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# Regulation of surfactant-like particle secretion by Caco-2 cells

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**Abstract**

Surfactant-like particle (SLP) is a phosphatidylcholine (PC)-rich membrane produced in the small intestine, and its secretion is increased by fat feeding. In Caco-2 cells known to produce SLP, preincubation with [$^3$H]palmitate labelled the SLP and was used as a marker for newly secreted membrane. SLP-associated PC and protein ($d$ = 1.07–1.08 g/ml in a linear non-equilibrium NaBr gradient) were secreted in parallel with triacylglycerols (TG) and at a rate about twice the control rate in response to feeding cells with an oleate/egg PC mixture. Cholesterol and apolipoprotein A-I identified only a small peak corresponding to high-density lipoprotein (HDL), but the largest peak corresponded with SLP ($d$ = 1.07–1.08). Palmitate incorporation into PC showed a similar small peak migrating at the density of HDL, but most labelled PC secreted from the cells was due to SLP. PC secretion, alkaline phosphatase activity, and newly synthesized immunoprecipitated SLP proteins from conditioned serum-free media migrated together at a density of ≥1.21 g/ml in a lipoprotein NaBr step gradient, and represented SLP. Glycerol incorporated into TG migrated at a peak density of 1.12 g/ml, consistent with HDL secretion from cells incubated in serum-free media. These data confirm that the secreted PC in SLP is distinct from lipoprotein particles. Incorporation of [$^3$H]palmitate into the PC fraction of either whole cell homogenate or isolated brush border membranes was not affected by oleate/egg PC feeding. Both Pluronic L-81, an inhibitor of chylomicron secretion, and BMS-197636-02, a microsomal triglyceride transfer protein inhibitor, blocked the secretion of both TG and PC. Elevation of intracellular cAMP levels that stimulate surfactant secretion from type II pneumocytes caused a 50% reduction in SLP and TG secretion from Caco-2 cells. These results confirm the SLP response to fat feeding found in vivo, further supporting a role for SLP in TG secretion from the enterocyte, and show that the regulation of SLP secretion differs from that of pulmonary surfactant. 

*Keywords:* Caco-2 cell; Lipid absorption; Lipid transport; Surfactant-like particle

## 1. Introduction

The surfactant-like particle (SLP) is a phospholipid- and protein-rich membrane lining the surface of the stomach, small intestine and colon [1–3]. SLP is enriched in intestinal alkaline phosphatase (IAP) and contains a high percentage of disaturated phosphatidylcholine (PC) relative to the apical membrane of the enterocyte [4]. The dominant fatty acid is the saturated 16-carbon fatty acid, palmitate, similar to pulmonary surfactant [4]. SLP also contains several proteins found in pulmonary surfactant (SP-A, SP-D), and is capable of lowering the surface tension at

Abbreviations: IAP, intestinal alkaline phosphatase; SLP, surfactant-like particle; PC, phosphatidylcholine; MTPI, microsomal triglyceride transfer protein inhibitor; BBM, brush border membrane; TG, triacylglycerol

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an air–water interface, although not to the same extent as pulmonary surfactant [3,4]. In the small intestine, SLP is secreted by the enterocyte into the basolateral compartment (lamina propria), from which it migrates either directly into the blood or via the tight junctions onto the apical surface of the mucosal cells [5].

A definitive function for SLP in the small intestine has not been confirmed. Several lines of evidence, however, point to its role in intracellular triacylglycerol (TG) transport. First, using IAP as a marker, the production of SLP in rat intestine was increased 2–3-fold after TG feeding, with increased particle concentration noted in the cell, the lamina propria, and the lumen [6]. Second, after fat feeding, intracellular lipid droplets were surrounded by IAP-containing lamellar membranes [7], which accompanied the lipid droplet to the region near the basolateral membrane of the cell [8]. Finally, inhibitors of TG secretion also inhibit SLP secretion [6]. The nonionic hydrophobic detergent, Pluronic L-81, which inhibits formation and secretion of chylomicrons in the enterocyte, and fenfluramine, which blocks chylomicron transport in fat-fed rats, both produced retention of large intracellular lipid droplets surrounded by IAP-containing lamellar membranes [8]. Decreases in serum TG levels and diminished SLP on the apical surface of the enterocyte accompanied these intracellular events.

Extracellular SLP binds uropathogenic *Escherichia coli*, suggesting a colonization site as a possible role for SLP in the intestinal lumen [9]. Although IAP has been used as a marker for intracellular SLP, it was only possible to measure the enzyme in extracellular SLP by first perfusing animals to remove the abundant phosphatidylinositol-specific phospholipase D present in serum [10]. Moreover, even when perfusion was performed, detection of increased IAP secretion on SLP was not sensitive to small amounts of fat feeding ($<$ mmolar) [6]. Thus, it seemed reasonable to use a continuous small intestinal cell line, and to study secretion of preformed phospholipids as a marker for SLP release in the presence of limited amounts of added fat, as has been done for secretion of pulmonary surfactant [11].

To confirm a role for SLP in TG secretion and to further investigate the regulation of SLP production in the small intestine, Caco-2 cells were used, since they are known to produce SLP [12]. This colonic, tumor-derived cell line differentiates post-confluence into a polarized monolayer of cells with properties of fetal enterocytes, retaining some colonocyte properties as well [13,14]. Caco-2 cells transfected with IAP produce more SLP [12] and have been extensively employed in studies of intestinal TG formation and secretion [15]. Using this model system, we now show that factors that affect TG secretion into the medium (such as oleate feeding, cyclic AMP (cAMP), Pluronic L-81, and microsomal triglyceride transfer protein inhibitor (MTPI)) also affect SLP secretion proportionately, while intracellular phospholipid synthesis was unaffected. These data demonstrate that Caco-2 cells reproduce the physiological events involving SLP that follow lipid feeding in vivo, and confirm that SLP and TG secretion are closely linked.

