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LIPID AND PROTEIN MEMBRANE COMPONENTS ASSOCIATED WITH CHOLESTEROL UPTAKE BY MYCOPLASMAS

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Summary

Membranes of *Mycoplasma* species take up 2—4 times more exogenous cholesterol than membranes of *Acholeplasma* species. To test whether the lower cholesterol uptake capacity of *Acholeplasma* is due to the high glycolipid content of their membranes, the phospholipids of *Acholeplasma laidlawii* and *Mycoplasma capricolum* membranes were hydrolyzed by phospholipase A_2. Digestion removed about 30% of the polar lipids of *A. laidlawii*, leaving the glycolipids and phospholglycolipids intact, and about 70% of the polar lipids of *M. capricolum*, the residue consisting mostly of sphingomyelin. Cholesterol uptake by the treated membranes from phosphatidylcholine/cholesterol vesicles decreased in rough proportion to the amount of polar lipid removed, indicating that the glycolipids in *A. laidlawii* membranes can participate in cholesterol uptake.

Trypsin digestion of growing cells and isolated membranes of *M. capricolum* decreased cholesterol uptake by about one-half. Similar treatment of *A. laidlawii* cells and membranes had no effect on cholesterol uptake. These findings suggest the existence of protease-sensitive receptors on the cell surface of *M. capricolum* responsible for tighter contact with the cholesterol/phosphatidylcholine vesicles. It is proposed that the ability of *Mycoplasma* species to take up large quantities of exogenous cholesterol and phospholipids depends on the presence of protein receptors for cholesterol donors, receptors which are absent in *Acholeplasma* species.

Introduction

Cholesterol is not uniformly distributed amongst the various membranes of mammalian cells. In general, plasma membranes are rich in cholesterol whereas

most internal membranes contain very low levels of cholesterol [1]. The reasons for these differences in cholesterol distribution are not clear. *Mycoplasma* species are wall-less procaryotes which require cholesterol for growth and incorporate large quantities of free and esterified cholesterol. The molar ratio of free cholesterol to esterified cholesterol in the organism investigated was 2 : 1. The free cholesterol comprises about 20% by weight of the total membrane lipid. The related *Acholeplasma* species do not require cholesterol and are much more restricted in their capacity to incorporate cholesterol (up to 10% of total membrane lipid [2,3]). Elucidation of the factors responsible for the significant differences in cholesterol binding capacity between *Mycoplasma* and *Acholeplasma* species may obviously lead to better understanding of the mechanism contributing to the differences in cholesterol distribution among biological membranes. Mycoplasma and acholeplasma membranes are eminently suitable for studying this problem as all of their membrane cholesterol is of exogenous origin and remains chemically unmodified [3].

The notion that the amount of cholesterol incorporated is determined by the polar lipid composition of the membrane has been repeatedly raised [4,5]. *Acholeplasma* membranes differ from those of *Mycoplasma* species in their high content of glycosyldiglycerides and phosphoglycolipids [6]. Several recent reports claim significant differences in cholesterol binding ability among different phospholipid and glycolipid species [5,7,8]. It seemed therefore of interest to examine the effect of the selective removal of phospholipids on the cholesterol-binding capacity of representative *Mycoplasma* and *Acholeplasma* membranes. Pancreatic phospholipase A_2 was found to hydrolyse effectively the phospholipids of *Acholesplasma laidlawii* membranes leaving the glycolipids and phosphoglycolipids intact [9]. The same enzyme has been shown also to hydrolyze phospholipids of *Mycoplasma capricolum* membranes (Gross, Z. and Rottem, S., unpublished data).

The possibility that the protein component of mycoplasma membranes plays a role in cholesterol uptake has attracted little attention so far [10]. The membrane protein composition of *Acholeplasma* species is totally different from that of *Mycoplasma* species [11]. It can be hypothesized that the great difference in cholesterol-binding ability between *Mycoplasma* and *Acholeplasma* species depends on the presence of specific protein receptors for cholesterol donors on the surface of *Mycoplasma* species only. As a first step to test this hypothesis we examined the effect of proteolytic digestion of mycoplasma membranes on cholesterol uptake.

The data presented in this communication support the notion [4] that the amount of cholesterol incorporated into mycoplasma membranes is correlated with the amount of membrane polar lipids, including the phosphoglycolipids and glycolipids of *A. laidlawii* membranes. However, the proteolytic digestion experiments indicate that a protease-sensitive membrane component plays an important role in cholesterol uptake by *M. capricolum* but not by *A. laidlawii*, supporting the idea of protein receptors for cholesterol donors on *Mycoplasma* membranes.

