Phosphatidylcholine Hydrolysis Is Required for Pancreatic Cholesterol Esterase- and Phospholipase A₂-facilitated Cholesterol Uptake into Intestinal Caco-2 Cells*

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Pancreatic secretion is required for efficient cholesterol absorption by the intestine, but the factors responsible for this effect have not been clearly defined. To identify factors involved and to investigate their role in cholesterol uptake, we studied the effect of Viokase®, a porcine pancreatic extract, on cholesterol uptake into human intestinal Caco-2 cells. Viokase is capable of facilitating cholesterol uptake into these cells such that the level of uptake is 5-fold higher in the presence of solubilized Viokase. This stimulation is time-dependent and is dependent on the presence of bile salt. However, bile salt-stimulated pancreatic cholesterol esterase, which has been proposed to mediate cholesterol uptake, is not fully responsible. The major cholesterol transport activity was purified and identified as pancreatic phospholipase A₂. Anti-phospholipase A₂ antibodies abolished virtually all of the phospholipase A₂ and cholesterol transport activity of solubilized Viokase. We demonstrate that both phospholipase A₂ and cholesterol esterase increase cholesterol uptake by hydrolyzing the phosphatidylcholine that is used to prepare the cholesterol-containing micelles. In the absence of cholesterol esterase or phospholipase A₂, uptake of cholesterol from micelles containing phosphatidylcholine is not as efficient as uptake from micelles containing phosphatidylinositol is not as efficient as uptake from micelles containing phosphatidylcholine. These results indicate that phospholipase A₂ may mediate cholesterol absorption by altering the physical-chemical state of cholesterol within the intestine.

The serum cholesterol level is determined mainly by cholesterol synthesis in the liver and clearance of cholesterol-containing lipoproteins and also by the amount of cholesterol absorbed from the intestine. This is demonstrated by the fact that inhibition of cholesterol absorption can decrease serum cholesterol, specifically low density lipoprotein cholesterol (1, 2). The intestinal cholesterol pool has two sources; typically one-third comes from the diet, and the remainder is endogenous cholesterol from bile (3, 4). Cholesterol absorption is not complete and varies widely among individuals; in humans, the percent of cholesterol load absorbed in the intestine has been estimated to vary from 15 to 75% (4), and individuals respond differently to changes in dietary cholesterol (5). This variation suggests that metabolic or genetic factors regulate absorption.

Although cholesterol absorption has been widely studied, the multiple factors involved are not fully understood. However, the absolute requirement for bile is established; bile salts are necessary for solubilization of cholesterol from the oil phase into micelles, from which it is available for absorption (6, 7). Pancreatic secretions also appear to be required. Many studies have shown that giving pancreatic enzymes as a dietary supplement increases fat absorption in patients with pancreatic insufficiency, and one report has specifically demonstrated that enzyme supplementation increases cholesterol absorption in these patients (8). Pancreatectomized dogs and humans have low plasma cholesterol, which can be increased by feeding raw pancreas or pancreatin, a pancreatic extract preparation (9, 10).

Of the pancreatic proteins, cholesterol esterase (CEase),¹ also known as bile salt-stimulated lipase and carboxyl ester lipase, has received most attention as having a potential role in cholesterol absorption. CEase has a wide substrate specificity, hydrolyzing tri-, di-, and monoglycerides and phospholipids in vitro (11). It also hydrolyzes cholesterol esters, which form a small part of dietary cholesterol and cannot be absorbed without prior hydrolysis to free cholesterol (12). Its role in absorption of free cholesterol has been under debate for many years with conflicting evidence regarding its importance in vivo (13, 14). In vitro, human intestinal Caco-2 cells have been used as a model for cholesterol uptake into the intestinal mucosa. Lopez-Candales et al. (15) reported that CEase stimulated cholesterol uptake from egg phosphatidylcholine (PC) vesicles by Caco-2 cells, whereas Huang and Hui (16) found no stimulation using a similar system. However, the latter study was performed at suboptimal concentrations of bile salt (15). Shmira et al. (17) also found no indication that CEase increased unesterified cholesterol uptake from egg PC or monoolein vesicles. Disruption of the CEase gene in mice confirmed the role of CEase in hydrolysis of cholesterol ester but found no evidence of a role for CEase in the absorption of free cholesterol (18).

The demonstrated importance of pancreatic proteins, combined with increasing data against a role for CEase in unesterified cholesterol uptake, motivated this study to investigate the presence in pancreas of proteins other than CEase which facilitate absorption of free cholesterol. A commercially available porcine pancreatic extract, Viokase®, was used to study cholesterol uptake into Caco-2 cells. Viokase has been used to increase lipid absorption in patients with pancreatic insufficiency (19). We describe the identification of the major cholesterol transport activity in the extract as pancreatic phospho-

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¹ The abbreviations used are: CEase, cholesterol esterase; PC, phosphatidylcholine; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
lipase $A_2$ and investigate the mechanism by which this enzyme facilitates cholesterol uptake in this model system.