## 2. Materials and methods

### 2.1. Materials

[9,10-$^3$H]Palmitate and [1,2,3-$^3$H]glycerol were purchased from Dupont NEN (Boston, MA, USA) and [$^{35}$S]methionine/cysteine was purchased from Amersham-Pharmacia Biotech (Piscataway, NJ, USA). Egg PC, oleic acid, dibutyryl cAMP, isobutylmethylxanthine, forskolin, and fatty acid-poor bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Murakami's serum-free medium [13] was prepared by the Washington University Tissue Culture Center. All other reagents were reagent grade or better. The MTPI, BMS-197636-02, was generously supplied by Dr. Nicholas Davidson (Washington University, St. Louis, MO, USA).

### 2.2. Cell culture techniques

Caco-2 cells, originally obtained from Dr. Jeffrey Field (University of Iowa), were grown in 35 mm plastic dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, 0.1 mM non-essential amino acids, 4 mM L-glutamine, penicillin (50 μg/ml), streptomycin (50 μg/ml), gentamicin (50 μg/ml), and 10 mM HEPES, pH 7.4.

Cells were seeded at $1 \times 10^6$/dish, were fed every 48 h, incubated in an atmosphere of 5% $CO_2$, and used after either 14 or 21 days in culture. For studies examining direction of secretion, Caco-2 cells were grown on filters in 6-well Costar Transwell clusters.

### 2.3. Incubation conditions

In experiments designed to measure lipid release, Caco-2 cells were grown in culture for 21 days post-confluence, when the small intestinal phenotype was optimized [14]. The Caco-2 cells were washed three times with supplemented DMEM (minus calf serum) to remove surface lipid and incubated for 30 min in the same medium at 37°C in a 5% $CO_2$ atmosphere. The cells were subsequently incubated for 4 h in 2 ml of Murakami's serum-free medium alone (control) or in Murakami's medium with 500 μM oleic acid and 500 μM egg PC (lipid-fed) [16]. To harvest enough SLP to measure cholesterol content from a single experiment (see Fig. 1), cells were transiently transfected with cDNA encoding rat IAP-II, using the calcium phosphate method, to produce large amounts of SLP, as described previously [12]. Transient transfections were not used for other studies, as the Caco-2 cells were only 48 h post-confluence (relatively undifferentiated), and might not have responded optimally to fat feeding.

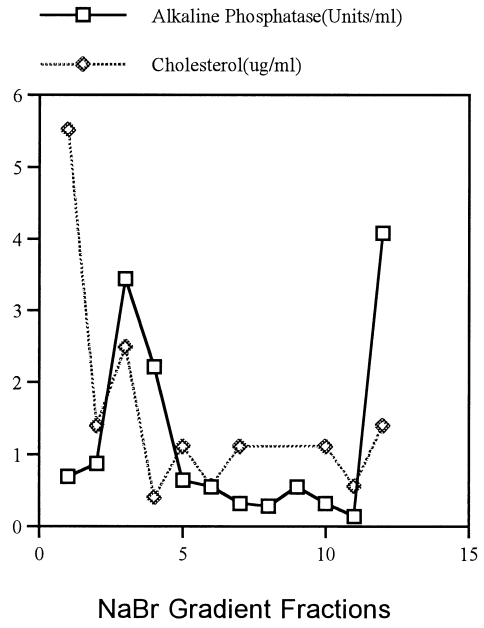


Fig. 1. NaBr gradient of media from Caco-2 cells transfected with IAP-II cDNA, and harvested 48 h after confluency. The conditioned media were concentrated 15-fold and applied to a continuous NaBr gradient (0.49–1.46 M). Alkaline phosphatase activity and cholesterol content were assayed as described in Section 2.

To estimate SLP secretion, cells were pre-labelled by adding 10 μCi/ml of [$^3$H]palmitate or 30 μCi/ml of [$^{35}$S]L-methionine/cysteine (Pro-Mix, 70% methionine, 30% cysteine, Amersham-Pharmacia Biotech, Arlington Heights, IL, USA) to 2 ml of supplemented DMEM with fetal calf serum, and incubated overnight. The purpose of this incubation was to label the phospholipids (or protein) in SLP sufficiently for easy detection when fat secretion was stimulated in serum-free medium, as has been done using type II alveolar cells [11]. To measure TG secretion, 10 μCi/ml of [$^3$H]glycerol was added to both control and fat-fed incubations, only for the 4 h of incubation in serum-free medium. In this way the newly formed TG would have little or no labelled palmitate, whereas the released phospholipids (largely SLP) would contain little or no labelled glycerol. The following morning the cells were washed three times with DMEM (minus fetal calf serum) and incubated as outlined above with or without added lipid (oleate/egg PC). Pluronic L-81 and the MTPI were added at the beginning of the incubation period in DMEM. Neither inhibitor had any effect at the concentrations used on release of lactate dehydrogenase (LDH) activity from Caco-2 cells.

The method chosen for detection of secreted SLP was release of pre-labelled phospholipids, as described above. This method was selected over release of labelled SLP protein, because the proteins had to be immunoprecipitated with attendant losses, and the degree of incorporated label was lower than that achieved by pre-labelling phospholipids. Moreover, initial experiments showed that SLP proteins and PC were increased in the medium after lipid feeding to approximately the same degree, and secretion was blocked by Pluronic L-81 and by the MTPI to the same degree. Thus, the simpler method of measuring labelled PC was used for most experiments.

