Transport of cholesterol from the endoplasmic reticulum to the plasma membrane is constitutive in CaCo-2 cells and differs from the transport of plasma membrane cholesterol to the endoplasmic reticulum

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Abstract The transport of newly synthesized cholesterol from its site of synthesis, the endoplasmic reticulum, to the plasma membrane was studied in CaCo-2 cells. The appearance of newly synthesized cholesterol on the cell surface was rapid. By 30 min, 50% of the total labeled cholesterol was observed in the plasma membrane. The arrival of cholesterol at the plasma membrane was independent of new protein synthesis, a functional Golgi apparatus, or microtubular function. Progesterone, verapamil, and trifluoperazine, inhibitors of p-glycoprotein which are known to inhibit cholesterol transport from the plasma membrane to the endoplasmic reticulum, reduced the amount of newly synthesized cholesterol reaching the plasma membrane. The p-glycoprotein inhibitors, however, caused the accumulation of sterol intermediates in the plasma membrane, suggesting that sterol trafficking to the plasma membrane remained intact, but that trafficking from the plasma membrane to the endoplasmic reticulum was disrupted. In contrast, nigericin, another potent inhibitor of cholesterol movement from the plasma membrane to the endoplasmic reticulum, did not alter the transport of newly synthesized cholesterol to the plasma membrane. Moreover, promoting cholesterol transport from the plasma membrane to the endoplasmic reticulum by sphingomyelin hydrolysis or by micellar cholesterol influx did not alter the percent of newly synthesized cholesterol transported to the plasma membrane. Likewise, preventing plasma membrane cholesterol from reaching the endoplasmic reticulum by incubating cells with lysophosphatidylcholine, filipin, or digitonin did not alter the arrival of newly synthesized cholesterol to the plasma membrane. The results suggest that the amount of cholesterol moving to the plasma membrane from the endoplasmic reticulum is constitutive and regulated at the level of cholesterol synthesis and not at the level of the transport process. The pathways of cholesterol transport to and from the plasma membrane are distinct.—**Field, F. J., E. Born, S. Murthy, and S. N. Mathur.** Transport of cholesterol from the endoplasmic reticulum to the plasma membrane is constitutive in CaCo-2 cells and differs from the transport of plasma membrane cholesterol to the endoplasmic reticulum. *J. Lipid Res.* 1998. **39:** 333–343.

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Most cholesterol within a cell is localized to the plasma membrane (1–3). Intracellular sorting of newly synthesized cholesterol and trafficking of plasma membrane cholesterol into the cell interior, therefore, cannot be a random process. To date, most evidence would suggest that cholesterol is transported from the plasma membrane to the endoplasmic reticulum in special transport vesicles likely originating from the plasma membrane itself (3, 4). This influx of cholesterol from the plasma membrane to the endoplasmic reticulum is important for maintaining cholesterol homeostasis. For example, in response to an accumulation of cholesterol in the plasma membrane, more cholesterol influxes to the endoplasmic reticulum to be esterified by acyl-coenzyme A: cholesterol acyltransferase, thereby preventing potentially harmful changes to membrane structure and function (5–7). Moreover, in times of an immediate need for cholesterol, the plasma membrane can serve as a readily available and accessible source. In steroid-hormone producing cells, plasma membrane cholesterol is preferentially used for hormone synthesis when demand is high (8–10). Likewise, when the demand for cholesterol is increased in intestinal cells during lipoprotein assembly,

Abbreviations: MDR, multiple-drug resistance protein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acyl-CoA: cholesterol acyltransferase; CHO, Chinese hamster ovary.

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the cholesterol secreted in these lipoproteins is derived predominately from the plasma membrane (5). MDR, or multiple-drug resistance protein, a member of the p-glycoprotein ATPase binding cassette transport family, has been implicated in intracellular cholesterol transport. It has been shown that progesterone, a recognized inhibitor of p-glycoprotein function, as well as other sterol and nonsterol inhibitors of p-glycoprotein, interfere with the trafficking of plasma membrane cholesterol to the endoplasmic reticulum (11–13).

The movement of newly synthesized cholesterol from its site of synthesis, the endoplasmic reticulum, to the plasma membrane is also important for maintaining cholesterol homeostasis. In the intestinal absorptive cell, for example, it could be argued that during lipoprotein assembly and secretion, which will result in the loss of cholesterol from the plasma membrane, the movement of newly synthesized cholesterol to the plasma membrane is necessary to replenish the membrane with cholesterol in order to maintain its normal lipid composition. This would ensure proper structure and function of the plasma membrane and also provide a continuous supply of accessible cholesterol. Results from other cell types would suggest that cholesterol is transported from its site of synthesis to the plasma membrane in unique transport vesicles by a route that differs from the secretory pathway (14, 15). Unlike other cells, however, the intestinal cell is unique. It can absorb cholesterol and, therefore, perhaps utilize dietary or biliary cholesterol for the purpose of replenishing the lost cholesterol from the plasma membrane. There is no information in intestinal cells as to whether this flux of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane is a regulated process, whether it differs from the vesicular pathway of plasma membrane cholesterol influx, or whether p-glycoprotein is involved.