**Experimental Procedures**

**Materials**—Colonial adenocarcinoma Caco-2 cells were from the American Type Culture Collection. 10–20% polyacrylamide Tricine gels were from NOVEX, Novel Experimental Technology. Fast Stan was purchased from Zoion Biotechnology. Viokase brand of pancrelipase USP was obtained from A. H. Robins (0.7 g contained 16,800 USP units of lipase, 70,000 USP units of protease, and 70,000 USP units of amylase. This latter preparation for scintillation spectrometry (TLS) plates was used was purchased from Silica Gel G from Analtech Inc. Porcine pancreatic phospholipase $A_2$, egg yolk 3-sn-phosphatidylcholine (egg PC), 1-palmitoyl, sn-glyceryl-3-PC, t- and n-dipalmitoyl PC, bovine serum albumin, Sephadex G-100, S-Sepharose, and heparin-agarose were all from Sigma Chemical Co. Tissue culture supplies were from Life Technologies, Inc. Bovine pancreatin CEase was purified to homogeneity, and anti-bovine CEase antibody was prepared in rabbits as described previously (20, 21). Porcine pancreatic phospholipase A2 or 100 nM CEase, or buffer alone in a final volume of 0.25 ml of buffer (final concentrations 1 mM leupeptin, and the indicated amount of enzyme or control buffer (25 mM acetate buffer, pH 5.1)). The protein-containing supernatant was used in the cholesterol transport assay. Immunoblotting of phospholipase $A_2$ and Viokase with anti-phospholipase $A_2$ antibodies was carried out as described previously (20).

**Purification of Transport Activity**—A sample of 12.5 ml (20 mg/ml) of Viokase solubilized in PBS containing protease inhibitors was applied to a 500-ml Sephadex G-100 column (100 × 2.5 cm) and eluted with approximately 600 ml of PBS at a flow rate of 12 ml/h. Protein eluted in three well separated peaks, and fractions were tested for cholesterol transport activity. All activity was found in the first peak, just after the void volume, which was estimated to contain less than 5% of total protein. Active fractions were pooled, dialyzed in 25 mM acetate buffer, pH 5.1, and approximately 3 mg of total protein was applied to an S-Sepharose column (25 ml bed volume). The column was washed with 25 mM acetate buffer, pH 5.1, then washed with a gradient of buffer containing 0–1,000 mM NaCl (total wash volume 200 ml) at a flow rate of 10 ml/h. Four peaks of protein eluted with approximately 60, 180, 270, and 370 mM NaCl. Fractions were tested for cholesterol transport activity, and the peaks eluting with 180 and 370 mM NaCl, were found to have transport activity. The active fractions eluting with 180 or 370 mM NaCl were pooled separately (pool 1 and pool 2) and contained 400 and 250 μg of protein, respectively. Both pools were dialyzed in 25 mM acetate buffer, pH 5.1.

Each of the two pools was then applied separately to a 10-ml bed volume heparin-agarose column in 25 mM acetate buffer, pH 5.1, at a flow rate of 15 ml/h and washed with buffer at the same rate. For pool 1, 380 μg of protein was loaded and the activity did not bind to the heparin column. From the 20-ml flow-through a 10-μl sample was active in the transport assay. Hereafter, this protein is referred to as Hep-P1. With pool 2, 225 μg of protein was applied to the heparin column. The activity bound to the heparin column and was eluted with a step wash of buffer containing 200 mM NaCl. Antibodies to Hep-P1 were raised in rabbits by Josman Laboratories, and the IgG fraction of immunoglobulin and nonimmune IgG were pooled, dissolved in 25 mM acetate buffer, pH 5.1, and approximately 3 mg of total protein was applied to a DEAE Affi-Gel Blue according to standard procedures (22). The antibodies reacted with both the major and minor isoforms of porcine phospholipase $A_2$. In some experiments, the active fractions from the Sephadex G-100 column were extracted by shaking for 3 min with 2 volumes of ice-cold diisopropylether:n-butyl alcohol (1.5:1.0, v:v). After phase separation by centrifugation, the solvent phase was recovered and dried under N₂ at 40 °C. Residue was dissolved in 20 μl of chloroform and was subjected to TLC as described below.

**Extraction of Lipids and Thin Layer Chromatography**—Cholesterol transport assays were performed as described above. After the specified time, incubation medium was removed from each well and placed on ice. Chloroform:methanol (5.0 ml of 2:1, v:v) and 1.0 ml of acid H₂O (H₂O containing 1% H₂SO₄) were added to the tubes containing 40 ml of the incubation medium. After mixing the tubes, 1 ml of chloroform:methanol:acetic acid:water (65:35:8:4) was added and the mixture shaken well. The layers were separated by low speed centrifugation at 4 °C for 10 min. The lower organic layer was recovered and dried under N₂ at 40 °C. Residue was dissolved in 20 μl of chloroform and was chromatographed using chloroform:methanol:acetic acid:water (65:35:8:4) as solvent. The solvent front was halfway up the plate. The plate was dried and then placed in a second chamber containing methanol:methanol:n-propanol:water (70:70:30:10) so that the solvent front was at the top of the plate. The plate was dried, and the lipids were stained with I₂ vapor.