At the end of the incubation period, medium was removed and centrifuged at $1500 \times g$ to remove any free cells or cellular debris. The media were concentrated 5–12-fold using a dialysis concentrator (Schleicher&Schuell, Keene, NH, USA) prior to application

to density gradients. Cells were washed three times with 10 mM Tris–HCl, pH 7.4, and harvested by scraping into the same buffer. Homogenates were prepared by sonicating the cells for 30 s at the maximum microtip setting on a VibraCell sonicator (Sonics and Materials, Inc., Danbury, CT, USA). Both cells and media were stored at −20°C prior to separation of labelled PC and TG.

### 2.4. Preparation of lipid medium

Oleic acid was added to a glass test tube and dried under a stream of $N_2$. The fatty acid was solubilized in 100% ethanol and the sodium salt prepared by adjusting the pH with 1 N NaOH to approximately 8. After evaporating the ethanol with $N_2$, egg PC dissolved in chloroform:methanol (1:1) was added and the solvent once again evaporated with $N_2$. Murakami's serum-free medium containing 0.15 mM fatty acid-poor bovine serum albumin was added with gentle shaking, the pH of the medium adjusted to 7.4–7.5 if necessary, and the mixture allowed to sit at 37°C for 1 h before use.

### 2.5. Biochemical analyses

Alkaline phosphatase activity was measured in both media and cell homogenates by hydrolysis of *p*-nitrophenyl phosphate [17]. Sucrase-isomaltase and leucine aminopeptidase were assayed as previously described [12]. LDH activity was assayed by the method of Bergmeyer and Bernt [18], and brush border membranes (BBMs) prepared as described by Schmitz et al. [19]. Total cholesterol was determined using a commercial kit (Boehringer Mannheim, Indianapolis, IN, USA). Lipids were isolated by a modification of the Folch method [20], and individual lipids were separated by thin layer chromatography (TLC) using a solvent system of hexane:diethylether:acetic acid (70:30:1) for TG, or chloroform:methanol:acetic acid:water (25:15:4:2) for PC. Lipids were visualized by iodine vapor, the spots were scraped into scintillation vials containing 5 ml of ScintiVerse liquid scintillation cocktail (Packard), and the radioactivity was counted in a Packard Model 4530 counter. In some experiments cpm were corrected to dpm by comparison to a quench curve. Protein was determined by the BCA method (Pierce). Immunoprecipitation of SLP proteins was carried out as previously described [21] using antibodies against human small bowel SLP proteins [22].

### 2.6. Gradient analyses

SLP was isolated from Caco-2 cell media on a continuous NaBr gradient (0.49–1.46 M) as described previously [3,4] in which SLP migrates at a density of 1.07–1.08 g/ml, and brush borders are found at $d = 1.17$ g/ml in 18 h at $100\,000 \times g_{av}$. SLP and lipoproteins were also separated by adjusting media to $d = 1.25$ g/ml with NaBr. This fraction was then overlaid with layers of $d = 1.063$, 1.019, and 1.006 g/ml. This step gradient was centrifuged at $136\,000 \times g_{av}$ for 24 h [23]. The linear gradient is more limited in the volume that can be applied, was not designed to separate lipid-containing particles, and is not taken to equilibrium. On the other hand, the discontinuous gradient is centrifuged harder and longer to equilibrium, and was designed to separate lipoproteins on the basis of their TG content. Carrier PC and TG (approximately 250 nmol) were added to each gradient fraction prior to lipid extraction, so that these lipids could be better visualized on TLC. Human apolipoprotein A-I was detected by Western blot of concentrated gradient fractions (antibody provided by courtesy of Dr. Gus Schonfeld, Washington University School of Medicine).

Caco-2 cells have been used to study TG secretion in the form of very low-density lipoproteins (VLDLs) and chylomicrons, i.e. $d < 1.020$ g/ml [24,25]. These cells secrete both SLP [12] (which contains dipalmitoyl PC and cholesterol but not TG [4]) and lipoproteins that contain about 15% of the secreted PC as well. The major lipoprotein class produced by Caco-2 cells under basal conditions is high-density lipoprotein (HDL) ($d = 1.063$–1.21) [26]. Moreover, HDL is the dominant lipoprotein for cells grown in serum-free medium [27], and different from VLDL ($d = < 1.006$ g/ml) seen in serum-containing medium. When Caco-2 cells are grown in serum [27] or palmitate is the fatty acid added [28], secreted lipoproteins appear at LDL density (1.020–1.067 g/ml), indicating TG-depleted or phospholipid-enriched lipoproteins. Palmitate is not efficiently incorporated into TG by Caco-2 cells, but rather is incorporated preferably

Table 1
Secretion of lipid from Caco-2 cells

| Lipid class | Secreted into media (%) | |
|---|---|---|
| | Apical | Basolateral |
| TG | 32.1 | 66.9 ± 3.0 (12) |
| PC | 70.2 | 29.8 ± 4.2 (12) |

Cells were grown on filters for 21 days, labelled with either [$^3$H]glycerol or [$^3$H]palmitate for TG or PC, respectively, and secretion assessed in the presence of the egg PC/oleic acid mixture (500 μM each), as described in Section 2. The data represent the mean ± S.E.M. of (*n*) dishes.

into phospholipids [28]. We chose palmitate as the labelled fatty acid to enhance labelling of PC, and exposed cells for 4 h to serum-free medium.

## 3. Results

### 3.1. Caco-2 cells as a model system to study the secretion of TG and SLP

When Caco-2 cells were grown on filter disks, labelled with either glycerol or palmitate, 2/3 of the secreted TG was confined to the basolateral compartment (Table 1), a finding consistent with other studies [24]. In contrast, only about 1/3 of PC was detected basolaterally (Table 1), consistent with the observation that most of the SLP is transported to the apical chamber in Caco-2 cells by way of the tight junction [5,12].