The results of this study, performed in cultured human intestinal cells, CaCo-2, show that inhibitors of p-glycoprotein function interfere with newly synthesized cholesterol reaching the plasma membrane by disrupting the return of cholesterol intermediates from the plasma membrane to the endoplasmic reticulum for completion of biosynthesis. Trafficking of newly synthesized cholesterol to the plasma membrane is independent of the Golgi apparatus, microtubular function, or protein synthesis. Interfering with cholesterol influx from the plasma membrane to the endoplasmic reticulum does not alter cholesterol transport from the endoplasmic reticulum to the plasma membrane, suggesting that the two transport pathways are dissimilar. Cholesterol absorption (influx) or cholesterol efflux, both of which cause marked changes in the rates of cholesterol biosynthesis, do not alter the efficiency in which newly synthesized cholesterol reaches the plasma membrane, suggesting that trafficking of newly synthesized cholesterol to the plasma membrane is a constitutive process. The amount of cholesterol arriving at the plasma membrane from the endoplasmic reticulum, therefore, is regulated by changes in the rates of cholesterol synthesis and not by steps involved in this transport process.

MATERIALS AND METHODS

[^{14}C]acetate was purchased from New England Nuclear (Boston, MA). Cholesterol, cholesterol oxidase, cholestenone, acetate, sodium taurocholate, verapamil, and progesterone were from Sigma Chemical Co. (St. Louis, MO). Trifluoperazine was purchased from Smith Kline Beecham (Philadelphia, PA).

Cell culture

CaCo-2 cells were grown in T-75 flasks as described previously (5). They were subcultured on polycarbonate micropore membranes inserted in Transwells (Costar, Cambridge, MA). Cells were used 14 days after plating and medium was changed every 2 days. At this time, CaCo-2 cells have typical epithelial cell morphology and secrete lipoproteins predominantly to the basolateral side (5, 16). All treatments were only added to the apical side.

Oxidation of plasma membrane cholesterol

The cholesterol oxidase methodology used to estimate the amount of newly synthesized cholesterol found on the cell surface of CaCo-2 cells has been previously described (5). After the incubation as described for each experiment, the cells were washed twice with ice-cold phosphate-buffered saline. After washing two more times with 10 mm sodium phosphate buffer (pH 7.4), the cells were incubated for 10 min at 4°C with water. The water was removed and the cells were fixed by incubating them for 10 min at 4°C with 1% glutaraldehyde. After removing the glutaraldehyde, the cells were washed twice with 10 mm sodium phosphate buffer (pH 7.4) containing 310 mm sucrose that had been warmed to 37°C. They were then incubated in this buffer for 15 min at 37°C. Cholesterol oxidase, 100 IU/ml, was added and the cells were incubated for 45 min at 37°C. The cells were again washed twice with cold phosphate-buffered saline and the lipids were extracted with hexanes–isopropanol–water 3:2:0.1 (v/v). Unlabeled cholestenone was added as a carrier. The lipid extract was dried under a stream of nitrogen and the lipids were saponified by heating at 80°C in an alkaline methanol solution. The lipids were extracted with hex-

ane and washed three times with an equal volume of alkaline water–methanol 1:1 (v/v) to remove fatty acids. Cholesterol, cholestenone, and other non-saponifiable sterols were separated by thin-layer chromatography. The plates were eluted twice in heptane–diethyl ether–acetic acid, 85:15:1 (v/v) as the elution solvent. Bands corresponding to cholesterol and cholestenone were scraped from the plate and counted. Using this procedure, recoveries for radiolabeled cholestenone were between 92 and 95%. The radioactivity in the cholesterol and cholestenone bands was added to determine the amount of total newly synthesized cholesterol. The amount of cholesterol on the cell surface was calculated by dividing the radioactivity in the cholestenone fraction by the total cholesterol.

Estimation of cholesterol synthesis

Cholesterol synthesis was estimated by the incorporation of [^{14}C]acetate into cholesterol as previously described (17, 18). The final acetate concentration was 60 μm with a specific activity of 130 dpm/pmol. The data are given as pmol of [^{14}C]acetate incorporated into sterols.

Subcellular fractionation of CaCo-2 cells

CaCo-2 cells grown on micropore membranes were incubated for 5 h with 60 μm [^{14}C]acetate (130 dpm/pmol) with or without treatments. Labeled cells from six filters for each treatment were combined with cells from one T-75 flask to provide cell mass for the sucrose gradient. The cells were suspended in 2 ml of hypotonic buffer, pH 7.4, containing 1 mm Tris, 75 mm sucrose, 0.5 mm dithiothreitol, 42 μm leupeptin, and 0.5 mg/ml soybean trypsin inhibitor. The cell suspensions were homogenized in a Potter-Elvehjem homogenizer with 50 downward strokes. The total membranes were sedimented by ultracentrifugation at 100,000 *g* for 1 h. The total membrane pellet was resuspended in 2.2 ml of 60% sucrose/1 mm Tris-HCl solution and layered in a 13.2-ml tube on a cushion of 1.2 ml 66% sucrose solution followed by 1.2 ml of 55–20% sucrose solutions at a 5% interval. The gradients were centrifuged in a Beckman SW 41 Ti rotor at 118,610 *g* for 18 h. The fractions were collected from the top in 0.5 ml-aliquots, kept on ice, and diluted with cold 1 mm Tris-HCl to a total volume of 1.5 ml. Aliquots were taken for determining: protein, cholesterol mass, RNA, newly synthesized cholesterol, and alkaline phosphatase activity. Newly synthesized cholesterol was measured by thin-layer chromatography as previously described. Alkaline phosphatase activity was estimated by measuring absorbance of *p*-nitrophenol at 405 nm generated by the hydrolysis of 6 mm disodium *p*-nitrophenyl phosphate in a buffer of pH 10.4 containing 0.1 m glycine, 1 mm magnesium chloride, and 1 mm zinc chloride. Protein was estimated using Eosin B dye to reduce interference by sucrose as described by Waheed and Gupta (19). For RNA estimation (20), 1 ml of ice-cold 6% perchloric acid was added to 500 μl of the diluted fraction and kept on ice for at least 3 h. The precipitate was collected by centrifugation at 16,000 *g* for 7 min and washed once with 3% perchloric acid. The sediment was dissolved in 200 μl 1 N NaOH by incubation at 37°C for 1 h. The protein and DNA were precipitated by adding 800 μl ice-cold 3% perchloric acid and centrifugation at 16,000 *g* for 10 min. The RNA in the supernatant was estimated at 260 nm. Cholesterol mass was determined using a total cholesterol kit (Catalog #352-100) from Sigma, St. Louis, MO.

