independently of their effects on the transport of plasma membrane cholesterol to the ER and their effects on cholesterol esterification.

The results, however, do implicate a possible role for p-glycoprotein or a member of the ATP-ase binding cassette family in the normal transport of plasma membrane cholesterol to the ER and in the secretion of triacylglycerol-rich lipoproteins by the intestine. P-glycoprotein is expressed in absorptive cells of the small intestine, but the normal function for this transporter remains in question (16, 25). Progesterone, trifluoperazine, and verapamil are known to interact with p-glycoprotein to modulate its function (15); and, in fact, verapamil has recently been used in CaCo-2 cells to inhibit the function of p-glycoprotein causing a decrease in the basolateral to apical transport of vinblastine (26). These modulators, however, have other intracellular actions that make it difficult to specifically define their effects only to interfering with p-glycoprotein function. While this may be true, the observed relationship between the effects of a wide range of agents, all recognized to interfere with p-glycoprotein (Table 4), on cholesterol transport and apoB secretion is highly suggestive that p-glycoprotein is involved. As it is believed that intracellular cholesterol is transported within vesicles and that lipoprotein secretion is dependent upon normal vesicular function, the data would suggest that p-glycoprotein may be important in the normal movement of transport vesicles. CFTR, a member of the ABC binding cassette family, has been implicated in regulating cAMP-dependent endocytic trafficking (27). Because CFTR expression is turned off and p-glycoprotein expression turned on as the intestinal cell moves up the villus (28), perhaps p-glycoprotein functions to regulate vesicular trafficking and thus, lipid transport in the mature absorptive cell. Moreover, p-glycoprotein, as is CFTR, acts as a chloride channel and functions to control cell volume and possibly endosome acidification (29, review). Defective organelle acidification can lead to impaired endocytosis, protein processing, and pro-

tein glycosylation, any of which could cause a decrease in apoB secretion and interfere with cholesterol transport. Support for this possibility was demonstrated by the effects of nigericin. Nigericin, a potassium ionophore that promotes the exchange of potassium for hydrogen ions causing alkalinization of cells and subcellular compartments (30), on a mole-to-mole basis, was significantly more potent in decreasing cholesterol transport and apoB secretion than were progesterone, trifluoperazine, or verapamil. In data not shown, nigericin also potently inhibited the secretion of newly synthesized triacylglycerols. Further studies would be required, however, to definitively determine whether p-glycoprotein functions to maintain organelle acidification in CaCo-2 cells.

It also seemed reasonable to assume that cholesterol taken up from bile-salt micelles would equilibrate with the apical plasma membrane of the cell and be transported together with the plasma membrane cholesterol pool into the cell. The observation that verapamil, trifluoperazine, and progesterone interfered with the transport of micellar cholesterol suggests that normal vesicular trafficking is required for the esterification of absorbed cholesterol. As ACAT inhibitors have been shown to interfere with the absorption of cholesterol (31, 32), it is quite possible that agents that interfere with the intracellular trafficking of cholesterol might also inhibit cholesterol absorption by decreasing the sterol's entry into the ACAT pool.

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