**Phospholipase Assay**—Vesicles containing 1-palmitoyl-2-[1-3H]-cholesterol PC were prepared by mixing 2.0 μCi of radiola beled phospholipid with 12.5 μg of unlabeled 1-palmitoyl-2-oleyl PC and drying the mixture under N₂. A volume of 2.5 ml of 150 mM Tris·HCl, pH 7.5, was added and the mixture was sonicated, centrifuged, and the supernatant by the cholesterol transport assay in the presence and absence of 37 mM phospholipase $A_2$ or 100 nM CEase as described above.

**Cholesterol Oleate Hydrolysis Assay**—Release of [1-14C]oleic acid over a 5- or 10-min period from vesicles containing cholesteryl [1-14C]oleate and egg yolk PC was measured in 100 mM Tris·HCl, pH 7.5, and 8 mM taurocholate, as described previously (21).

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting**—Polyacrylamide 10–20% gradient gels were run in SDS running buffer (24 mM Tris, 192 mM glycine, 0.1% SDS). For recovery of protein, two samples of approximately 12 μg each were electrophoresed and half of the gel stained with Fast Stan. From the lane loaded on the unstained half of the gel, 0.5- or 1.0-cm slices were excised and placed in separate tubes. The slices were washed with a pipette tip, and 60 μl of 25 mM acetate buffer, pH 5.1, was added. The slices were incubated at 4 °C overnight and then centrifuged at 2,400 × g for 5 min. The protein-containing supernatant was used in the cholesterol transport assay. Immunoblotting of phospholipase $A_2$ and Viokase with anti-phospholipase $A_2$ antibodies was carried out as described previously (20).
stored at 4 °C. The specific activity of the vesicles was approximately $4 \times 10^5$ dpm/mg phospholipid. Reaction mixtures contained a final volume of 0.25 ml of Hanks’ buffered salt solution supplemented with 2.0 mM taurochenodeoxycholate, 1.0% bovine serum albumin, 1.0 mM CaCl$_2$, pH 7.4, 150 μg of 1-palmitoyl-2-[1-14C]oleoyl PC vesicles, and the indicated amount of enzyme diluted in PBS. After incubation for 30 min at 37 °C, each sample was extracted for total lipids, as described above, after the addition of 20 μg each of lyso-PC and oleic acid as carriers. The lipids were separated by TLC using the two solvent system described above, the lipid-containing areas were visualized with I$_2$ vapor, scraped, and radioactivity was determined by scintillation counting. The effects of anti-phospholipase A$_2$ antibodies on 1-palmitoyl-2-[1-14C]oleoyl PC hydrolysis were assessed by preincubating the assay mixtures described above with 12.0 μg/ml solubilized Viokase, 50 μg/ml soybean trypsin inhibitor, 100 μM leupeptin, and the concentrations of antibodies listed in the figures. After 15 min at 4 °C, the radiolabeled phospholipid vesicles were added, and hydrolysis of 1-palmitoyl-2-[1-14C]oleoyl PC was measured as described above.

Other Methods—Cell protein was measured using the Bio-Rad protein assay kit with bovine serum albumin as the standard. Phospholipids were measured by the method of Bartlett (23). Amino-terminal protein sequence was obtained at the Protein and Nucleic Acid Facility of Stanford University School of Medicine. Statistical analyses were performed using the computer program InStat 2.01 and an unpaired, two-tailed Mann-Whitney test.

RESULTS

Cholesterol Transport and Cholesteryl Oleate Hydrolysis Are Distinct Activities in Pancreatic Extract—In the Caco-2 model system used, with cholesterol presented in mixed micelles of egg PC and bile salt, there is a background level of uptake of cholesterol using buffer alone. Stimulation of cholesterol uptake above this background level is referred to as cholesterol transport activity. The ability of Viokase to stimulate uptake of cholesterol into Caco-2 cells was examined by performing the cholesterol transport assay in the presence of increasing amounts of solubilized Viokase. Uptake was stimulated in a concentration-dependent manner, reaching a plateau at approximately 5-fold stimulation over buffer alone. The effects of anti-phospholipase A$_2$ antibodies on 1-palmitoyl-2-[1-14C]oleoyl PC hydrolysis were assessed by preincubating the assay mixtures described above with 12.0 μg/ml solubilized Viokase, 50 μg/ml soybean trypsin inhibitor, 100 μM leupeptin, and the concentrations of antibodies listed in the figures. After 15 min at 4 °C, the radiolabeled phospholipid vesicles were added, and hydrolysis of 1-palmitoyl-2-[1-14C]oleoyl PC was measured as described above.
time points up to 4 h, facilitated transport was maintained at 5-fold over background (Fig. 1C).