Separation of non-saponifiable lipids by HPLC

Non-saponifiable lipids were separated on an All-Tech/Applied Science Adsorbosphere C18, 3 micron (4.6 × 150 mm) column. The lipids were eluted with 15% water in methanol at a flow rate of 0.7 ml/min. The radioactivity in the column effluent was determined using a β-RAM radioactivity detector from IN/US Systems, Inc., Tampa, FL. A Gilson Datamaster system was used to integrate the detector signal and control the pumps.

Statistical analysis

The data were analyzed by Dunnett's method to compare treatment groups with the control group at alpha value of 0.05 using SIGMASTAT software from Jandel Scientific (San Rafael, CA).

RESULTS

Cholesterol synthesis and trafficking to the plasma membrane

To address the rate of movement of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane in CaCo-2 cells, cells were incubated for 5 h with labeled acetate. After the incubation, cells were fixed and the amount of label incorporated into total cholesterol and cholesterol on the cell surface was estimated after the addition of cholesterol oxidase (**Fig. 1**). At 30 min, the earliest time point which provided sufficient label in cholesterol, 47% of the total label was found on the cell surface. By 5 h, 72% of the newly synthesized cholesterol was found on the cell surface. In experiments to be described later, there is good correlation between the cholesterol oxidase methodology for estimating the percent of newly synthesized cholesterol in the plasma membrane and subcellular fractionation.

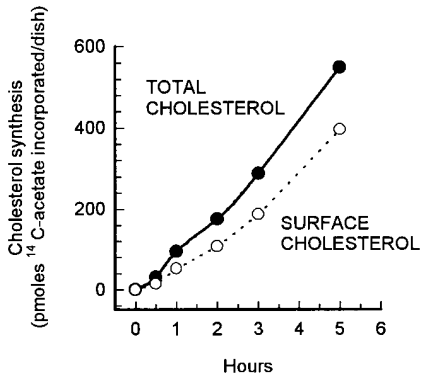

Fig. 1. Transport of newly synthesized cholesterol to the cell surface. CaCo-2 cells grown on micropore membranes were incubated for up to 5 h with 60 μm [^{14}C]acetate (130 dpm/pmol). At each time point indicated, the cells were harvested and radioactivity incorporated into total and surface cholesterol was estimated as described in Methods. The amount of protein per insert was 1 ± 0.05 mg. The values are an average of duplicate filters at each time point. This experiment is representative of three separate experiments.

TABLE 1. Effect of inhibition of Golgi function, microtubules, and protein synthesis on the incorporation of [^{14}C]acetate into total and surface cholesterol

Treatment	Cholesterol		
	Total	Surface	Percent on Surface
	pmol [^{14}C]acetate incorporated/6 h		*%*
Control	707 ± 17	561 ± 17	79 ± 1
Brefeldin A	338 ± 22[a]	255 ± 18[a]	76 ± 1
Monensin	403 ± 15[a]	291 ± 10[a]	72 ± 1
Colchicine	606 ± 11[a]	449 ± 14[a]	74 ± 1
Cycloheximide	448 ± 14[a]	327 ± 11[a]	73 ± 1

CaCo-2 cells grown on micropore membranes were incubated for 6 h with buffer alone, 0.25 μg/ml brefeldin A, 10 μm monensin, 500 μm colchicine, or 5 μm cycloheximide. To label newly synthesized cholesterol, 60 μm [^{14}C]acetate (130 dpm/pmol) was added with the treatment at the beginning of the incubation. The amount of radioactivity incorporated into total cholesterol and cholesterol present on the cell surface was estimated using cholesterol oxidase as described in methods. The values are the mean ± SEM of four filters.

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

Effect of inhibitors of Golgi, microtubules, and protein synthesis

In non-intestinal cells, transport of cholesterol from the endoplasmic reticulum to the plasma membrane is thought to be independent of the secretory pathway (14, 15). To investigate whether the transport of newly synthesized cholesterol to the plasma membrane required a normal secretory process in intestinal cells, cells were incubated with agents that are known to disrupt the function of Golgi (brefeldin A and monensin), microtubules (colchicine), or protein synthesis (cycloheximide). The effect of these agents on cholesterol synthesis and the transport of newly synthesized cholesterol to the cell surface was then estimated (**Table 1**). Both brefeldin A and monensin caused a significant decrease in the synthesis of total cholesterol. The percent of newly synthesized cholesterol reaching the plasma membrane, however, was similar to that observed in control cells. Colchicine modestly decreased total cholesterol synthesis, but it too did not alter the transport of cholesterol to the plasma membrane. Likewise, for newly synthesized cholesterol to reach the plasma membrane, new protein synthesis was not required. Compared to control cells, cells incubated with cycloheximide had a similar percent of labeled cholesterol found on the cell surface.

