Phosphatidylcholine Hydrolysis Is Required for Pancreatic Cholesterol Esterase- and Phospholipase A\textsubscript{2}-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\textsuperscript{®}, 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 A\textsubscript{2}. Anti-phospholipase A\textsubscript{2} antibodies abolished virtually all of the phospholipase A\textsubscript{2} and cholesterol transport activity of solubilized Viokase. We demonstrate that both phospholipase A\textsubscript{2} 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 A\textsubscript{2}, uptake of cholesterol from micelles containing phosphatidylcholine is not as efficient as uptake from micelles containing phosphatidylcholine A\textsubscript{2}-hydrolytic products. These results indicate that phospholipase A\textsubscript{2} may mediate cholesterol absorption by altering the physical-chemical state of cholesterol within the intestine.

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),\textsuperscript{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). Shamir 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\textsuperscript{®}, 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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\textsuperscript{1} The abbreviations used are: CEase, cholesterol esterase; PC, phosphatidylcholine; Tricine, N\textsubscript{2}-hydroxy-1,1-bis(hydroxymethyl)ethyglycine; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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lipase A₂ 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 Stain was purchased from Zoon Biotechnology. Viokase brand of pancreatic lipase 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. Thin layer chromatography (TLC) plates used were Whatman Silica G F from Analtech Inc. Porcine pancreatic phospholipase A₂, egg yolk 3-sn-phosphatidylcholine (egg PC), 1-palmitoyl-sn-glycero-3-PC, l- and d-n-palmitoyl 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 pancreatic CEase was purified to homogeneity, and anti-bovine CEase antibody was prepared in rabbits as described previously (20, 21). Tissue culture supplies were from Life Technologies, Inc. Bovine pancreatic CEase was purified to homogeneity, and anti-bovine CEase antibody was prepared in rabbits as described previously (20, 21).

Enzyme-facilitated Cholesterol Uptake

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stored at 4 °C. The specific activity of the vesicles was approximately $4 \times 10^5$ dpm/μg 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 Cholesterol 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 with 6 μg of Viokase protein (Fig. 1A). The effect was dependent on presence of a narrow range of bile salt concentrations (Fig. 1B) and was rapid, with stimulation of uptake seen within 15 min; at

FIG. 1. Effect of Viokase on uptake of micellar cholesterol in Caco-2 cells. Each well of Caco-2 cells was incubated with bile salt/egg PC micelles containing $[^{3}H]$cholesterol (0.06 μCi) as described under "Experimental Procedures." Panel A, incubation in the presence of the indicated amount of Viokase protein. Uptake of $[^{3}H]$cholesterol was measured after a 1.5-h incubation at 37 °C. Each point is the average of duplicate dishes of cells. Panel B, cells were incubated in the presence (●) or absence (○) of 6.0 μg of Viokase protein in buffer containing the indicated concentration of taurochenodeoxycholate (TCDC). Cholesterol uptake was determined after incubation for 1.5 h at 37 °C. Panel C, cells were incubated with 6.0 μg of Viokase protein (●) or buffer only (○). After the indicated time at 37 °C, uptake of $[^{3}H]$cholesterol was measured. Each point represents the mean ± S.D. of triplicate dishes of cells.
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 as described under “Experimental Procedures.” The data represent the average of duplicate determinations and are representative of two separate experiments.

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 diluted into 25}

![Fig. 2](http://www.jbc.org/)

**FIG. 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 (○) 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 (○) 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.

![Fig. 3](http://www.jbc.org/)

**FIG. 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.

![Table I](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Temperature</th>
<th>Uptake of [3H]cholesterol</th>
<th>Cholesteryl [14C]oleate hydrolysis</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></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>85.8</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>

Effect of elevated temperature on cholesterol uptake and cholesteryl oleate hydrolytic activities of Viokase extract and purified CEase

Viokase (180 μg of protein) or CEase (34 μg of protein) was incubated for 10 min at either 4 °C or 100 °C in 25 mM acetate buffer, pH 5.1. Precipitated protein was removed by low speed centrifugation, and aliquots, containing 3 μg of Viokase or 1.7 μg of CEase, were removed for measurement of [3H]cholesterol uptake in 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>
<thead>
<tr>
<th>Addition</th>
<th>Temperature</th>
<th>Uptake of [3H]cholesterol (dpm/μg cell protein)</th>
<th>Hydrolysis of cholesteryl [14C]oleate (dpm/10 min)</th>
</tr>
</thead>
<tbody>
<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>
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<td>49</td>
</tr>
</tbody>
</table>

Enzyme-facilitated Cholesterol Uptake

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 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 diluted into 25
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 40m amide gradient gels and transferred to nitrocellulose strips. Each strip mass standards in kDa are listed on the with alkaline phosphatase-conjugated secondary antibodies. Molecular nonimmune antiserum, and the immune complexes were visualized antibodies on Viokase-facilitated uptake of [3H]cholesterol in Caco-2

