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THE EFFECT OF PHOSPHOGLYCERIDES ON THE INCORPORATION OF CHOLESTEROL INTO ISOLATED BRUSH-BORDER MEMRBANES FROM RABBIT SMALL INTESTINE

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The incorporation of cholesterol from bile salt micelles into brush-border membranes was found to be similar whether these originated from jejunum, ileum or whole small intestine. This incorporation, however, was appreciably lower in membranes obtained from duodenum. Studies pursued with membranes from whole small intestine revealed that dipalmitoyl or dioleoylphosphatidylcholine, when micellized together with the sterol and taurocholate markedly inhibited the incorporation. The didecanoyl and dilauroyl analogues of this lipid class were without significant effect. Preincubation of the membranes for 30 min at 37°C with or without dipalmitoylphosphatidylcholine had no effect on cholesterol incorporation. Again in this case, suppression of sterol uptake could be seen only when phosphatidylcholine was admixed with the sterol. In contrast, dipalmitoyl and dilauroylphosphatidylethanolamines were found to be stimulatory rather than inhibitory. Addition of palmitic acid to the sterol-bile salt micelles had no effect on the uptake of cholesterol; however, this fatty acid could completely reverse the inhibition of cholesterol uptake by dipalmitoylphosphatidylcholine. The present study supports the conclusion that cholesterol incorporation into isolated brush-border membranes is governed largely by factors which affect the partitioning of the sterol out of the bile salt micelle.

Introduction

The incorporation of lipids into isolated rabbit brush-border membranes was reported recently [1]. A variety of neutral lipids and phosphoglycerides were found to be incorporated into these membranes via a process markedly stimulated by Ca^{2+} and other divalent cations [1]. Other factors influencing the incorporation were not studied in detail although the composition of the exogenous lipid suspensions used appeared to be an important factor.

It is well known that the rate of incorporation of cholesterol from micellar suspensions into isolated whole tissue preparations of small intestine is markedly diminished by the presence of phosphatidylcholine [2–5]. The mechanism by which the suppression occurs remains unclear. The possibility has been considered that phosphatidylcholine causes an increase in size as well as changes in shape and charge of the micelles in such a manner as to retard diffusion across the unstirred water layer. However, changes in micellar size and diffusion rate following phosphatidylcholine insertion appear to be quite small [2,8]. Because of this, other explanations have been suggested, namely that the suppression is due to a direct effect of phosphatidylcholine on the cell membrane or to a retention of the sterol within the micellar phase. The present investigation defines the effects of certain phosphoglycerides and palmitic acid on the incorporation of cholesterol into isolated intestinal

brush-border membranes and attempts to clarify further the mechanism of suppression of cholesterol absorption by phosphatidylcholine.

Methods

Phosphoglycerides, fatty acids and cholesterol (over 98% pure) were purchased from Sigma Chemicals. 1-Monoolein was obtained from Serdary Chemicals (London, Ontario). All these lipids gave a single spot when checked by TLC, except dioleoylphosphatidylcholine and monoolein which were found to contain trace amounts of free fatty acids. [7(n)-^{3}H]Cholesterol was purchased from New England Nuclear and diluted to required specific activity.

Preparation of brush-border membranes. Rabbit intestinal brush-border membranes were prepared from strips of small intestine which included parts of the duodenum, the jejunum and the ileum. The latter two portions furnished the bulk of the membranes. In one experiment, these three portions were separated and membranes were isolated from each for comparison. The isolation procedure used was that described by Selhub and Rosenberg [9]. The purity of the membranes was checked by assay of sucrase [10] and alkaline phosphatase [11] as marker enzymes, the respective specific activities of which were found to be, typically, 30-fold and 15-fold higher than those found for homogenate. On the other hand, the NADH oxidase [12] decreased 10-fold in the isolated membranes when compared to homogenate. Electron microscopic examination of the fractions established the membranes to be free of organelles and undissociated brush-border structures.