To determine whether the secreted PC represented apolipoprotein or SLP or both, NaBr density gradients were performed. The density of SLP (1.07–1.08 g/ml) fell between the densities of LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml), as expected (Fig. 1). Because both lipoproteins and SLP contain cholesterol, the cholesterol and apolipoprotein A-I content was examined in conditioned media from cells overproducing SLP following transfection with rat IAP-II cDNA, but without the addition of oleate/egg PC. Fig. 1 shows that most of the cholesterol was found either at the top of the gradient ($d < 1.02$) where chylomicrons and VLDL migrate, or in frac-

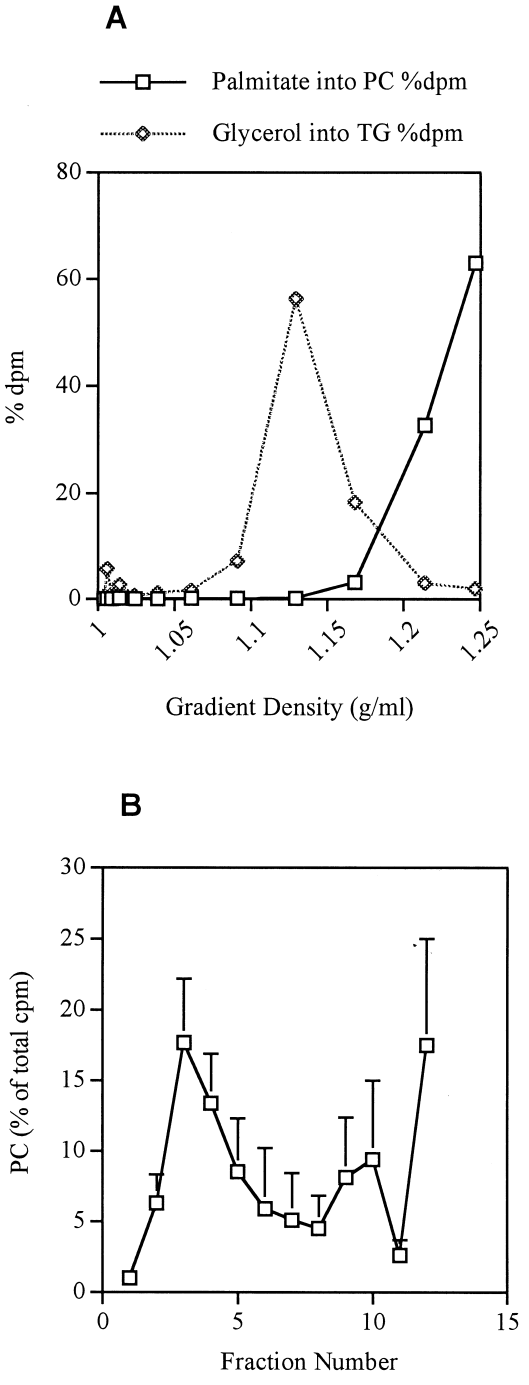


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Fig. 2. NaBr gradients of media from oleate/egg PC-fed Caco-2 cells. Cells were grown in culture for 21 days and incubated for 4 h in serum-free medium as described in Section 2. PC and TG were isolated from the individual fractions and isolated by TLC following lipid extraction. Individual TG and PC spots were visualized with iodine vapor and assayed for radioactivity as outlined in Section 2. (A) PC and TG content of individual fractions after centrifugation on a NaBr step gradient. (B) The PC radioactivity assayed in the fractions of a continuous NaBr gradient. All gradients were poured and centrifuged as described in Section 2.

tion 3 ($d = 1.07$) corresponding with the peak in alkaline phosphatase activity identifying SLP. More than 90% of the glycerol-labelled TG produced by these transfected cells grown in serum was consistently recovered in fraction 1 of the NaBr gradients (data not shown). A broad low peak of cholesterol was found in that part of the gradient ($d = 1.08$–$1.21$) where HDL migrates. Fractions 7–10, but no other gradient fractions, contained small amounts of apolipoprotein A-I, consistent with migration of HDL in those fractions.

To further separate the lipoproteins from SLP, conditioned media after oleate/egg PC feeding of non-transfected Caco-2 cells were applied to a stepwise NaBr gradient, in which SLP isolated on a linear gradient migrates to the bottom of the gradient with a density $\geq 1.21$ g/ml (data not shown). In this gradient, palmitate incorporation into PC also migrated almost entirely in fractions $\geq 1.21$ g/ml (Fig. 2A). Glycerol incorporation into TG peaked at $d = 1.12$ g/ml, with no incorporation at densities $\geq 1.21$ g/ml (Fig. 2A). These data are consistent with the high palmitate and absent TG content of SLP [22], and with the expected preponderance of high-density lipoproteins (HDLs) secreted from Caco-2 cells maintained in serum-free medium [27]. Fig. 2B shows the results of palmitate incorporation into PC on the linear NaBr gradient. The major peak of palmitoyl PC was in fractions 3 and 4, corresponding to densities of 1.07–1.08 g/ml. The small peak of palmitoyl PC in fractions 8–10 migrates in the position of cholesterol-containing HDL (Fig. 1), and constituted only 17% of the total labelled PC recovered in the gradient. The fraction at the bottom of the tube contained neither sucrase-isomaltase nor leucine aminopeptidase activity. This result differs from that of Caco-2 cells grown in serum, in which brush border fractions were found in fraction 12 [12]. When fractions 3 and 4 were collected from Fig. 2B and separated again on an identical linear gradient, the same pattern was found, with a peak of labelled PC in the bottom of the tube (data not shown). This finding is consistent with some of the SLP migrating with a density $> 1.17$.