Effect of decreasing cholesterol influx from the plasma membrane

Depending upon the cell's immediate requirement for cholesterol, it would seem reasonable to assume that an intestinal cell could regulate the amount of newly synthesized cholesterol arriving at its plasma membrane by altering the efficiency of transport of the sterol to the surface. To investigate this possibility, agents were used that are known to interfere with the influx of plasma membrane cholesterol to the cell's interior. It is postulated that the cell would interpret this as a cholesterol deficit because less cholesterol would be entering the regulatory pools of cholesterol within the endoplasmic reticulum. Cells were incubated with labeled acetate and filipin or digitonin, two agents that

TABLE 2. Effect of decreasing the influx or increasing the efflux of plasma membrane cholesterol on the incorporation of [^{14}C]acetate into total and surface cholesterol

Treatment	Cholesterol		
	Total	Surface	Percent on Surface
	pmol [^{14}C]acetate incorporated/6 h		*%*
Control	438 ± 13	344 ± 13	78 ± 1
Digitonin	601 ± 23[a]	460 ± 17[a]	77 ± 1
Filipin	783 ± 27[a]	631 ± 23[a]	80 ± 1
Taurocholate	381 ± 11	308 ± 10	81 ± 1
Taurocholate + LPC	588 ± 17[b]	419 ± 16[b]	71 ± 1

CaCo-2 cells grown on micropore membranes were incubated for 6 h with buffer alone, 5 μg/ml digitonin, or 5 μg/ml filipin. Another set of cells was incubated for 6 h with 5 mm taurocholate or 5 mm taurocholate + 250 μm lysophosphatidylcholine (LPC). To label newly synthesized cholesterol, 60 μm [^{14}C]acetate (130 dpm/pmol) was added with the treatment at the beginning of the incubation. Radioactivity incorporated into total and surface cholesterol was estimated using cholesterol oxidase as described in methods. The values are the mean ± SEM of six filters.

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

[b] $P < 0.05$ vs. taurocholate.

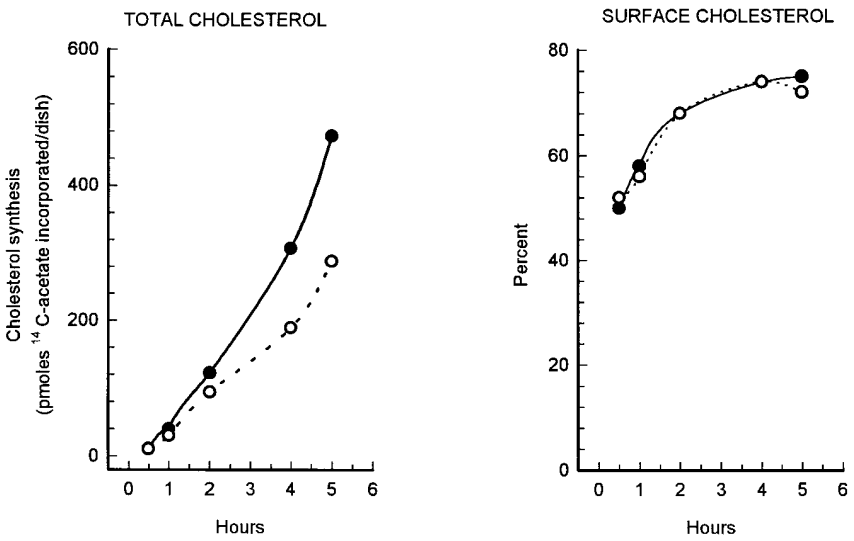

Fig. 2. Effect of sphingomyelinase on the transport of cholesterol to the cell surface. CaCo-2 cells grown on micropore membranes were incubated for up to 5 h with 60 μm [^{14}C]acetate (130 dpm/pmol) with or without 100 mU/ml sphingomyelinase. At each time point indicated, the cells were harvested and radioactivity incorporated into total and surface cholesterol was estimated as described in Methods. The values are an average of duplicate filters at each time point. Control ●; sphingomyelinase ○.

avidly bind cholesterol. The incorporation of labeled acetate into total and surface cholesterol was then estimated (**Table 2**). As expected, compared to control cells, cells incubated with filipin or digitonin synthesized significantly more cholesterol, suggesting that the cell perceived less available incoming cholesterol. The percent of total cholesterol found in the plasma membrane, however, was similar between control cells and cells incubated with the cholesterol-binding agents. In another set of cells, cholesterol efflux was promoted by incubating cells with lysophosphatidylcholine solubilized in taurocholate micelles. This treatment has been demonstrated to cause significant cholesterol efflux leading to an increase in HMG-CoA reductase activity and a decrease in ACAT activity (21, 22). Again, as expected, compared to cells incubated with taurocholate alone, cholesterol synthesis was significantly increased in cells incubated with lysophosphatidylcholine. The percent of newly synthesized cholesterol reaching the plasma membrane, however, was similar to that observed in control cells.

Effect of increasing cholesterol influx from the plasma membrane

Using the same rationale as described above, we studied whether the cell would alter its transport of cholesterol to the plasma membrane under conditions of increased cholesterol influx. In the first experiment, cells

TABLE 3. Effect of micellar cholesterol on the incorporation of [^{14}C]acetate into total and surface cholesterol

Treatment	Cholesterol		
	Total	Surface	Percent on Surface
	pmol [^{14}C]acetate incorporated/6 h		*%*
5 mm Taurocholate + 50 μm oleate	762 ± 68	527 ± 43	69 ± 1
+ 200 μm cholesterol	488 ± 12[a]	319 ± 13[a]	65 ± 1
+ 2.5 μm 25-OH cholesterol	265 ± 12[a]	171 ± 9[a]	64 ± 1

CaCo-2 cells grown on micropore membranes were incubated for 6 h with 5 mm taurocholate + 50 μm oleate, 5 mm taurocholate + 50 mm oleate + 200 μm cholesterol, or 5 mm taurocholate + 50 μm oleate + 2.5 μm 25-hydroxycholesterol. To label newly synthesized cholesterol, 60 μm [^{14}C]acetate (130 dpm/pmol) was added with the treatment at the beginning of the incubation. Radioactivity incorporated into total and surface cholesterol was estimated using cholesterol oxidase as described in methods. The values are the mean ± SEM of six filters.