Preparation of lipid suspensions. [^{3}H]-Cholesterol-containing lipid mixtures were prepared by first dissolving the components together in chloroform. The solutions were then evaporated to dryness by prolonged exposure to a stream of nitrogen. A step, allowing removal of trace amounts of solvent, which involved storage of the residues in vacuo overnight prior to aqueous suspension was found to have no significant effect on any of the results and was subsequently dropped after having been introduced in experiments. The residue was suspended in 10 mM Tris buffer (pH 7.4) containing various concentrations of taurocholate depending on the experiment and sonicated at a power output of 250–300 W with the large probe of an Ultrasonics sonifier. Oleic acid (0.6 mM) and 1-monoolein (0.3 mM) usually added to the lipid mixture, helped considerably the dispersion of cholesterol in aqueous media. Suspensions containing phosphoglyceride were sonicated at temperatures slightly above the transition temperature of the phospholipid. Other suspensions were prepared at room temperature. Following sonication, completely clear suspensions were obtained by centrifugation at $10^7\ g \cdot$ min in a Beckman ultracentrifuge equipped with a 65 or a 50.2 Ti rotor.

Incorporation of lipids by membrane preparations. Incubation mixtures contained, in 1 ml volume, brush-border membranes (0.10–0.25 mg protein), Tris buffer, 10 mM (pH 7.4) and lipid sonicated in taurocholate-containing Tris buffer, 10 mM (pH 7.4) as specified in the text. After incubation with agitation at 37 °C for 30 min, the suspensions were centrifuged for 15 min at 12000 $\times\ g$ and the sediments obtained were washed once with Tris buffer. The final pellets were suspended in 0.2 ml water and transferred together with a 0.2 ml wash to counting vials containing toluene/PCS (Amersham), (1 : 1, v/v). Tritium was counted in a Beckman LS133 spectrometer using a channels ratio method. Controls without membranes added, were prepared in an identical manner and usually yielded counts corresponding to less than 1% of the added lipid.

Analytical procedures. Lipid concentrations in the clarified sonicated suspensions were determined by analysis of the lipid phosphorus according to the method of Bartlett [13] or by counting the amount of [^{3}H]cholesterol remaining. Since recoveries of cholesterol matched those of phosphoglycerides when these two lipids were sonicated together and centrifuged, routine recovery checks for these lipids were performed by counting the amount of [^{3}H]cholesterol remaining after centrifugation.

Reproducibility. All experiments were performed with membranes obtained from two rabbits or more, except for that experiment dealing with membranes isolated from different segments of intestine, where only one rabbit was used. However, variation between different rabbits and

determinations were relatively small (S.E. < 10%) and effects were always reproducible in each of the rabbits used.

Results

Results in Figs. 1 and 2 contrast the rates of cholesterol incorporation into membrane vesicles prepared from duodenum, jejunum and ileum. The amounts incorporated with time and the rates of incorporation with concentration were similar for the jejunum and ileum fractions but were considerably lower and did not increase above a cholesterol concentration of 0.05 mM in the case of membranes isolated from duodenum.

The rate of cholesterol incorporation into vesicles isolated from whole small intestine (Fig. 3) was found to vary with sterol concentration in a manner very similar to that seen for membranes isolated from jejunum and ileum. There seemed therefore to be no apparent advantage in working with fractions from distinct segments of the small intestine, and further experimentation was performed with membranes isolated from whole small intestine.

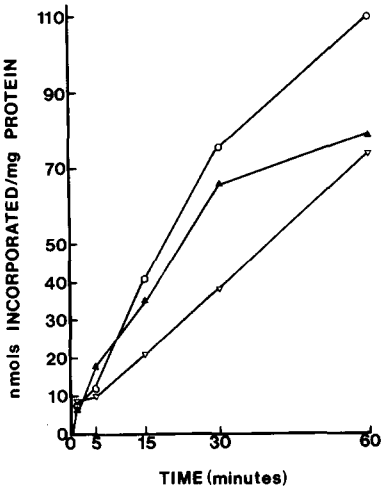

Fig. 1. Time-course of [^{3}H]cholesterol incorporation into brush-border membranes isolated from segments of small intestine. The incubation mixture was prepared by diluting 0.4 ml of a micellar solution containing 7.2 mM taurocholate, 0.26 mM [^{3}H]cholesterol, 0.6 mM oleic acid and 0.3 mM monoolein with 0.5 ml of 10 mM Tris buffer (pH 7.5) also containing 7.2 mM taurocholate and adding 0.1 ml membranes (0.13–0.35 mg protein). The mixtures were placed in a shaking water bath at 37 °C for the periods indicated. ○, Jejunum; ▲, ileum; ▽, duodenum. Each point represents an average of 2–5 determinations with membrane fractions from one rabbit.