To further confirm that the fractions containing palmitoyl PC ($d \geq 1.21$ g/ml in the discontinuous gradient) represented mostly SLP, newly synthesized proteins secreted in response to oleate/egg PC addi-

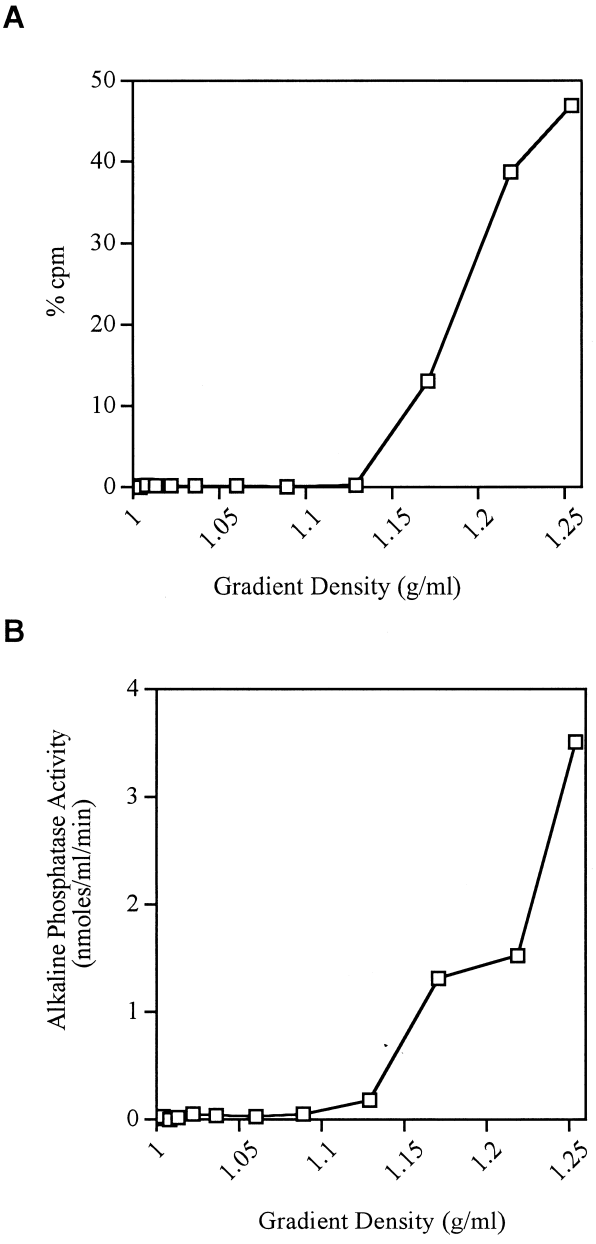


Fig. 3. NaBr step gradients of media from oleate/egg PC-fed Caco-2 cells. A: Profile of $^{35}$S-labelled SLP proteins immunoprecipitated with anti-human SLP small bowel antibody. Cells were labelled with [$^{35}$S]methionine/cysteine, as was done for palmitate, and the media harvested after 4 h in serum-free conditions prior to immunoprecipitation. B: Alkaline phosphatase activity in the same fractions after centrifugation on a step NaBr gradient. Cells were cultured and incubated and the media were centrifuged and assayed as outlined in Section 2.

tion were immunoprecipitated from the fractions after centrifugation, using antiserum against human small intestinal SLP. Fig. 3A shows that the distribution of these proteins was mostly in fractions 10 and 11 ($d \geq 1.21$ g/ml). Alkaline phosphatase, a marker for SLP not found in lipoproteins, also followed a similar distribution (Fig. 3B), while sucrase, an enzyme abundant in the apical membranes, was undetectable in any of the fractions (data not shown). Because these studies revealed that, under the condition used, SLP (reflected by 83% of the newly synthesized PC) was predominantly found in a fraction that could be completely separated from secreted HDLs (containing all the secreted TG), measurement of the secreted PC and TG was subsequently performed on lipids isolated from the media by TLC alone.

### 3.2. Regulation of SLP secretion by Caco-2 cells

Caco-2 cells increase their output of lipid in response to addition of fatty acids [24,25]. The mixture of oleate and egg PC increased TG release [29,30]. This finding was confirmed by the demonstration that [$^3$H]glycerol recovered in the TG TLC spot was increased by about 7.5-fold (Table 2). On the other hand, addition of dibutyryl cAMP, known to stimulate pulmonary surfactant release [11,31], produced a 57% inhibition of TG release. Extracellular IAP increased about 15% in apical medium in response to feeding oleic acid to Caco-2 cells [12],

Table 2
TG secretion from Caco-2 cells

| Additions to medium | TG in medium (cpm/mg protein) |
|---|---|
| None (control) | 409 ± 82 (6) |
| Egg PC/oleic acid (500 μM each) | 3065 ± 363 (6)* |
| Dibutyryl cAMP | 174 ± 34 (6)# |

Cells were grown and labelled as described in Section 2. The data represent the mean ± S.E.M. of ($n$) samples. *$P < 0.05$ vs. control (no addition). #$P < 0.001$ vs. control (no addition).