Pancreatic CEase has been shown to stimulate cholesterol uptake in this model system (15) and is expected to be present in Viokase. Therefore, a series of studies was performed to evaluate whether the transport activity was the result of CEase. The bile salt-dependent cholesteryl oleate hydrolytic activity of both purified bovine pancreatic CEase and Viokase was assessed. On a mass basis, purified bovine pancreatic CEase was found to be about 1,000-fold more active than the impure Viokase (Fig. 2A). To test whether this hydrolytic activity in Viokase corresponded to cholesterol transport activity, equivalent amounts of cholesteryl oleate hydrolytic units of both purified bovine CEase and Viokase were used to measure cholesterol uptake into Caco-2 cells. With increasing amounts of hydrolytic activity, Viokase stimulated cholesterol uptake whereas even at the highest amount of hydrolytic units used, equivalent to 0.2 nM enzyme, CEase showed no stimulation above background (Fig. 2B). Thus, CEase, measured by cholesteryl oleate hydrolytic activity, does not account for all of the cholesterol transport activity of solubilized Viokase.

Further evidence that cholesteryl oleate hydrolysis and cholesterol transport are distinct activities in the pancreatic extract was a difference in sensitivity to boiling. Solubilized Viokase and purified bovine pancreatic CEase were used to measure cholesterol uptake into Caco-2 cells as described under “Experimental Procedures.” Samples of Viokase or CEase were removed for measurement of hydrolysis of cholesteryl [14C]oleate as described under “Experimental Procedures.” The data represent the average of duplicate determinations and are representative of two separate experiments.

Table I

<table>
<thead>
<tr>
<th>Addition</th>
<th>Temperature</th>
<th>Uptake of [3H]cholesterol</th>
<th>Hydrolysis of cholesteryl [14C]oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>dpm/µg cell protein</td>
<td>dpm/10 min</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>24.4</td>
<td>47</td>
</tr>
<tr>
<td>Viokase</td>
<td>4</td>
<td>96.7</td>
<td>2,265</td>
</tr>
<tr>
<td>Viokase</td>
<td>100</td>
<td>85.5</td>
<td>42</td>
</tr>
<tr>
<td>CEase</td>
<td>4</td>
<td>76.4</td>
<td>1,358</td>
</tr>
<tr>
<td>CEase</td>
<td>100</td>
<td>22.4</td>
<td>49</td>
</tr>
</tbody>
</table>

Figure 2. Determination of specific activity of Viokase and CEase for hydrolysis of cholesteryl [14C]oleate and comparison of transport activities. Panel A, hydrolysis of cholesteryl [14C]oleate was measured as described under “Experimental Procedures” in the presence or absence of the indicated amount of purified bovine pancreatic CEase (C) or Viokase (●). Each point represents the average of duplicate determinations and is representative of two separate experiments. The specific activities of CEase and Viokase calculated from these data were 4,241 and 8.1 units/µg of protein, where 1 unit = 10 dpm of free fatty acid released/5 min at 37 °C. Panel B, each well of cells was incubated with the indicated units of cholesteryl [14C]oleate hydrolytic activity of bovine pancreatic CEase (C) or Viokase (●). The uptake of [3H]cholesterol was measured after a 1.5-h incubation at 37 °C. Each point represents the average of duplicate dishes of cells and is representative of two separate experiments.

Figure 3. Recovery of cholesterol transport activity from SDS-PAGE. Two 12-µg samples of Hep-P1 were subjected to SDS-PAGE on a 10–20% polyacrylamide gel. Panel A, stained half of the gel, with molecular mass markers shown in kDa. Panel B, an unstained lane from the same gel was used to prepare proteins from gel slices as described under “Experimental Procedures.” Samples of 10 µl were tested in the cholesterol transport assay. The graph shows [3H]cholesterol dpm plotted as a function of the distance of the center of each gel slice from the top of the gel. This is aligned with the stained gel in panel A.
mM acetate buffer, pH 5.1, and incubated at 100 °C for 10 min. This inactivated the cholesteryl oleate hydrolytic activity of both preparations and also the cholesterol transport activity of CEase, whereas there was little reduction of Viokase-facilitated cholesterol transport (Table I). These combined results suggested that cholesteryl oleate hydrolysis and cholesterol transport activity were exhibited by different proteins.

Purification of the Cholesterol Transport Activity from Viokase and Identification as Phospholipase A₂—Sequential column chromatography was used to purify the cholesterol transport activity from Viokase. The first step was size exclusion chromatography on Sephadex G-100 in which the cholesterol transport activity eluted from the column just after the void volume. The active fractions were pooled and further purified by ion exchange chromatography on S-Sepharose. Two separate peaks of cholesterol transport activity eluted, one with 180 mM NaCl and the second with 370 mM NaCl. Only the peak eluting with 370 mM NaCl exhibited cholesteryl oleate hydrolysis activity. When the first peak was further purified by chromatography on heparin-agarose, cholesterol transport activity did not bind to the column. The active flow-through fractions were pooled and designated Hep-P1. In contrast, cholesterol transport activity from the second S-Sepharose peak bound to heparin-agarose and was eluted with 200 mM NaCl (designated Hep-P2). Hep-P1 and Hep-P2 were analyzed by immunoblotting with bovine CEase antibody, and immunoreactivity was demonstrated in Hep-P2 but not Hep-P1. Thus, the heparin-binding cholesterol transport activity was attributed to CEase (but see later in this section). Bovine CEase has previously been shown to bind to heparin-agarose (21).