Materials and Methods

Organisms and growth conditions. Acholeplasma laidlawii (oral strain) and *Mycoplasma capricolum* (California kid, ATCC 27434) were grown in a modified Edward medium [12] supplemented with 0.5% (w/v) glucose, 0.2% (w/v) bovine serum albumin (Fraction V, Sigma), 25 μg/ml elaidic acid and 0.1 μCi/ml [^{3}H]palmitate (Amersham). For growth of *M. capricolum*, 0.3% (v/v) horse serum was added. The low serum concentration which enabled only suboptimal growth of *M. capricolum* resulted in cells with a low cholesterol content, while the *A. laidlawii* cells cultivated in the total absence of serum had no cholesterol in their membrane. The organisms were harvested after 20—24 h of incubation at 37°C, when the absorbance of the culture reached about 0.17 at 640 nm for *M. capricolum* and 0.30 for *A. laidlawii*. The sedimented organisms were washed twice and resuspended in 0.25 M NaCl. Cell membranes were isolated by osmotic lysis of the organisms, as described in detail by Razin and Rottem [12]. To facilitate the isolation of *M. capricolum* membranes free of DNA, pancreatic deoxyribonuclease (20 μg/ml) was added to the lysed cell preparation.

Phospholipase A_2 treatment of membranes. Membranes were treated with phospholipase A_2 (pancreatic, Boehringer) in a buffer containing 0.25 M NaCl, 100 mM Tris-HCl, pH 7.4, 50 mM $CaCl_2$. 0.25% (w/v) albumin and 1 unit of enzyme per mg membrane protein. After 2 h of incubation at 37°C, samples were withdrawn and analyzed for free fatty acids by a modified Dole procedure [13]. The reaction in the remaining mixture was stopped by the addition of an equal volume of 0.1 M EDTA. To remove the free fatty acids and lyso compounds, the treated membranes were collected by centrifugation, resuspended in 0.05 M phosphate buffer, pH 7.4, containing 4% (w/v) albumin [14] and incubated for 1 h at 37°C. The membranes were then sedimented and resuspended in 0.05 M NaCl.

Proteolytic digestion of membranes. Membranes in 0.05 M phosphate buffer, pH 7.0, were incubated with 50 μg/ml of trypsin (Sigma, diphenylcarbamyl chloride treated) or pronase (Sigma) for 2 h at 37°C. The membranes were sedimented and washed twice with 0.25 M NaCl.

Cholesterol donors. Cholesterol/phosphatidylcholine (0.9 : 1.0, molar ratio) vesicles were used as donors. Lipid vesicles were prepared by sonication from cholesterol (Sigma) labeled with [4-^{14}C]cholesterol (Amersham) and egg phosphatidylcholine (egg lecithin, Makor Chemicals, Israel) as described in detail by Razin et al. [15]. Over 95% of the lipid in the vesicle suspension was retained on filtration through a Sepharose 2B column [16], indicating that it consisted primarily of unilamellar vesicles. The vesicle suspension was kept at 4°C and used within 2—3 days after preparation.

Measurement of cholesterol uptake. Cholesterol uptake from vesicles was tested with untreated or enzymatically treated membranes of *M. capricolum* grown with 0.3% horse serum, and with membranes of *A. laidlawii* grown with no serum. The uptake mixture consisted of 0.05 M phosphate buffer, pH 7.0, containing cholesterol/phosphatidylcholine vesicles (to yield 20 μg cholesterol/ml) and membranes (0.1 mg protein/ml). The suspension was incubated statically at 37°C for 4—5 h, and 25-ml samples were withdrawn at various time

intervals. The membranes were collected by centrifugation at 38 000 × *g* for 25 min, washed once in 0.25 M NaCl and resuspended in 0.05 M NaCl. The centrifugation employed did not cause the sedimentation of the vesicles, as was evidenced by the absence of phosphatidylcholine from the pellet of *A. laidlawii* membranes incubated with the vesicles [15]. The washed membranes were analyzed for their protein, radioactivity and lipid content. Cholesterol uptake from vesicles was also tested with untreated or trypsin-treated *M. capricolum* cells grown with 0.3% (v/v) horse serum and *A. laidlawii* grown with no serum. After 23 h of incubation at 37°C, the culture was divided into two equal parts. Trypsin (100 μg/ml) was added to one part and incubation was continued for another 2 h at 37°C. Cholesterol/phosphatidylcholine vesicles were then added to both parts to raise the cholesterol concentration in the medium by 20 μg/ml, and incubation was continued for another 5 h at 37°C. Aliquots were taken for absorbance measurements and for viable counts [17]. The cells were then collected by centrifugation at 12 000 × *g* for 15 min, washed once in 0.25 M NaCl and analyzed for their protein, radioactivity and lipid content.