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

were incubated with labeled acetate and a bacterially derived sphingomyelinase (**Fig. 2**). Sphingomyelinase, which causes the hydrolysis of plasma membrane sphingomyelin, enhances the amount of plasma membrane cholesterol influxing to the endoplasmic reticulum (23–25). The explanation for why this occurs is not entirely clear, but is likely related to an alteration of the sphingomyelin/cholesterol ratio in the membrane. With an increase in the influx of cholesterol, cells incubated with sphingomyelinase demonstrated less cholesterol synthesis. The percent of newly synthesized cholesterol found on the plasma membrane, however, was similar.

Cells were then incubated 6 h with labeled acetate plus taurocholate and oleic acid or taurocholate and oleic acid containing either 200 μm cholesterol or 2.5 μm 25-hydroxycholesterol. The incorporation of acetate into total and surface cholesterol was again estimated (**Table 3**). As expected, compared to control cells incubated with micelles without sterol, cholesterol synthesis was significantly decreased in cells incubated with cholesterol and more so with 25-hydroxycholesterol. Despite the down-regulation of cholesterol synthesis due to increased cholesterol influx, the efficiency with which the cell transported newly synthesized cholesterol to the plasma membrane was unaltered.

Effect of p-glycoprotein inhibition

We and others have shown that inhibitors of p-glycoprotein function, such as progesterone, trifluoperazine, and verapamil, interfere with the influx of plasma