When the Hep-P1 fraction was concentrated and a sample (12 μg) electrophoresed under denaturing conditions, only one major band, with an apparent molecular mass of 14 kDa, was observed (Fig. 3A). To confirm that this band represented the cholesterol transport activity, slices were excised from an unstained SDS-polyacrylamide gel, and protein recovered from each slice was used to measure cholesterol transport. Only the slice corresponding to the 14-kDa band stimulated uptake of cholesterol into Caco-2 cells (Fig. 3), demonstrating that the 14-kDa band was responsible for the observed cholesterol transport activity.

A sample (approximately 100 pmol) of Hep-P1 was subjected...
was incubated with 40 mAmide gradient gels and transferred to nitrocellulose strips. Each strip
mass standards in kDa are listed on the
with alkaline phosphatase-conjugated secondary antibodies. Molecular
antibodies on Viokase-facilitated uptake of [3H]cholesterol in Caco-2

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13385
to amino-terminal sequencing and was identified as the minor
isofrom of phospholipase A2. Phospholipase A2 is secreted from
the pancreas as a zymogen, which is activated by proteolytic
cleavage of the amino-terminal 7 amino acids. Pig pancreas has
two isofroms of phospholipase A2 which differ by only 4 resi-
dues, of which 2 occur within the amino-terminal 17 residues of
the mature protein (24). The sequence obtained corresponded
exactly to the 17 amino-terminal amino acids of the active,
minor isofrom, which is about 5% by weight of total pancreatic
phospholipase A2 (24).

To confirm the result that phospholipase A2 was responsible
for cholesterol transport, SDS-PAGE and Western blotting
with anti-porcine pancreatic phospholipase A2 antiserum was
performed and revealed an immunoreactive band of 14 kDa
from both Hep-P1 and phospholipase A2 purchased from
Sigma. Phospholipase A2 (Sigma) was tested for cholesterol
transport activity, and both this and Hep-P1 showed a 5-fold
stimulation of cholesterol uptake with an EC50 of 6–10 nm.
Sigma phospholipase A2 gives multiple bands on SDS-PAGE
data not shown). This material was further purified by hydro-
phobic chromatography on phenyl-Sepharose eluted with an
inverse gradient of (NH4)2SO4, followed by affinity chromatog-
raphy on heparin-agarose. Cholesterol transport activity was
found in two separate peaks; the first was the flow-through,
which represented unbound material, and the second was ma-
terial that bound to the heparin column and eluted with ap-
proximately 150 mM NaCl. Both were subjected to amino-ter-
rrnal sequencing. The unbound portion gave a mixed sequence
but contained the sequence of the minor phospholipase A2
isoform. The heparin-bound portion contained a single band of
14 kDa by SDS-PAGE and gave a sequence identical to the 18
amino-terminal residues of the major isofrom of phospholipase A2.
This demonstrates that both the major and minor isoforms
of phospholipase A2 exhibit cholesterol transport activity,
although they have different affinity for heparin. Subsequent
experiments showed that during the purification from Viokase,
the major isofrom of phospholipase A2 eluted with CEase in the
370 mM NaCl wash from the S-Sepharose column and with
CEase when Hep-P2 was chromatographed on heparin-agar-
ose. It had thus been attributed to CEase during purification
from Viokase.

Phospholipase A2 hydrolyzes phospholipids, cleaving the
fatty acid at the sn-2 position to give free fatty acid and sn-1
acyllyso-PC (25). To demonstrate conclusively that Hep-P1 is
phospholipase A2, we performed an in vitro hydrolysis assay
with micelles containing 1-palmitoyl-2-[1-14C]oleoyl PC and
quantitated radioactivity released as fatty acid or lyso-PC.
When increasing amounts of Hep-P1 were used, the amount of
radioactivity released as fatty acid increased, with little or no
radioactivity released as lyso-PC (Fig. 4A). This demonstrated
that Hep-P1 cleaves at the sn-2 and not the sn-1 position, i.e. it
exhibits phospholipase A2 activity. When CEase was used in
the same assay, radioactivity was found in lyso-PC, with little
radioactivity in free fatty acid, demonstrating that CEase acts
as phospholipase A1 (Fig. 4B). Solubilized Viokase was found to
have phospholipase A2 activity, with radioactivity released as
fatty acid and little radioactivity released as lyso-PC (Fig. 4C).

The 100% value for 1-palmitoyl-2-[1-14C]oleoyl PC hydrolysis was
12,000 dpm and for [3H]cholesterol uptake was 54 dpm/µg cell
protein. For 1-palmitoyl-2-[1-14C]oleoyl PC hydrolysis, each point represents a
single measurement and is representative of two separate experiments.
For [3H]cholesterol uptake, each point represents the mean ± S.D. of
three dishes of cells from two separate experiments.

