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THE PRESERVATION OF *MYCOPLASMA CAPRICOLUM* CELL INTACTNESS AFTER PHOSPHOLIPASE A_2 TREATMENT

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(1) By treating *Mycoplasma capricolum* cells with phospholipase A_2 about 80% of membrane phospholipids were rapidly hydrolyzed. The rate and extent of hydrolysis (at 37°C) were the same in intact cells and in isolated unsealed membranes. (2) Due to the low endogenous lysophospholipase activity detected in *M. capricolum*, phospholipase A_2 treatment resulted in the accumulation of lysophospholipids and free fatty acids. The free fatty acids were efficiently extracted from the cells by 1% bovine serum albumin whereas the lysophospholipids were almost fully retained within the cell membrane. (3) Following phospholipase A_2 treatment in the presence of 1% bovine serum albumin, cell intactness was preserved as indicated by the constant absorbance of the cell suspension and the retention of nucleic acids and NADH dehydrogenase activity within the cells. The treated cells showed, however, a slight decrease in K^+ content and a decrease in cell viability. Viability was fully preserved after phospholipase A_2 treatment of cells grown with exogenous sphingomyelin. (4) Adapting *M. capricolum* to a cholesterol-poor medium resulted in a marked decrease in the cholesterol to phospholipid molar ratio (from about 1.1 to 0.3). Phospholipase A_2 treatment of the cholesterol-poor cells resuted in cell lysis. Cell lysis was induced in the cholesterol-rich cells by hydrolysing the lysophospholipids accumulated following phospholipase A_2 treatment. (5) It is suggested that after phospholipase A_2 treatment of *M. capricolum* cells, a relatively stable cell membrane is maintained and cell intactness is preseved due to the interaction of cholesterol, present in high amount in this membrane, with the lysophospholipids formed.

Introduction

Studies on the effect of phospholipase A_2 on membrane phospholipids of mycoplasmas have been carried out with *Acholeplasma laidlawii* [1] and *Mycoplasma gallisepticum* [2] and the results were taken as a suggestion of a transbilayer distribution of phosphatidylglycerol in these organisms. A prerequisite for such studies is that throughout the treatment the cells remain intact. It was suggested that cell intactness will be maintained in mycoplasmas that possess an active membrane bound lysophospholipase activity [1,3]. This enzyme will minimize membrane perturbations, due to lysophospholipids that tend to disrupt the lipid bilayer. Recent ^{31}P-NMR studies with erythrocytes, however, indicate that following hydrolysis by phospholipase A_2 and accumulation of lysophospolipids the bilayer structure is preserved [4]. This preservation is obtained by other membrane constituents that are playing an important role in stabilizing the structure of the erythrocyte membrane [4,5]. The phospholipids of *Mycoplasma capricolum* have been the subject of detailed studies in our laboratory [6]. Applying phospholipase A_2 in an attempt to gain insight into the phos-

pholipid topography of this organism failed so far due to cell lysis. In the present study we describe the preservation of intactness and viability of *M. capriocolum* cells after degradation of membrane phospholipids by phospholipase A_2 treatment and the factors controlling it.

Materials and Methods

Organism and growth conditions. Mycoplasma capricolum (California kid) was grown in an Edward medium [7] containing 0.5% bovine serum albumin (Fraction V, Sigma, St. Louis, MO), a mixture of palmitic and oleic acids (10 μg/ml of each) and 20 μg/ml of cholesterol (Sigma). In some experiments, bovine brain sphingomyelin (Sigma) was added to the growth medium to a final concentration of 25 μg/ml. The sphingomyelin was dispersed in the growth medium by ultrasonic irradiation for 3 min at 0 °C in a sonicator (W-350 Heat systems) operated at 50% duty cycle at 200 W. To label membrane phospholipids, 0.002 μCi of [1-^{14}C]palmitate (55 Ci/mol) or 0.02 μCi of tritiated oleic acid (500 Ci/mol), products of the Radiochemical Center, Amersham, U.K. were added per ml of medium. To test for cell leakiness, 0.1 μCi/ml of [*methyl*,6-^{3}H]thymidine (35.2 Ci/mol, Nuclear Research Center, Negev, Isreal) was added to the medium. The cultures were incubated at 37 °C for 14–28 h and growth was followed by measuring the absorbance of the culture at 640 nm. The cells were harvested at the mid-exponential phase of growth (A_{640} = 0.18–0.25) by centrifugation at 12000 × *g* for 15 min, washed once and resuspended in 0.4 M sucrose solution containing 50 mM Tris-HCl (pH 7.5) and 25 mM $CaCl_2$ (referred to as sucrose-Tris-$CaCl_2$ buffer). *M. capricolum* membranes were obtained by osmotic lysis of cells [7]. Membranes from *M. gallisepticum* cells were obtained by ultrasonic irradiation as previously described [8].