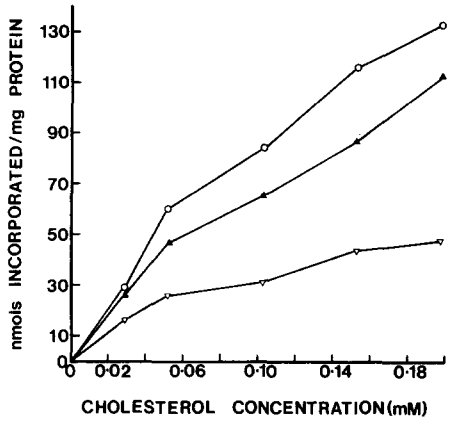

Fig. 2. Effect of [^{3}H]cholesterol concentration on incorporation into brush-border membranes. The conditions were as stated in Fig. 1, except that the incubation was for 30 min and the concentration of cholesterol was varied as indicated. ○, Jejunum; ▲, ileum; ▽, duodenum. Each point represents the average of three determinations with membrane fractions from one rabbit.

Whereas dipalmitoyl- and dioleoylphosphatidylcholines had a marked inhibitory effect on the rate of cholesterol incorporation (Fig. 4 and Table I), didecanoyl- and dilauroylphosphatidylcholines were without marked effect. The C_{16} and C_{18}

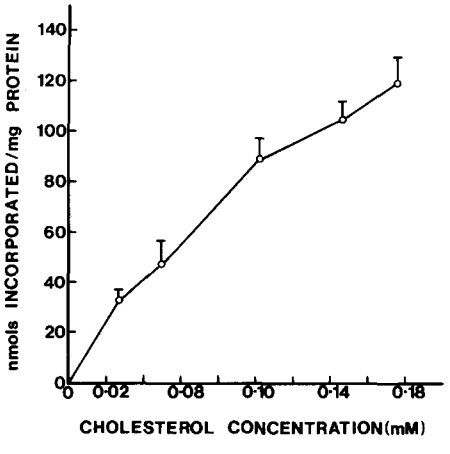

Fig. 3. The effect of [^{3}H]cholesterol concentration on incorporation into brush-border membranes isolated from whole small intestine. Each point represents the average ± S.E. of six determinations with membrane fractions obtained from two rabbits.

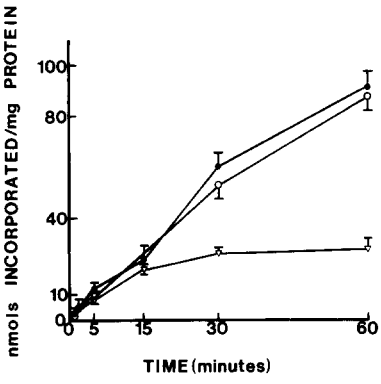

Fig. 4. The effect of phosphatidylcholines on the amount of [^{3}H]cholesterol incorporated with time. The conditions were the same as in Fig. 1, except that the micellar solution contained when indicated, 0.6 mM phosphatidylcholine prior to dilution. The values represent the average ± S.E. of 4–8 determinations on membranes obtained from 2–4 rabbits. ●, With dilauroylphosphatidylcholine; ○, without phosphatidylcholine; ▽, with dipalmitoylphosphatidylcholine.

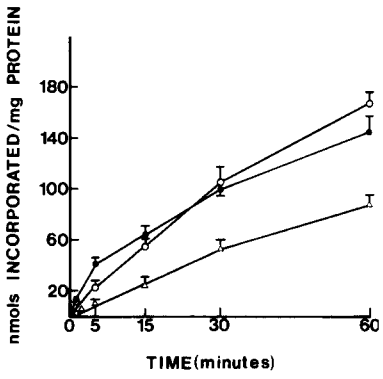

Fig. 5. The effect of phosphatidylethanolamines on the amount of [^{3}H]cholesterol incorporated with time. The conditions were as stated for Fig. 4, except that phosphatidylethanolamines replaced the choline analogues. The values are averages ± S.E. of four determinations on membranes from two rabbits. ○, Dilauroylphosphatidylethanolamine; ●, dipalmitoylphosphatidylethanolamine; △, no phosphatidylethanolamine.

diacylphosphatidylcholines inhibited incorporation by 60% or more at a concentration of 0.48 mM. In contrast, dilauroyl- and dipalmitoylphosphatidylethanolamines stimulated cholesterol incorporation quite significantly when these lipids were added to the bile salt micelles (Fig. 5).