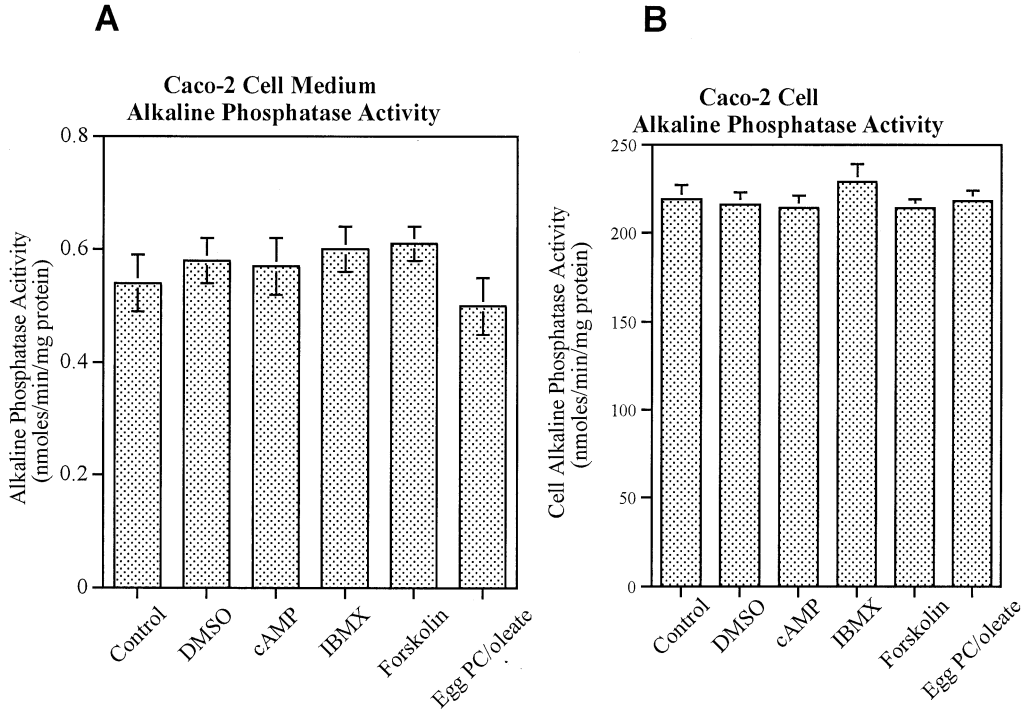


Fig. 4. Alkaline phosphatase activity in media (A) and in Caco-2 cells (B). Cells were grown in culture for 21 days and incubated in serum-free medium for 4 h as described in Fig. 2 with the following additions: dimethylsulfoxide (DMSO) as carrier control (0.5%), dibutyryl cAMP (100 μM), isobutylmethylxanthine (IBMX) (100 μM), forskolin (5 μM), or egg PC/oleic acid (500 μM each). The forskolin and IBMX were dissolved in DMSO 0.5%. Control incubations contained the fatty acid-poor bovine serum albumin used in the oleate/egg PC incubations. Specific activities were calculated from the total protein of the scraped cell layer.

but led to some shedding of BBMs. Therefore, experiments were performed in Caco-2 cells to see if the same response could be measured by assaying the media for alkaline phosphatase activity following oleate/egg PC feeding, a combination that maximizes TG secretion [29,30]. In contrast with the data following oleate feeding alone, the addition of an oleate/egg PC mixture sufficient to stimulate TG release in differentiated Caco-2 cells did not have a measurable effect on either cellular or medium alkaline phosphatase activity (Fig. 4). However, the percent of secreted alkaline phosphatase (compared with cell content) was only 0.25%, less than that observed (~3%) when cells are incubated in serum-containing medium [12,36]. Agents that elevate intracellular cAMP and increase pulmonary surfactant secretion (dibutyryl cAMP, isobutylmethylxanthine, or forskolin) in type II pneumocytes were added in the absence of the oleate/egg PC mixture, and did not significantly alter alkaline phosphatase activity in Caco-2 cells or media.

When preformed SLP release was analyzed using the pulse-chase model in which the amount of [$^3$H]PC released into the medium was used as a marker of newly synthesized SLP, the addition of the egg PC/oleate mixture caused a 2.2-fold increase

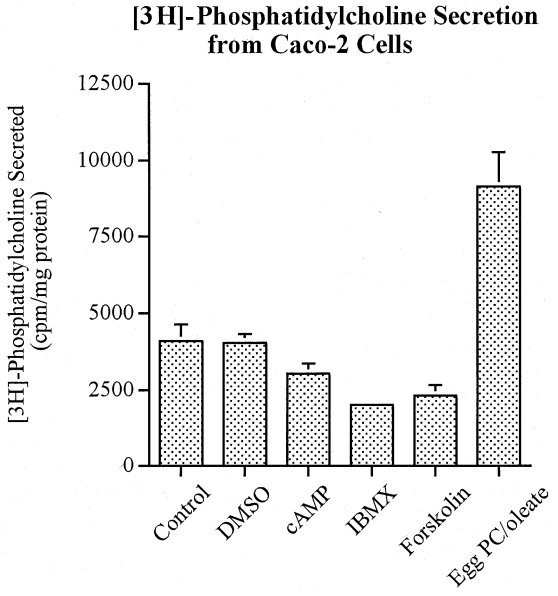


Fig. 5. [$^3$H]Palmitate-labelled PC secretion into media from Caco-2 cells. Caco-2 cells were grown, labelled, and incubated, and PC isolated and assayed as noted in Figs. 2 and 4.