Enzyme-facilitated Cholesterol Uptake

FIG. 5. Effect of anti-phospholipase A2 antibodies on the phospholipase A2 and cholesterol uptake activities of Viokase. Panel A, a 0.4-μg sample of the purified major isoform 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-polyacrylamide 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
to amino-terminal sequencing and was identified as the minor isoform 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 isoforms of phospholipase A2 which differ by only 4 residues, 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 isoform, 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 hydrophobic chromatography on phenyl-Sepharose eluted with an inverse gradient of (NH4)2SO4, followed by affinity chromatography 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 material that bound to the heparin column and eluted with approximately 150 mM NaCl. Both were subjected to amino-terminal 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 isoform 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 isoform 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-agarose. 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).

Effect of Anti-phospholipase A2 Antibodies on the Phospholipase A2 and Cholesterol Transport Activities of Viokase—To evaluate what portion of Viokase-facilitated cholesterol uptake

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, 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,
 sn-glycero-3-PC (lyso-PC), and oleic acid were prepared to mimic the effect of phospholipase A2 hydrolysis of egg PC. Micellar cholesterol, bile salt, and total lipid concentrations were maintained to be the same as the vesicles previously used, but increasing amounts of PC were replaced with the molar equivalents of oleic acid and lyso-PC, which are the end products of phospholipase A2 hydrolysis. Cholesterol uptake into Caco-2 cells was determined, in the presence or absence of CEase or phospholipase A2 (Fig. 7A). In the absence of added enzyme, increasing amounts of oleic acid and lyso-PC increased cholesterol transport to the level seen with egg PC plus enzyme. Thus, with micelles containing oleic acid and lyso-PC but no PC, background cholesterol transport is increased, with no further increase caused by the addition of enzymes.

Thus it appears that cholesterol transport was stimulated by the presence of lyso-PC and/or free fatty acid rather than being stimulated by the enzyme, phospholipase A2, or CEase, itself. To test whether lyso-PC or fatty acid or both are responsible for this effect, vesicles were prepared containing 20% the normal concentration of egg PC plus either 80% oleic acid or 80% lyso-PC. The total lipid content was therefore not maintained, but the amount of products relative to PC was maintained. With both these mixtures, increased transport was seen even in the absence of CEase or phospholipase A2 (Fig. 7A). An additional increase with 20:80, PC:oleic acid vesicles was observed when phospholipase A2 or CEase was added.

The importance of phospholipid hydrolysis was confirmed by using vesicles prepared with dipalmitoyl PC instead of egg PC. The L-isomer of this phospholipid is hydrolyzed by phospholipase A2, whereas the D-isomer is not (26). In the presence of phospholipase A2, uptake was stimulated with L-dipalmitoyl PC vesicles but not with D-dipalmitoyl PC vesicles (data not shown). Hydrolysis of L-dipalmitoyl PC and the lack of D-dipalmitoyl PC hydrolysis were confirmed by extraction of lipids from the assay media and separation by TLC. CEase, which hydrolyzes both isomers, stimulated uptake with both. In addition, inactivation of the phospholipase A2 active site histidine residue with p-bromophenacylbromide (27) abolished both phospholipase A2-facilitated cholesterol transport and phospholipase A2-mediated hydrolysis of 1-palmitoyl-2-[1-14C] oleoyl PC with IC50 values of 10–20 μM.

As described above, phospholipase A2-facilitated cholesterol transport is dependent on phospholipase A2 hydrolytic activity. Although pancreatic phospholipase A2 does not interact well with biological membranes (28), it was possible that 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 [3H]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 multilamellar vesicles containing lipolytic products pinch off the surface of the emulsion as triglyceride within it is hydrolyzed. With bile salt present, these multilamellar 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 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 monoo lein; PC inhibited cholesterol uptake in the presence of 2-fold molar excess of monoo lein 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 monoo lein micelles was used. Although monoo lein is a substrate for CEase, the enzyme has a relatively low reactivity with this substrate and especially with the sn-2 ester compared 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 monoo lein 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, monoo lein 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 monoo lein (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, acyl-coenzyme A-cholesterol acyltransferase, in a variety of cell types (41). Therefore, it is possible that free fatty acid, released by hydrolysis of PC, induces acylcoenzyme A-cholesterol 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 A-cholesterol acyltransferase. Although cholesterol also induces acylcoenzyme A-cholesterol acyltransferase, the cholesterol concentration used in these experiments was too low to induce acylcoenzyme A-cholesterol 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.

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Phosphatidylcholine Hydrolysis Is Required for Pancreatic Cholesterol Esterase- and Phospholipase A\textsubscript{2}-facilitated Cholesterol Uptake into Intestinal Caco-2 Cells

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