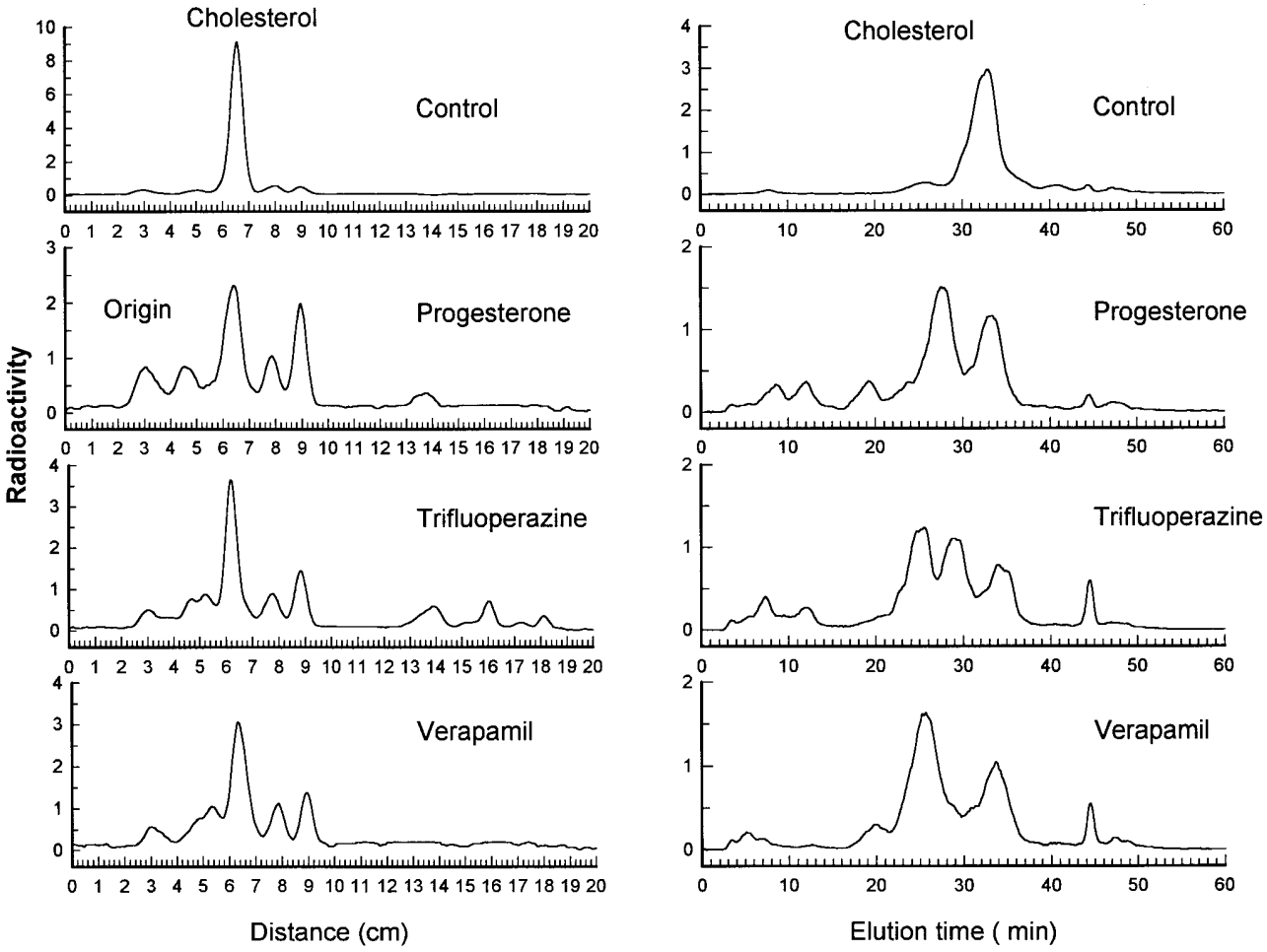

Fig. 3. Effect of p-glycoprotein inhibitors on the incorporation of labeled acetate into non-saponifiable sterols. CaCo-2 cells grown on micropore membranes were incubated for 6 h with 60 μm [^{14}C]acetate (130 dpm/pmol) with or without progesterone (10 μg/ml), trifluoperazine (50 μm), or verapamil (50 μm). The cells were harvested and the total non-saponifiable fraction was isolated as described in Methods. Left panel: representative thin-layer chromatograms of non-saponifiable sterols on silica G plates using solvent mixture: heptane–diethyl ether–acetic acid 85:15:1 (v/v). The plate was developed twice in the same solvent to separate the sterols. The figure shows the radioactivity in the various sterols determined by scanning of the plate by AMBIS 100 scanner (AMBIS, Inc., San Diego, CA). R_f values for sterols were: cholesterol, 0.20; desmosterol, 0.20; lanosterol, 0.34; and cholestenone, 0.38. Right panel: representative high-pressure liquid chromatography profiles of non-saponifiable sterols eluted from a C18 column with 15% water in methanol, 0.7 ml/min.

membrane cholesterol to the endoplasmic reticulum (11–13). To investigate whether p-glycoprotein could be involved in the transport of newly synthesized cholesterol to the plasma membrane, cells were first incubated for 6 h with progesterone, trifluoperazine, or verapamil and the incorporation of labeled acetate into total nonsaponifiable sterols was estimated. The left panel of **Fig. 3** shows a representative thin-layer chromatography scan demonstrating the effect of the p-glycoprotein inhibitors on sterol synthesis. Compared to control cells that synthesized predominately cholesterol, cells incubated with either progesterone, trifluoperazine, or verapamil accumulated several other intermediate sterols. To ensure adequate separation of the sterols and to confirm these results from thin-layer chromatography, the sterol products were also separated by high-pressure liquid chromatography (right panel). It is again evident that compared to control cells, cells incubated with the p-glycoprotein inhibitors accumulated intermediate sterol products. These data are summarized in **Table 4**. In control cells, cholesterol accounted for 72% of the sterols synthesized. In cells incubated with progesterone, there was a modest decrease in total sterols synthesized. Compared to control cells, however, most of the sterols synthesized were intermediates accounting for 69% of the total. Cholesterol synthesis was decreased by more than 2.5-fold. Trifluoperazine increased the incorporation of acetate into total nonsaponifiable sterols, whereas the effect of verapamil on total sterol synthesis was not dissimilar to that of progesterone. Both nonsterol p-glycoprotein inhibitors, however, decreased the synthesis of cholesterol and caused the accumulation of sterol intermediates within the cell. Because the p-glycoprotein inhibitors caused the accumulation of several cholesterol intermediates within the cell, it was not possible to use cholesterol oxidase to estimate the amount of sterols on the plasma membrane. To circumvent this problem, we performed subcellular fractionation using sucrose-density gradient ultracentrifugation to separate the plasma membrane fraction from intracellular membranes. Cells were incubated for 6 h with labeled acetate in the presence or absence of either progesterone or trifluoperazine representing a sterol and nonsterol inhibitor of p-glycoprotein, respectively. After the incubation, subcellular fractionation was performed (**Fig. 4**). Plasma membrane fractions were identified by enrichment of cholesterol mass and alkaline phosphatase activity. RNA enrichment was used to identify the fractions corresponding to the rough endoplasmic reticulum (Fig. 4A). Figure 4B shows the intracellular distribution of label found in cholesterol (left column) or sterol intermediates (right column). In cells incubated with progesterone, the sterol products from each fraction were separated by thin-layer chromatography and high-pressure liquid chromatography. As the results using either method were similar for progesterone-treated cells, sterol products from cells incubated with trifluoperazine were analyzed by high-pressure liquid chromatography only. In control cells, approximately 80% of the label found in nonsaponifiable sterols was present in cholesterol and enriched in the plasma membrane fractions. In contrast, in cells incubated with progesterone or trifluoperazine, although the label found in nonsaponifiable sterols was concentrated in the plasma membrane, most of the label was not present in cholesterol. These data suggest that p-glycoprotein inhibitors interfere with the arrival of newly synthesized cholesterol to the plasma membrane, not by disrupting sterol movement from the endoplasmic reticulum to the plasma membrane, but by preventing the return to the endoplasmic reticulum of cholesterol intermediates within the plasma membrane. As further evidence to support the notion that the two cholesterol pathways within the cell were distinct, nigericin, a potassium ionophore that alkalinizes intracellular compartments and causes a marked inhibition of cholesterol influx from the plasma membrane to the endoplasmic reticulum (11, 26), caused an increase in the rate of cholesterol synthesis but did not alter the transport of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane (data not shown).

TABLE 4. Effect of p-glycoprotein inhibitors on the incorporation of [^{14}C]acetate into nonsaponifiable sterols

Treatment	Total Sterols	Intermediates	Cholesterol
	pmol [^{14}C]acetate incorporated/dish/6 h		
Control	1610 ± 38	445 ± 10	1165 ± 34
Progesterone	1387 ± 37[a]	954 ± 34[a]	433 ± 11[a]
Trifluoperazine	2119 ± 19[a]	1455 ± 13[a]	665 ± 12[a]
Verapamil	1066 ± 49[a]	674 ± 29[a]	392 ± 27[a]

CaCo-2 cells grown on micropore membranes were incubated for 6 h with buffer alone, 50 μm verapamil, 50 μm trifluoperazine, or 10 μg/ml progesterone. To label newly synthesized sterols, 60 μm [^{14}C]acetate (130 dpm/pmol) was added with the treatment at the beginning of the incubation. The total nonsaponifiable lipid extract was analyzed by thin-layer chromatography or high-pressure liquid chromatography to determine radioactivity in newly synthesized cholesterol and sterol intermediates. The values are the mean ± SEM of six filters.