Fig. 5. Effect of anti-phospholipase A2 antibodies on the phos-
pholipase A2 and cholesterol uptake activities of Viokase. Panel
A, a 0.4-µg sample of the purified major isofrom of porcine pancreatic
phospholipase A2 (lanes 1 and 3) or a 10.0-µg sample of solubilized
Viokase (lanes 2 and 4) was electrophoresed on 10–20% SDS-polyacryl-
amide gradient gels and transferred to nitrocellulose strips. Each strip
was incubated with 40 µl of either anti-phospholipase A2 antiserum or
nonimmune antiserum, and the immune complexes were visualized
with alkaline phosphatase-conjugated secondary antibodies. Molecular
mass standards in kDa are listed on the left. Panel B, effect of anti-
phospholipase A2 antibodies on Viokase-facilitated hydrolysis of
1-palmitoyl-2-[1-14C]oleoyl PC. Panel C, effect of anti-phospholipase A2
antibodies on Viokase-facilitated uptake of [3H]cholesterol in Caco-2
cells. The 100% value for 1-palmitoyl-2-[1-14C]oleoyl PC hydrolysis was
12,000 dpm and for [3H]cholesterol uptake was 54 dpm/µg cell protein.

For 1-palmitoyl-2-[1-14C]oleoyl PC hydrolysis, each point represents a
single measurement and is representative of two separate experiments.
For [3H]cholesterol uptake, each point represents the mean ± S.D. of
three dishes of cells from two separate experiments.
was caused by phospholipase A₂, antibodies directed against phospholipase A₂ were raised in rabbits. Antibody specificity was assessed by Western blotting, and the effects on cholesterol uptake were determined. The anti-phospholipase IgG specifically recognized the 14-kDa phospholipase A₂ on Western blots of the purified major isoform of porcine pancreatic phospholipase A₂ (Fig. 5A, lane 1) and recognized one protein of identical size in the solubilized extract of Viokase (Fig. 5A, lane 2). No reactivity was observed with nonimmune IgG (Fig. 5A, lanes 3 and 4). These data demonstrate that the antibodies are specific for phospholipase A₂. Incubation of solubilized Viokase with increasing amounts of anti-phospholipase A₂ IgG produced a concentration-dependent decrease in Viokase-mediated phospholipase A₂ activity, whereas equivalent amounts of nonimmune IgG had little or no effect (Fig. 5B). Similarly, incubation of solubilized Viokase with anti-phospholipase A₂ IgG produced a concentration-dependent decrease in Viokase-facilitated cholesterol uptake (Fig. 5C), whereas nonimmune IgG was without effect. Cholesterol uptake was reduced >95% by 280 μg/ml anti-phospholipase A₂ IgG. These data demonstrate that phospholipase A₂ was responsible for virtually all of the cholesterol uptake activity of Viokase in this system.

Mechanism of Phospholipase A₂-stimulated Cholesterol Transport—Identification of the cholesterol transport activity as phospholipase A₂ suggested that hydrolysis of micellar phospholipids may play a role in the observed transport. We thus tested whether or not micellar PC is hydrolyzed under the conditions used in the standard cholesterol transport assay (Fig. 6). The assay was performed for varying times in the presence of phospholipase A₂, purified bovine CEase, or control buffer, cells were solubilized, and [³H]cholesterol uptake was determined. In addition, the lipids in the assay media were extracted and separated by TLC. As before, 37 nM phospholipase A₂ or 100 nM CEase increased cholesterol uptake over background in a time-dependent manner (Fig. 6A). Within 15 min of incubation with phospholipase A₂ or CEase, hydrolysis of egg PC to lyso-PC and free fatty acid was observed (Fig. 6B). After incubation for 2 h, the majority of the egg PC was hydrolyzed. There was no significant hydrolysis of PC over this time course in the absence of enzyme. Although lipid staining with I₂ vapor was not quantitative and the results complicated by possible uptake of free fatty acid and lysophospholipid by the cells, this did provide an estimate of the relative amount of intact phospholipid in the extracellular media. The staining patterns of lipid remaining in the extracellular media were different for the incubations with CEase and phospholipase A₂. This is consistent with CEase cleaving at the sn-1 position, which in egg yolk PC is predominately saturated fatty acid, and phospholipase A₂ cleaving at the sn-2 position, which is predominately unsaturated fatty acid, since I₂ vapor stains the latter darker than the former.

These results suggested that the observed hydrolysis of micellar egg PC may directly cause the stimulation of cholesterol uptake observed with these enzymes. To investigate this, mixed micelles containing various ratios of egg PC, 1-palmitoyl...
Enzyme-facilitated Cholesterol Uptake

FIG. 8. Cholesterol uptake in cells pretreated with phospholipase A2 or CEase. Each dish of cells was pretreated with media containing 37 nm phospholipase A2, 100 nm CEase, or buffer alone in the absence of radiolabeled sterol as described under “Experimental Procedures.” The uptake of \([^{3}H]\)cholesterol was determined in the second incubation as described in the presence and absence of enzymes as indicated. Each bar represents the mean ± S.D. of four dishes of cells from two separate experiments. The level of \([^{3}H]\)cholesterol uptake was also measured in parallel cultures that were not pretreated. In the presence of buffer alone, 37 nm phospholipase A2, or 100 nm CEase, sterol uptake values for these cells were 33.3 ± 2.1, 137.2 ± 22.9, and 121.5 ± 13.3 (dpm/µg of cell protein, mean ± S.D., n = four dishes of cells) respectively.