Analytical procedures. Protein was determined according to the method of Lowry et al. [18]. Lipids were extracted from cells or membranes with $CHCl_3$/CH_3OH (2 : 1, v/v [19]). The lipid extracts were analyzed for cholesterol by using the colorimetric technique of Rudel and Morris [20] or by radioactivity measurements of [^{14}C]cholesterol using a toluene-Triton scintillation mixture [21]. Lipid phosphorus was determined by using the method of Ames [22] after digestion of the sample by an ethanolic solution of $Mg(NO_3)_2$. Polar lipids were estimated according to radioactivity derived from [^{3}H]palmitate. Thin-layer chromatography of poalr lipids was performed on silica gel HR (Merck) plates [15]. For quantitation of polar lipids, the area of the spots was scraped off the plate and radioactivity and phosphorus were determined directly on the collected silica gel material. Neutral lipids were separated on silica gel G (Merck) plates and the amounts of free and esterified cholesterol were determined as described in detail previously [15].

Results

Cholesterol-binding capacity of phospholipase A_2-treated membranes

Phospholipase A_2 hydrolyzed about 30% of the polar lipids of *A. laidlawii* membranes and about 70% of those of *M. capricolum* membranes. Treatment of the hydrolyzed membranes with albumin [14] removed effectively the hydrolysis products consisting of free fatty acids and lyso compounds, as was evidenced by thin-layer chromatography and by [^{3}H]palmitate and lipid P_i determinations. In *A. laidlawii*, only phosphatidylglycerol and diphosphatidylglycerol, which comprise about 30% of the polar lipids, were hydrolyzed by phospholipase A_2. The phosphoglycolipids and glycolipids, which comprise the major fraction of *A. laidlawii* polar lipids, were unaffected by the enzyme. Of the polar lipids of *M. capricolum* membranes, only sphingomyelin remained intact (Table I). The other lipid components, including cholesterol and cholesteryl esters in *M. capricolum* and carotenoids in *A. laidlawii*, were not attacked by the enzyme, nor was the protein component of these membranes affected.

Fig. 1. shows that phospholipase A_2 treatment decreased the cholesterol-up-

TABLE I

LIPID SPECIES HYDROLYZED BY PHOSPHOLIPASE A_2 TREATMENT OF *A laidlawii* AND *M. capricolum* MEMBRANES

Lipid species	*A. laidlawii*		*M. capricolum*	
	% of polar membrane lipid *	% hydrolyzed	% of polar membrane lipid **	% hydrolyzed
Phosphatidylglycerol	13.4	94	23.5	87
Diphosphatidylglycerol	15,6	90	32.4	95
Sphingomyelin	0	0	12.1	0
Phosphatidylcholine	0	0	31.8	80
Phosphoglycolipids	36.6	0	0	0
Glycolipids	34.4	0	0	0

* According to radioactivity derived from [^{3}H]palmitate.
** According to lipid phosphorus.

take capacity of *A. laidlawii* membranes by about 55% and that of *M. capricolum* membranes by about 60%. The amount of lipid P_i and [^{3}H]palmitate-labeled lipids in the phospholipase A_2-treated or untreated membranes of both organisms did not change during the exposure of the membranes to the cholesterol/phosphatidylcholine vesicles. Thin-layer chromatography showed no increase in the amount of phosphatidylcholine in the membranes, indicating that the lipid vesicles used as cholesterol donors neither adhered to nor fused with the membranes.

Cholesterol-binding capacity of trypsin-treated membranes

Trypsin or pronase treatment of *A. laidlawii* and *M. capricolum* membranes resulted in the digestion and removal of 45—50% of the membrane protein components without affecting the lipid content of the membranes. Fig. 2 shows that trypsin treatment of *A. laidlawii* membranes did not affect their cholesterol-binding capacity, whereas the amount of cholesterol taken up by the digested *M. capricolum* membranes was only about one-half of that taken up by the untreated membranes. Similar results were obtained with pronase. In

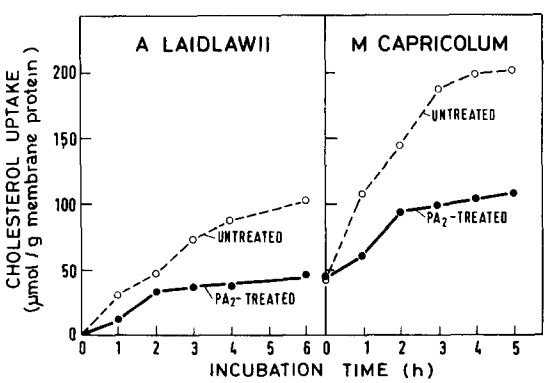

Fig. 1. Cholesterol uptake by phospholipase A_2-treated (PA_2-treated) mycoplasma membranes.