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

DISCUSSION

In times of dietary fatty acid influx, the intestinal absorptive cell must rapidly assemble the necessary com-

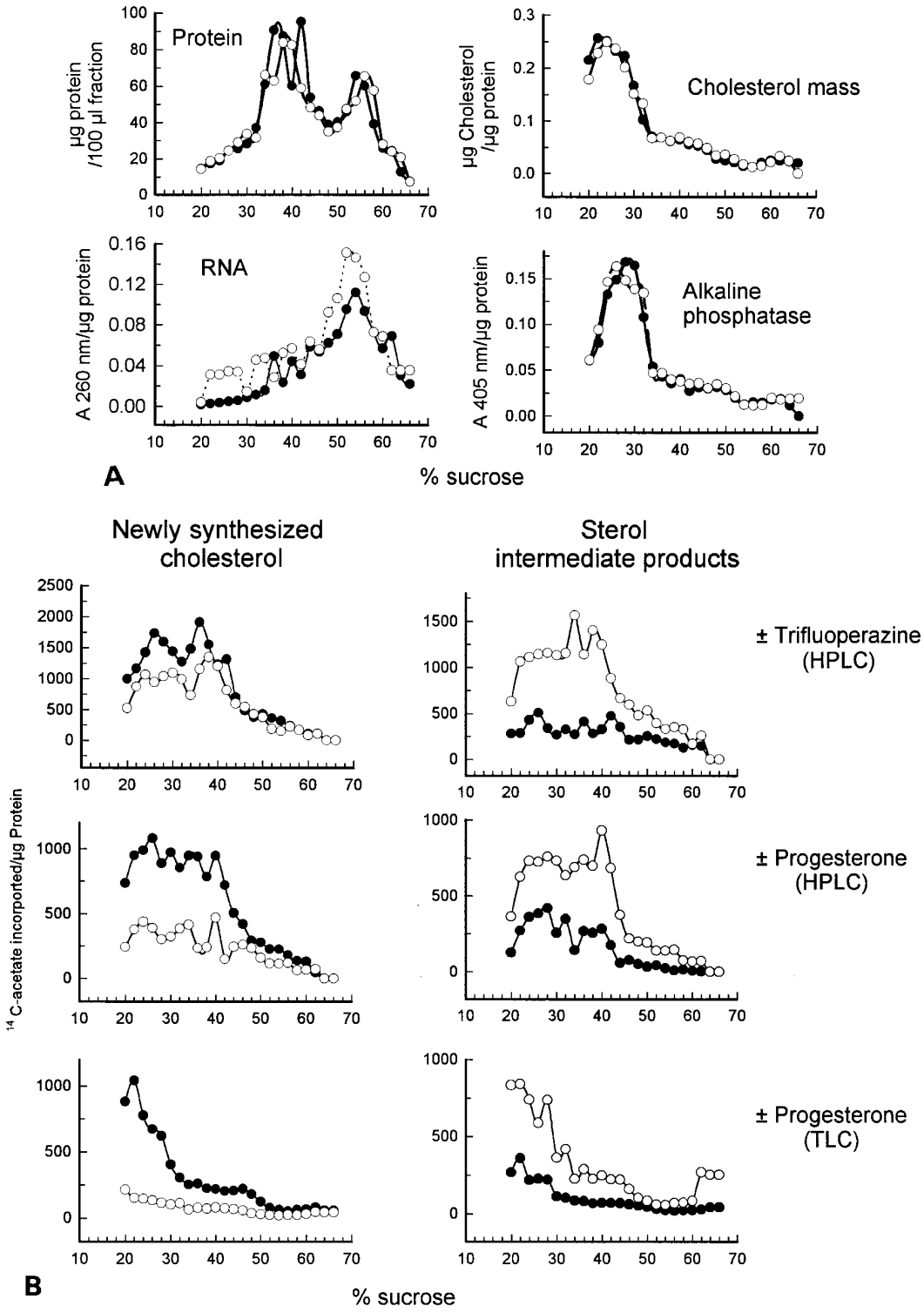

Fig. 4. Effect of progesterone or trifluoperazine on the distribution of newly synthesized nonsaponifiable sterols in subcellular fractions. CaCo-2 cells were incubated for 5 h with [^{14}C]acetate in the presence or absence of progesterone (10 µg/ml) or trifluoperazine (50 µm). Total membranes were prepared and separated on a 66–20% sucrose gradient. A: Distribution of protein, RNA, cholesterol mass, and alkaline phosphatase within each fraction. B: Newly synthesized cholesterol and intermediate sterols in the fractions as estimated by thin-layer chromatography (TLC) and/or high-pressure liquid chromatography (HPLC) as described in Methods. The data are a composite of four separate experiments for progesterone and one experiment for trifluoperazine. Control ●; progesterone ○, Trifluoperazine ○.

ponents of a lipoprotein particle and secrete the packaged triacylglycerols into the lymphatics as part of a triacylglycerol-rich lipoprotein. Normally, triacylglycerols do not accumulate within absorptive cells after the ingestion of a fatty meal suggesting that this process is, indeed, very efficient. We have previously shown that the cholesterol required for the normal assembly of the triacylglycerol-rich lipoprotein particle is derived predominately from the plasma membrane (5). Newly synthesized cholesterol and dietary micellar cholesterol contribute little to the cholesterol found in the secreted lipoprotein particle during fatty acid influx. Under these conditions, therefore, when cholesterol requirements are high, cholesterol is being depleted from the plasma membrane. To maintain membrane integrity, this cholesterol needs to be replenished. The transport of newly synthesized cholesterol from its site of synthesis, the endoplasmic reticulum, to the plasma membrane is likely very important in replenishing and maintaining the normal cholesterol content of the membrane. In the present study, in CaCo-2 cells, newly synthesized cholesterol reaches the plasma membrane rapidly. Within 30 min of adding labeled acetate to cells, approximately 50% of the labeled cholesterol is already found within the plasma membrane. This rapid movement of newly synthesized cholesterol to the plasma membrane agrees with the results obtained in other cell lines in which the $T_{1/2}$ for this transport process ranges from 10 to 20 min (3, 14). Moreover, in intestinal cells, as has been observed in CHO cells (3, 15), the transport of newly synthesized cholesterol from its site of synthesis to the plasma membrane does not require a functioning Golgi apparatus, microtubules, or new protein synthesis.