As described above, phospholipase A2 or CEase induced a change in the Caco-2 cell membrane, leading to enhanced cholesterol uptake. To test this, Caco-2 cells were preincubated for 1.5 h at 37 °C with cholesterol uptake buffer containing phospholipase A2, CEase, or control buffer alone. After preincubation, cells were washed extensively; and cholesterol uptake buffer, containing fresh additions of phospholipase A2, CEase, or control buffer, was added, and cholesterol transport was determined. Pretreatment of Caco-2 cells with enzymes had no effect on \([^{3}H]\)cholesterol uptake measured in the subsequent incubation (Fig. 8). This suggested that the effect of both enzymes on cholesterol transport was limited to the effect on the lipid composition of the mixed micelles.

**DISCUSSION**

Four lipolytic enzymes, gastric lipase, triglyceride lipase, phospholipase A2, and CEase, are involved in the digestion of dietary and endogenously produced fat prior to absorption. Gastric lipase starts the hydrolysis of triglycerides in the stomach and hydrolyzes about 30% into mainly diglyceride and free fatty acid (29, 30). The other three enzymes are produced in the pancreas and are secreted into the intestine in pancreatic juice. Triglyceride lipase, also called pancreatic lipase, with its cofactor colipase, continues hydrolysis of triglycerides (31). Pancreatic CEase has a wide substrate specificity and may aid in the hydrolysis of some triglycerides as well as hydrolyzing cholesterol esters (11). Phospholipase A2 hydrolyzes fatty acids from phospholipids at the sn-2 position (26).

Here, we investigated the role of pancreatic secretory proteins in cholesterol transport into Caco-2 cells and presented results demonstrating that hydrolysis of micellar egg PC increases cholesterol transport in these cells. Identification of the major cholesterol transport activity of Viokase as phospholipase A2 explained both the observed heat stability of the activity and the retention of activity after denaturing gel electrophoresis, because phospholipase A2 is a small compact protein with seven disulfide bonds and is known to be heat-stable (25). Purification of phospholipase A2 has been described previously (25, 32). Despite its low molecular weight, phospholipase A2 eluted with an apparently high molecular weight from size exclusion chromatography. The most likely explanation is that complexes were formed with lipids present in Viokase. The fractions containing the transport activity were turbid and, after performing lipid extraction and TLC, were shown to contain phospholipid and cholesterol.
Dietary lipid is composed mostly of triglyceride with phospholipid estimated to be less than 3%. However, a larger source of intestinal phospholipid is derived from bile, and this is almost completely PC (33). Thus, the final ratio of triglyceride to phospholipid in the upper small intestine is approximately 10:1 (34). Like cholesterol, these lipids are insoluble in water and must be solubilized prior to hydrolysis. The relative proportions and the physical chemical state of lipids during digestion have been studied by Hernell et al. (35). Lipids were present in various physical states. An oil phase consisted of an emulsion of mostly unhydrolyzed triglyceride and cholesterol ester, with smaller amounts of fatty acid and phospholipid. Phospholipid stabilizes triglyceride emulsions but on its own may prevent hydrolysis by triglyceride lipase by forming an external coat that prevents access of enzyme to the triglyceride (34). However, this inhibition may not be seen in vivo because fatty acids, derived initially from the action of gastric lipase, become partially ionized and act as emulsifiers (36). This emulsification may be further enhanced by the lipolytic products, lyso-PC and monoglyceride, formed via the action of phospholipase A2 and triglyceride lipase, respectively. An aqueous phase was also present, consisting of mixed micelles of bile salt, fatty acid, monoglyceride, phospholipid, and cholesterol coexisting with unilamellar liquid crystalline vesicles containing the same lipids. Hernell et al. (35) suggested that multimellar vesicles containing lipolytic products pinch off the surface of the emulsion as triglyceride within it is hydrolyzed. With bile salt present, these multimellar vesicles form small unilamellar vesicles and micelles. The vesicles spontaneously dissolve to micelles, enriching the micelles with lipolytic products. This aqueous micellar phase has been shown to be necessary for absorption of both cholesterol and the lipolytic products (36).