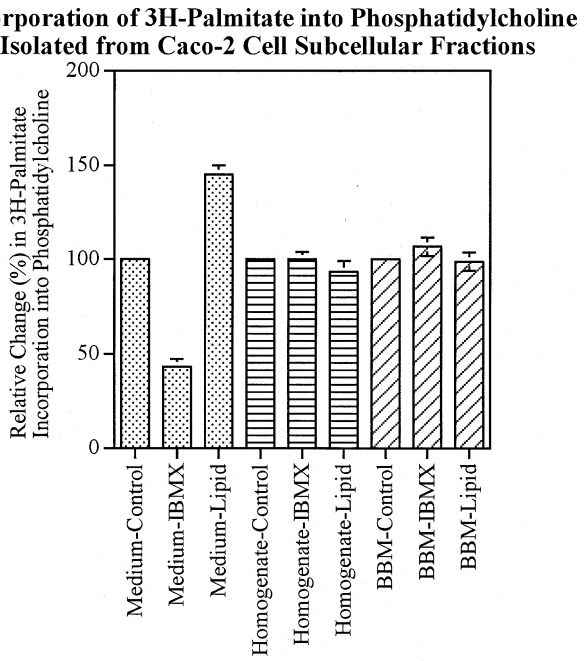


Fig. 6. Incorporation of [$^3$H]palmitate into PC fractions of Caco-2 cell homogenates and BBMs. Caco-2 cells were grown, labelled, and incubated as noted in Fig. 2. PC was isolated and assayed as described in Fig. 2. BBMs were prepared as described in Section 2.

in PC secretion (Fig. 5). In contrast, elevated cAMP, whether induced by extracellular dibutyryl cAMP or by isobutylmethylxanthine or forskolin, produced significant decreases (26–52%) in labelled PC (SLP) release from Caco-2 cells. The direction of the response of SLP secretion to both fat feeding and increased intracellular cAMP levels was the same as that for TG secretion (Table 2). These effects were not due to cell death, producing decreased secretion, or to cell damage, producing enhanced release, as neither medium LDH nor cell protein content were affected by any of these additions to the medium (LDH activity in the medium as a percent of total cell activity was 0.25–0.35% for all conditions).

Although TG release does not reflect any plasma membrane components, it was possible that labelled PC in the media might reflect 'solubilized' BBMs. Fig. 6 shows that incorporation of palmitate into the whole cell homogenate and into the BBM after overnight labelling was not altered in the presence of either inhibitors or stimulators of SLP release.

To determine if particle proteins and PC were coordinately secreted, SLP proteins were pre-labelled

Table 3
Effect of Pluronic L-81 and MTPI on Caco-2 cell lipid secretion and mannitol transport[a]

| Medium additions | [$^3$H]Triglyceride (dpm/mg protein) | [$^3$H]PC (dpm/mg protein) | Mannitol transport (%) |
|---|---|---|---|
| Control | 958 ± 178 (7) | 9837 (2) | 7.51 ± 0.44 (3) |
| +Lipid | 10 061 ± 1 894 (7)[#] | 21 921 ± 1 816 (8) | 7.75 ± 0.51 (4) |
| Lipid+Pluronic L-81 | 4 267 ± 1 820 (8)[+] | 9 255 ± 664 (8)[&] | 7.86 ± 0.81 (4) |
| Control | 1 612 (1) | 7 169 (1) | N.D. |
| +MTPI | 112 (1) | 5 091 (1) | N.D. |
| +Lipid | 46 060 (2) | 18 527 (2) | N.D. |
| Lipid+MTPI | 348 (2) | 5 706 (2) | N.D. |

[#] $P < 0.005$ compared to control; [+] $P < 0.05$ compared to lipid; [&] $P < 0.001$ compared to lipid; N.D. = not determined.
[a]Data represent the mean ± S.E.M. of (*n*) determinations. Lipid medium contained 500 μM oleic acid and 500 μM egg PC. Control medium contained no added lipids. Pluronic L-81 medium contained 500 μM oleic acid, 500 μM egg PC and 0.005% Pluronic L-81. Control+MTPI medium consisted of medium without added lipids and 150 μM MTPI. Lipid+MTPI medium contained 500 μM oleic acid, 500 μM egg PC, and 150 μM MTPI.

by overnight incubation of Caco-2 cells with [$^{35}$S]methionine/cysteine in a manner similar to that used for palmitate, and the pre-labelled cells were exposed to lipid. Media were concentrated, applied to a NaBr density gradient, and the membranes separated. SLP proteins were subsequently immunoprecipitated with anti-human small bowel SLP antibodies. Fat feeding led to a 1.8-fold increase in newly synthesized particle protein secreted into the medium that was comparable (1.8/2.2 or 81 ± 19%, mean ± S.E.M.) to the 2.2-fold increase observed for PC secretion (Fig. 5).

### 3.3. Effect of Pluronic L-81 and MTP inhibition on PC secretion in Caco-2 cells

Caco-2 cells were incubated with Pluronic L-81 to confirm the effect of this inhibitor of chylomicron secretion on SLP secretion. Pluronic L-81 produced a maximal 58% inhibition of both TG and PC release into the medium at a concentration of 0.005%. The amount of label in apical BBMs and whole cell homogenates was not altered (data not shown). Pluronic L-81 did not alter the permeability properties of the cellular monolayer, as assessed by mannitol transport (Table 3). Concentrations of the detergent greater than 0.005% sometimes adversely affected cellular integrity, as monitored by LDH activity, but the effect was not consistent. The MTPI, used at a concentration (150 μM) that nearly completely blocks apolipoprotein B100 secretion [32], decreased SLP and TG release after fat feeding by approximately 60% and 99%, respectively.

## 4. Discussion

The current study explores the factors that regulate secretion of PC from the fetal-like enterocytic cell line, Caco-2. PC is a component of membranes, but free PC is not secreted from cells [32]. In type II pneumocytes dipalmitoyl PC (pulmonary surfactant) is secreted in a process driven at least partially by cAMP [11,31,33]. Another PC-rich membrane, the SLP, is secreted by enterocytes in response to fat feeding [6] and by the Caco-2 cell in response to transfection with IAP [12]. However, enterocytes and Caco-2 cells also secrete lipoproteins in response to fatty acid feeding, among which are HDL that are rich in phospholipids [24]. When sufficient oleic acid is provided to Caco-2 cells in the presence of bile acids, they produce both small and large chylomicrons [34]. Thus, Caco-2 cells were chosen as a potential model to study the regulation of SLP secretion in response to the stimulation of fat feeding noted in vivo.