It has been suggested that the movement of plasma membrane cholesterol to the endoplasmic reticulum is distinct from the pathway of cholesterol trafficking from the endoplasmic reticulum to the plasma membrane (3, 27). The present results in cultured intestinal cells would agree with that conclusion. The movement of plasma membrane cholesterol to the endoplasmic reticulum is a regulated process. In CaCo-2 cells, driving lipoprotein production increases the percent of plasma membrane cholesterol transported to the endoplasmic reticulum (5). In addition, the absorption of micellar cholesterol also leads to an increased influx of plasma membrane cholesterol (5). In contrast to this regulated transport process, however, neither fatty acids nor cholesterol absorption altered the efficiency or percent of newly synthesized cholesterol reaching the plasma membrane. Because of the recognized affinity between sphingomyelin and cholesterol, the sphingomyelin content of a particular membrane has been proposed to play a role in cholesterol trafficking (27). One could argue, therefore, that by depleting the plasma membrane of sphingomyelin, the transport of cholesterol to the plasma membrane might be disrupted. This turned out not to be the case, however. After sphingomyelin hydrolysis, a condition which increases the percent of plasma membrane cholesterol moving to the endoplasmic reticulum, the percent of newly synthesized cholesterol moving to the plasma membrane was unaltered. Further support for the concept that the two pathways are distinct comes from the results with monensin and nigericin. Both monensin and nigericin potently interfere with trafficking of plasma membrane cholesterol to the endoplasmic reticulum (11, 26); however, neither affected cholesterol transport to the plasma membrane.

It seemed reasonable to assume that if p-glycoprotein plays a role in cholesterol trafficking from the plasma membrane to the endoplasmic reticulum that p-glycoprotein might also have a role in cholesterol transport from its site of synthesis to the plasma membrane. After all, both pathways are believed to involve specific transport vesicles. Recently, Metherall, Waugh, and Li (28), demonstrated that at high concentrations of progesterone (40–100 μm), cholesterol synthesis in CHO cells and a variety of other cell types, including CaCo-2 cells, could be abolished. They observed that progesterone also caused the accumulation of lanosterol and several other cholesterol intermediates within the cell. Using differential ultracentrifugation to separate cells into a low- and high-speed pellet, these investigators found that the cholesterol intermediates were enriched in the low-speed pellet containing plasma membrane markers. Thus, it was concluded that progesterone was likely interfering with the transport of sterol intermediates from the plasma membrane to the endoplasmic reticulum. Our results would support and extend those of Metherall et.al. (28). Using much lower concentrations of progesterone so as to avoid abolishing cholesterol synthesis, we found that less newly synthesized cholesterol arrived at the plasma membrane in cells incubated with progesterone. Moreover, using sucrose-density gradient ultracentrifugation to fractionate cells, it was clear that these intermediates were, indeed, contained within the plasma membrane. Verapamil and trifluoperazine, two nonsterol p-glycoprotein inhibitors, also caused the accumulation of cholesterol intermediates and, in cells incubated with trifluoperazine, these intermediate sterols were also found in the plasma membrane. The results would suggest, therefore, that p-glycoprotein inhibitors interfere with the influx of sterols from the plasma membrane to the endoplasmic reticulum. Similar to the other modulators used in this study to alter cholesterol transport from the plasma membrane to the endoplasmic reticulum, p-glycoprotein

does not appear to play a role in trafficking of sterols from the endoplasmic reticulum to the plasma membrane. What is clear from the data, however, is that cholesterol movement from the plasma membrane to the endoplasmic reticulum is a regulated process depending upon the immediate needs of the cell for cholesterol. In contrast, there is no evidence to suggest that cholesterol transport from its site of synthesis to the plasma membrane is regulated and, therefore, appears to be a constitutive process.

In MA104 cells, there is recent evidence to suggest that caveolin, a small molecular weight membrane protein that lines the inner membrane surface of caveolae, plays a role in the transport of newly synthesized cholesterol to the plasma membrane (29). Caveolae, sphingomyelin/cholesterol-rich microdomains of invaginated plasma membrane of endothelial and epithelial cells, may be the cellular compartment involved in the influx and outward flux of cellular cholesterol (30, 31). Progesterone has been shown to decrease the amount of caveolin and cholesterol in caveolar fractions and to block the movement of cholesterol into the caveolar compartment (29). Thus, it has been proposed that the effect of progesterone on cellular cholesterol trafficking may be related to its effect on caveolin and/or caveolar function. CaCo-2 cells have microdomains consistent with caveolae but the existence of caveolin in CaCo-2 cells has been disputed (32, 33). Whether intestinal cells have caveolae and caveolin and whether these structures are involved in intestinal cholesterol transport is presently being pursued.

The results of this study demonstrate that the transport of newly synthesized cholesterol to the plasma membrane is likely a constitutive process and is distinct from the transport of plasma membrane cholesterol to the endoplasmic reticulum. The amount of newly synthesized cholesterol arriving at the plasma membrane is regulated at the level of cholesterol synthesis, not at the level of the transport process.

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