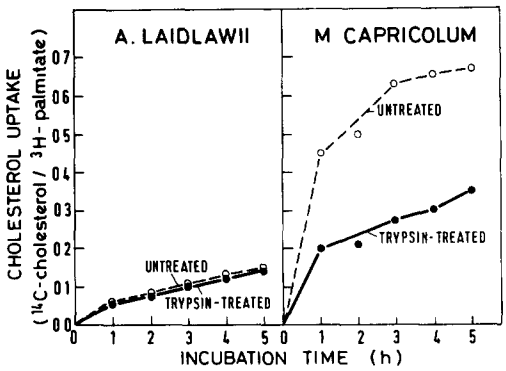

Fig. 2. Cholesterol uptake by trypsin-treated mycoplasma membranes.

one experiment, isolated *M. capricolum* membranes were treated with 5 μg/ml trypsin. The treated membranes, which lost only 30% of their membrane protein, also lost about 50% of their cholesterol-binding capacity. No change in the amount of lipid P_i and [^{3}H]palmitate-labeled lipids occurred in trypsin-treated and untreated membranes during incubation with the cholesterol/phosphatidylcholine vesicles. The lack of increase in the amount of phosphatidylcholine in the membranes indicated that lipid vesicles did not adhere to the digested membranes.

Cholesterol-binding capacity of trypsin-treated cells

Table II shows the effects of trypsin on growing cells. Trypsin had no effect on the growth of *A. laidlawii*, as indicated by the increase in absorbance of culture, cell protein and viable counts (not shown). The ratio of lipid P_i to cell protein remained constant. On the other hand, trypsin added to *M. capricolum* cultures inhibited cell reproduction, though it did not kill the cells. The number

TABLE II

EFFECTS OF TRYPSIN ON GROWING MYCOPLASMA MEMBRANES

The organisms were grown for 23 h in Edward medium with no serum for *A. laidlawii* and with 0.3% serum for *M. capricolum*. Trypsin (100 μg/ml) was added to one part of the cultures and incubation was continued for another 2 h at 37°C. Cholesterol/phosphatidylcholine vesicles were then added to both parts of the culture and incubation was continued for 5 h, during which aliquots were taken for measurements of absorbance, cell protein and lipid P_i. Zero time represents the time of vesicle addition.

Culture	Time (h)	*A laidlawii*		*M. capricolum*	
		Cell protein (mg/250 ml culture)	Lipid P_i (μmol/mg cell protein)	Cell protein (mg/250 ml culture)	Lipid P_i (μmol/mg cell protein)
Untreated	0	21.6	0.12	6.7	0.14
	2	27.0	0.12	10.4	0.25
	5	35.0	0.12	19.0	0.33
Trypsin-treated	0	17.5	0.12	5.2	0.19
	2	32.0	0.13	8.0	0.23
	5	38.1	0.12	8.2	0.28

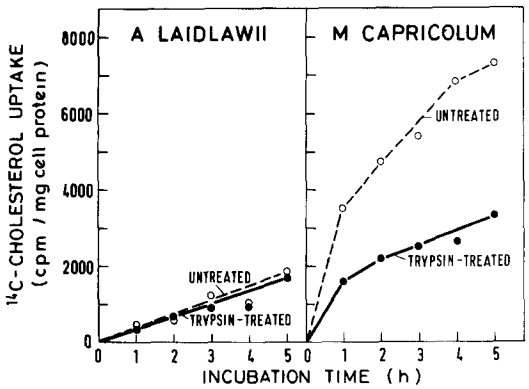

Fig. 3. Cholesterol uptake by trypsin-treated mycoplasma cells. For experimental procedures, see Materials and Methods.

of viable organisms, as determined by viable counts, remained at about $4 \cdot 10^8$ colony-forming units/ml in the trypsin-treated culture, whereas in the untreated culture it increased from $4.8 \cdot 10^8$ to $1.9 \cdot 10^9$ colony-forming units/ml at the end of the experiment. The ratio of lipid P_i to cell protein increased mostly due to the uptake of exogenous phosphatidylcholine [15]; the increase being higher in the untreated cells. Fig. 3 shows that, as in the case of isolated membranes, treatment of *A. laidlawii* cells by trypsin had no effect on cholesterol-binding capacity, whereas trypsin treatment of *M. capricolum* cells decreased cholesterol uptake by about one-half, expressed either as [^{14}C]-cholesterol per mg cell protein (Fig. 3) or per μmol lipid P_i (not shown).