Culture conditions were selected to maximize TG and phospholipid secretion, while diminishing release of apical BBMs. The mixture of egg PC and fatty acid was chosen because it stimulated maximal TG secretion in VLDL from Caco-2 cells [29,35]. Although taurocholate had been added in an earlier study [29], it was omitted from the present study because it was found to increase LDH release into the medium. The concentration of oleic acid used (500 μM) was sufficient to lead to chylomicron secretion [28]. Fetal calf serum was used at 20% concentrations for growth and labelling of cells to ensure

maximal lipoprotein [24,26] and SLP production [12]. Palmitate was chosen as the labelling fatty acid for PC secretion, because it is the major component of PC in SLP [4], and because unsaturated fatty acids stimulate more lipoprotein secretion and are incorporated more readily into lipoproteins compared with saturated fatty acids [9,34]. Although calf serum was not used for the short incubation periods employed (4 h) in order to detect PC release, the properties of the SLP and the amount of IAP secreted differed from those reported for Caco-2 cells grown in serum [12,36]. Thus, the specific findings reported here might differ in the presence of serum, but the relative effects of lipid feeding on TG and PC secretion should be similar.

Caco-2 cells make SLP at low levels, so the mass of secreted phospholipid is not evident visually following gradient separation. This is true even after transfection with IAP, which stimulates SLP production and secretion [12]. Although IAP is associated with SLP via its glycosylphosphatidylinositol anchor [9], only about 0.25% of Caco-2 alkaline phosphatase was secreted (Fig. 4), and the increase in PC secretion was only about 2-fold after fat feeding (Fig. 5). Thus, release of alkaline phosphatase activity is not a sensitive measure of SLP secretion in Caco-2 cells following the stimulus of fat feeding. For these reasons the release of pre-labelled phospholipid was used, a technique used to detect pulmonary surfactant release from type II cells [11,31].

Caco-2 cells grown on filters release 60–80% of their secreted TG in the basolateral direction, but 'equal amounts' of phospholipid to apical and basolateral compartments, although the specific percents were not provided [24]. The present finding that 70% of PC was found in the apical compartment is consistent with this observation, and clearly different from the major direction of TG secretion. Moreover, 90% of the lipoprotein produced is of the density of chylomicrons or VLDL ($d < 1.006$ g/ml) when cells are grown in serum [26–28], and most of the major HDL proteins (apolipoproteins A-I and A-IV) are also secreted basolaterally [24,28]. Thus, the relatively dominant appearance of PC in the apical compartment may largely be due to SLP, whose direction of secretion is predominantly apical, via the tight junction, in Caco-2 cells [5,12].

SLPs are produced in rat enterocytes at relatively high levels at birth and are probably elaborated in fetal enterocytes [37]. Caco-2 cells correspond to a fetal-like phenotype for enterocytes and are relatively defective in lipid secretion. However, the degree of TG and phospholipid secretion appears to be similar judged from a review of many studies, as $7.68 \pm 5.42\%$ (S.D.) and $5.57 \pm 5.05\%$ of the cellular content of TG and phospholipid, respectively, appear in the medium [15]. SLPs are produced in rat enterocytes at relatively high levels at birth [37] and are probably elaborated in fetal enterocytes. Moreover, Pluronic L-81, an inhibitor of chylomicron secretion from enterocytes [38], inhibits secretion of TG from Caco-2 cells. When lymph fistula rats were perfused with an emulsion missing monoacylglycerol to channel oleic acid to the α-glycero-phosphate pathway, Pluronic L-81 still markedly suppressed intestinal TG output [39]. As Caco-2 cells possess mainly the α-glycerol phosphate pathway for TG synthesis [25], one would predict that Pluronic L-81 would suppress TG secretion in that cell line. Thus, the expected normal physiology involving fat feeding and lipid release from adult enterocytes appears to be approximated in the Caco-2 model.

The data presented using this model system support the observation that SLP surrounds the lipid droplet and appears to be involved in the process of fat absorption [8]. The secretion of PC follows that of TG, whether the stimulus leads to increased or decreased secretion. The effects of agents that alter intracellular concentrations of cAMP had the opposite effect of SLP secretion (decreased) to that seen in type II pneumocytes for pulmonary surfactant, where they increase secretion about 3-fold [11,31,33]. The physiological significance of this regulatory influence on SLP secretion is not clear at present.

Further experimental support for the linkage of TG release and SLP secretion is provided by the incubation of Caco-2 cells with the inhibitor of MTP. The addition of this inhibitor abolished TG transport and secretion, while simultaneously decreasing the appearance of SLP in the medium by about 60%. The inhibitor used decreases secretion of apolipoprotein B100 and TG in hepatoma cells [40]. Similar inhibitors preferentially reduce secretion of TG-rich lipoproteins from Caco-2 cells [41,42], but not the rate of TG synthesis [42]. MTP appears to be

required for the first step of apolipoprotein B secretion, perhaps at the time of assembly of a primordial HDL precursor [43]. These findings are consistent with earlier morphological studies [8] that show a strong physical connection between lipid droplets and lamellar structures after a fatty meal, when SLP appears to surround the intracellular lipid droplet but is depleted from the Golgi during Pluronic L-81 treatment. Further experiments will be needed to determine the exact role of SLP in this process.

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