Although with disc preparations of small intestine it was reported that increasing the concentration of bile salt to 20 mM could abolish the inhibitory effect of phosphatidylcholine [7], in the present study, with isolated brush-border membranes, the inhibitory effect of 0.48 mM dipalmitoylphosphatidylcholine was seen to persist even in 20 mM taurocholate (Table I).

TABLE I

THE EFFECT OF PHOSPHATIDYLCHOLINES ON THE INCORPORATION OF CHOLESTEROL

The incubation conditions were as stated for Fig. 1, except that the membranes used were from whole small intestine, and the micellar mixtures contained, when specified, 1.2 mM phosphatidylcholine prior to final dilution in the incubation mixture. Values in parentheses were obtained with 20 mM instead of 7.2 mM taurocholate. The results represent averages ± S.E. of 4–8 determinations on membranes from two or more rabbits.

Lipid added	Cholesterol incorporated (nmol/mg protein)
None	59 ± 5 (113 ± 11)
Dipalmitoylphosphatidylcholine	24 ± 3 (36 ± 6)
Dioleoylphosphatidylcholine	15 ± 1
Didecanoylphosphatidylcholine	72 ± 3

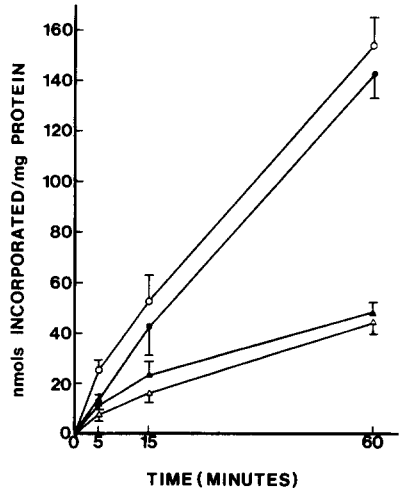

Fig. 6. The effect of preincubation with dipalmitoylphosphatidylcholine. Membranes were preincubated for 20 min under conditions stated for Fig. 1, except that the medium lacked cholesterol and either did not contain or did contain 0.3 mM choline phosphoglyceride (final concentration). The membranes were isolated, washed with 10 mM Tris buffer (pH 7.5) and reincubated for 30 min at 37 °C under conditions stated for Fig. 4. The values represent averages ± S.E. of four determinations on membranes from two rabbits. Preincubated with (○) or without (●) phosphatidylcholine, 0.48 mM, and reincubated without this lipid; preincubated with (△) or without (▲) phosphatidylcholine and reincubated with this lipid.

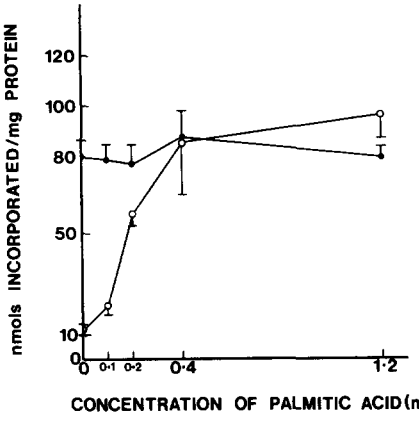

Fig. 7. The effect of palmitic acid on the rate of [^{3}H]cholesterol incorporation. The incubation conditions were as stated for Fig. 4, except that micelles were prepared with and without dipalmitoylphosphatidylcholine (1.0 mM prior to dilution) and various amounts of palmitic acid. The final concentrations of the fatty acid are indicated. The values represent averages ± S.E. of 5–7 determinations on membranes from three rabbits. ●, with; ○, without dipalmitoylphosphatidylcholine, 0.48 mM.

Results illustrated in Fig. 6 indicate that preincubation of membranes with and without dipalmitoylphosphatidylcholine followed by a single wash with Tris buffer, 10 mM (pH 7.4) and further incubation with cholesterol-bile salt micelles, had no effect on the incorporation of labelled sterol. Moreover, with such preincubated membranes, the inhibitory effect of dipalmitoylphosphatidylcholine could still be demonstrated provided this phosphoglyceride was in aggregate form with the sterol.