Discussion

Hydrolysis of mycoplasma membrane phospholipids by phospholipase A_2 and the subsequent removal of the hydrolysis products by albumin brought about a significant decrease in the polar lipid component, and an almost parallel decrease in the cholesterol-binding capacity of the treated membranes. This finding coroborates and extends our previous observations showing that the cholesterol-binding capacity of mycoplasma membranes is dependent on their polar lipid content [4,10]. Hence, the principle proposed by Cooper [23] for red cell membranes that the cholesterol content of the membrane is a function of the membrane phospholipid content appears to hold true also for mycoplasma membranes. Nevertheless, our data also indicate that *A. laidlawii* glycolipids also take part in cholesterol uptake, as the complete hydrolysis of the 'pure' phospholipids of *A. laidlawii* membranes decreased but did not abolish cholesterol binding. However, the finding that removal of the pure phospholipids, which comprise only 30% of the polar lipids, caused a decrease of about 55% in cholesterol uptake suggests that the glycolipids have a lower binding capacity for cholesterol. It is still not clear whether the residual cholesterol uptake is due to the phosphoglycolipids (glycerophosphoryldiglucosyldiglyceride, glycerophosphorylmonoglucosyldiglyceride) to the 'pure' glycolipids (monoglucosyldiglyceride, diglucosyldiglyceride) or to both. The monoglucosyldiglyceride

of *A. laidlawii* has some physical properties in common with phosphatidylethanolamine. These include low hydration capacity, relatively high transition temperature, and the presence of a reversed hexagonal phase structure [24]. If phosphatidylethanolamine shows a lower affinity for cholesterol than choline-containing phospholipids, as was claimed by van Dijck et al. [7] and by Demel et al. [8], then it can be argued that *A. laidlawii* glycolipids share also this property. However, purified *A. laidlawii* glycolipids solubilized about the same quantity of cholesterol as *A. laidlawii* phospholipids and phosphoglycolipids when sonicated in aqueous medium with cholesterol (Razin, S. and Greenberg, A., unpublished data). Similarly, lipids extracted from mitochondrial and myelin membranes, which naturally contain low and high levels of cholesterol, respectively, solubilized similar amounts of cholesterol when dispersed by sonication [5]. These results led McCabe and Green [5] to conclude that the polar lipid composition alone does not decide the sterol content of the various cell membranes.

Our finding that proteolytic digestion of *M. capricolum* cells and membranes decreases cholesterol uptake is of great interest. According to Cooper [23], the fraction of the membrane polar lipid tightly associated with membranes protein (boundary lipids) is not available for interaction with cholesterol. Hence, the digestion and removal of membrane proteins can be expected to increase cholesterol binding by releasing more polar lipids for this purpose. Our results show that in the case of *A. laidlawii* proteolysis has no effect on cholesterol uptake, whereas with *M. capricolum* proteolytic treatment of membranes actually decreases rather than enhances cholesterol uptake. This finding supports our hypothesis that *M. capricolum* possesses protease-sensitive receptors on its cell surface responsible for better contact of the phosphatidylcholine/cholesterol vesicles or serum lipoproteins, receptors which are absent in *A. laidlawii*. Our previous findings [15] that growing *M. Capricolum* cells differ from those of *A. laidlawii* in their ability to incorporate significant quantities of exogenous phospholipids and esterified cholesterol from serum lipoproteins or from phosphatidylcholine/cholesterol vesicles also support the chloser contact of the exogenous lipid donors with the *M. capricolum* cell membranes.

Protease-sensitive receptors, having high affinity for lipid vesicles [25] or for low-density lipoproteins [26], have been detected on the surface of some eucaryotic cells. The notion that the sterol-rich *Mycoplasma* species differ from the sterol-poor *Acholeplasma* species in possessing cell surface proteins with high affinity for exogenous lipid donors is therefore plausible. Experiments to obtain more support for this hypothesis are now underway.

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