Therefore, the mixed micelles from which cholesterol is absorbed appear to consist of free fatty acid, monoglyceride, phospholipid, bile salt, and cholesterol. The results presented in this paper demonstrate that hydrolysis of PC in bile salt/cholesterol/PC micelles increases uptake of cholesterol. In the Caco-2 cell system, background cholesterol uptake is probably limited by cholesterol availability from the PC mixed micelles. When PC is hydrolyzed, cholesterol uptake proceeds at a faster rate. However, the ratio of PC to lyso-PC and fatty acid may be important. We demonstrated that with micelles containing 30% PC, 70% lyso-PC, and 70% oleic acid, uptake in the absence of enzyme is only slightly reduced from the maximal level seen in the presence of 100% lyso-PC, 100% oleic acid micelles. This suggests that a small amount of PC in the presence of lyso-PC and fatty acid may not decrease cholesterol uptake in this system. In the intestine, however, the effect of phospholipase A2 and CEase may depend on the relative amounts of PC to lyso-PC and fatty acid. The relative concentration of bile salt to PC used in the present study was 2:0.3 mM, which is close to the relative concentration found in the micellar phase in vivo (35). The cholesterol concentration used in the experiments described in this paper was 0.72 μM, which is lower than the physiological concentration. However, when the cholesterol transport assay was performed with 20 or 50 μM cholesterol, a greater than 3-fold stimulation of cholesterol uptake was still observed in the presence of either 37 nM phospholipase A2 or 100 nM purified bovine CEase.

Several previous reports have shown an inhibitory effect of PC on micellar cholesterol absorption in rat intestinal sacs in vitro (37, 38). The experiments were performed in a relatively high concentration of oleic acid and monoolein; PC inhibited cholesterol uptake in the presence of 2-fold molar excess of monoolein and more than 4-fold molar excess of oleic acid. Those results combined with the results presented here suggest that the action of phospholipase A2 increases cholesterol absorption in vivo. CEase may also hydrolyze PC in vivo. Although the results presented with the anti-phospholipase A2 antibodies and pancreatic extract demonstrate that phospholipase A2 is quantitatively more important, the relative stabilities of the two enzymes in pancreatic extract powder may be different from those in vivo. To demonstrate any effect of PC on cholesterol absorption in an in vivo model, it may be necessary to inhibit both phospholipase A2 and CEase. Inhibiting phospholipase A2 in the intestine may result in general fat malabsorption because of decreased hydrolysis of triglyceride. Alternatively, triglyceride hydrolysis may not be affected because gastric lipase may provide sufficient fatty acid to allow the emulsification of triglycerides.

Two groups have previously failed to demonstrate any stimulatory effect of CEase on cholesterol uptake into Caco-2 cells (16, 17). The difference between their results and ours can be explained by differences in experimental systems. Huang and Hui (16) used 0.0625 mM taurocholate in the cholesterol uptake assay, which has been reported to be suboptimal for CEase facilitation of cholesterol uptake (15). Shamir et al. (17) used 100 nM human CEase in experiments where egg PC was used. The human enzyme has a lower specific activity for cholesterol ester hydrolysis than the bovine enzyme (20), and at 100 nM the human enzyme does not facilitate cholesterol uptake. However, in the majority of their experiments, cholesterol in monoolein micelles was used. Although monoolein is a substrate for CEase, the enzyme has a relatively low reactivity with this substrate and especially with the sn-1 ester (39). The sn-2 monoacylglycerol form will be present in vivo because triglyceride lipase cleaves preferentially at the sn-1 position (31). Although the form of monoolein used was not mentioned (17), either form may be resistant to hydrolysis under the conditions used. Alternatively, hydrolysis may not cause any change in the physical properties of cholesterol as appears to be the case for PC hydrolysis. On the other hand, monoolein is an emulsifying agent and may enhance cholesterol uptake (40), as we have shown for free fatty acid and lysophosphatidylcholine. This view is supported by the observation that egg PC micelles were not as efficient donors of cholesterol as micelles containing monoolein (17).

The data presented also provide a possible explanation for the result that CEase stimulates cholesterol esterification within enterocytes (15). CEase produces fatty acids from the hydrolysis of PC. Oleic acid has been shown to induce the enzyme that is responsible for cholesterol esterification, acylcoenzyme Acholesterol acyltransferase, in a variety of cell types (41). Therefore, it is possible that free fatty acid, released by hydrolysis of PC, induces acylcoenzyme Acholesterol acyltransferase in the Caco-2 cells, leading to increased cholesterol esterification. Background transport involves no hydrolysis of PC and therefore no resulting induction of acylcoenzyme Acholesterol acyltransferase. Although cholesterol also induces acylcoenzyme Acholesterol acyltransferase, the cholesterol concentration used in these experiments was too low to induce acylcoenzyme Acholesterol acyltransferase activity.

In conclusion, we have shown that hydrolysis of micellar PC increases cholesterol uptake into Caco-2 cells in vitro. Pancreatic CEase has been proposed to bind to intestinal cells and directly mediate cholesterol transport into these cells (15). Our results, however, suggest that both CEase and phospholipase A2 facilitate cholesterol transport by enzymatically modifying the micellar lipid composition. The lipid content of micelles in

the human intestine is clearly more complex than in our experimental system, and additional research, either with inhibitors in an in vivo model or with more complex systems in vitro, should clarify the roles of phospholipase A2 and CEase.

REFERENCES

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Phosphatidylcholine Hydrolysis Is Required for Pancreatic Cholesterol Esterase- and Phospholipase A2-facilitated Cholesterol Uptake into Intestinal Caco-2 Cells
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