Results shown in Fig. 7 illustrate the effect of palmitic acid on the incorporation of cholesterol in the presence and absence of dipalmitoylphosphatidylcholine. In the absence of phosphatide, palmitic acid had no effect, but when added to phosphatidylcholine-containing micelles, this fatty acid could completely reverse the inhibition due to phosphatidylcholine.

Discussion

The present study further defines the conditions influencing the incorporation of cholesterol into isolated brush-border membranes. In a previous study [1], it was shown that although Ca^{2+} addition to the incubation mixture greatly increased incorporation of various lipids, their association with membranes was too rapid to allow kinetic studies. Omission of divalent cations in the present investigation permitted an assessment of the rate of incorporation of cholesterol and factors which influence this rate.

The results indicate that the inhibitory effect of choline phosphatides on cholesterol incorporation, previously noticed in vitro with whole tissue preparations or in vivo [2–7] can also be seen with isolated membrane functions. Although the possibility exists that phosphatidylcholine acts directly on the membrane to decrease its permeability towards cholesterol, there is no evidence to support such a mechanism. In fact, our previous study established that incorporation of exogenous phosphoglycerides into brush-border membranes is greatly diminished in the absence of Ca^{2+} [1] and presently our results indicate that preincubation of the membranes with phosphatidylcholine did not affect the incorporation of cholesterol. It is only when phosphatidylcholine is intercalated within the cholesterol-bile salt micelles that inhibition occurs.

The possibility that phosphoglyceride may act by increasing the size and change the shape of the micelles sufficiently so as to decrease their rate of diffusion across the unstirred water layer has been recently tested and precluded as a major mechanism of inhibition [2]. In our present study, this type of mechanism certainly was not involved, since stirring the samples at 480 rpm (results not shown) did not enhance the incorporation of cholesterol and an unstirred water layer did not appear to be an intervening factor. Furthermore, the inhibitory effect cannot be ascribed to just any micelle-expanding factor regardless of the lipid class involved, since phosphatidylethanolamines actually stimulated cholesterol uptake, and again, addition of palmitic acid by itself had no effect on incorporation of the sterol but quite unexpectedly, could completely reverse the inhibitory effect of dipalmitoylphosphatidylcholine.

Our results as a whole indicate that addition of lipids affects primarily the partitioning of cholesterol out of the micelles. There is evidence that, with certain types of lipid-bile salt mixtures at least, the uptake or exchange of lipid by/or with membranes involves the monomer phase [14]. With other types of micelles, this process could

involve collision of the micelle [7] but not likely its fusion [14] with the membrane. Irrespective of the precise incorporation mechanism intervening in our case, increased retention of [^{3}H]cholesterol by the micellar phase would tend to decrease the pool of labelled sterol available for net uptake or exchange and this would decrease the rate of labelled cholesterol incorporation.

The ability of the micelle to retain cholesterol would be expected to vary greatly with the type of lipid added. In fact, the present study indicates that this ability depends not only on the acyl chain-length of the lipid but on the nature of its polar headgroup as well. A greater understanding of this phenomenon would require more detailed studies on the cholesterol-lipid-bile salt interactions within the micelle.

Acknowledgement

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References

1 Proulx, P., McNeil, J., Brglez, I. and Williamson, D.G. (1982) Can. J. Biochem. 60, 904–909
2 Rampone, A.J. and Machida, C.M. (1981) J. Lip. Res. 22, 744–752
3 Rampone, A.J. and Long, L.R. (1977) Biochim. Biophys. Acta 486, 500–510
4 Rodgers, J.B. and O'Connor, P.J. (1975) Biochim. Biophys. Acta 409, 192–200
5 Hollander, D. and Morgan, D. (1979) Lipids 15, 395–400
6 Greten, M., Raetzer, H., Stiehl, A. and Schettler, G. (1980) Atherosclerosis 36, 81–88
7 Thomson, A.B.R. and Cleland, L. (1981) Lipids 16, 881–887
8 Feldman, E.B. (1968) Biochem. Med. 2, 136–144
9 Selhub, J. and Rosenberg, I.H. (1981) J. Biol. Chem. 256, 4489–4493
10 Dahlqvist, A. (1964) Anal. Biochem. 7, 18–25
11 Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968) Biochem. J. 106, 381–390
12 Awruch, J. and Wallach, D.F.H. (1978) Biochim. Biophys. Acta 233, 334–337
13 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
14 Westergaard, H. and Dietschy, J.M. (1976) J. Clin. Invest. 58, 92–108