Phospholipase A_2 treatment. Intact *M. capricolum* cells (5 mg of cell protein were treated with 50 μg phospholipase A_2 from porcine pancreas (EC 3.1.1.4, Boehringer, Mannheim, F.R.G.) in 1 ml of sucrose-Tris-$CaCl_2$ buffer containing 1% of bovine serum albumin at 37 °C. At various time intervals, 0.1 ml aliquots were taken out and rapidly mixed with 0.1 ml of a 0.1 M solution of EDTA. In most experiments, phospholipid hydrolysis was determined by measuring the radioactivity derived from [^{14}C]palmitate in the free fatty acid fraction extracted from the cells. In some experiments hydrolysis was calculated from analyses of the residual undigested phospholipids in the phospholipase A_2 treated cells.

Determination of cell leakiness and viability. To determine whether the cells remained intact in the reaction mixture, absorbance (at 500 nm) and retention of [^{3}H]thymidine-labeled components were determined as described previously [9]. The Na^+ and K^+ concentrations were determined in cell extracts, obtained by boiling cells (5 mg cell protein) with 0.5 M HCl for 10 min. The cell residue was removed by centrifugation, and Na^+ and K^+ content were determined using the Perkin-Elmer model 403 Atomic Absorption Spectrophotometer. Calculations of ion concentrations were based on an average cell volume of 2 μl per mg cell protein. The viability of the cells throughout the phospholipase treatment was determined using the colony counting technique [10].

Lysophospholipase activity. Endogenous lysophospholipase activity in *M. capricolum* and *M. gallisepticum* membrane preparations was determined as described by Gatt et al. [11]. [^{3}H]Oleate labeled lysophospholipids were generated in the membranes by phospholipase A_2 treatment at pH 5.0. The membranes were then transferred to a pH 7.5 medium and lysophospholipase activity was determined at desired time intervals by measuring radioactive fatty acids released [12]. Hydrolysis of lysophospholipase in intact *M. capricolum* cells was obtained by adding *M. gallisepticum* membrane preparations to a final concentration of 100 μg protein per ml to phospholipase A_2 treated *M. capricolum* cells (5 mg of cell protein per ml) in sucrose-Tris-$CaCl_2$ buffer. Hydrolysis was estimated by measuring the radioactive fatty acids released.

Analytical methods. Protein was determined according to Lowry et al. [13] using bovine serum albumin as standard. NADH dehydrogenase in solubilized cell preparations was determined as previously described [14]. Total lipids were extracted by the method of Bligh and Dyer [15] and separated on silica gel HR (Kiesel gel 60 HR

Merck, Darmstadt, F.R.G.) plates, developed at 4°C with chloroform/methanol/water (65 : 25 : 4, by vol.). For determining radioactivity in the lipid spots they were scraped off the plates into scintillation vials containing 5 ml of toluene scintillation liquor. The radioactivity was determined in a Packard Tri-Carb scintillation spectrometer (model 2650). To determine phosphorus in the phospholipid spots, the spots were scraped off the plates into test tubes and digested with 0.5 ml of ethanolic $Mg(NO_3)_2$ solution in the presence of the silica gel. Phosphorus was determined by the method of Ames [16]. The total cholesterol concentration in the lipid extracts was measured calorimetrically [17].

Results

Hydrolysis of membrane phospholipids by phospholipase A

The phospholipid composition of *Mycoplasma capricolum* grown without serum consists of de-novo synthesized phospholipids, mainly phosphatidylglycerol (40–50% of total), diphosphatidylglycerol (30–40% of total), and small amounts of an aminophospholipid and an unidentified phospholipid [6]. These lipids were radiolabeled by growing the cells with radioactive fatty acids. As *M. capricolum* has an unusual positional distribution of fatty acids with saturated fatty acids located at position 2 and unsaturated fatty acids at position 1 of the *sn*-glycerol-3-phosphate [18], the [^{14}C]palmitate added to the growth medium will be incorporated mostly in position 2 whereas [^{3}H]oleate will be incorporated mostly in position 1.

Phospholipase A_2 of porcine pancreas hydrolyzed rapidly most membrane phospholipids. At 37°C the enzyme hydrolyzed the phospholipid of intact cells and isolated unsealed membranes at the same rate and to the same extent. Within 10 min of incubation about 60% of the phospholipids of intact *M. capricolum* cells were hydrolyzed, reaching maximal levels of 80–85% after 30 min of incubation. The phospholipase A_2 activity was determined by following the release of radioactivity in the free fatty acid fraction extracted from the [^{14}C]palmitate grown cells or by analyzing lipids extracted from isolated membranes by the method of Bligh and Dyer [15]. Extraction of intact cells, however, resulted in artifactual preparations containing low levels of diphosphatidylglycerol as described also with Gram-positive bacteria [19]. The phospholipid composition of the pool insusceptible to phospholipase A_2 (20% of the total) was essentially the same as that of the pool digested by the enzyme. Moreover, the fatty acid profile of the two pools were similar.

Lysophospholipase activity

The hydrolysis of *M. capricolum* phospholipids resulted in the formation of both free fatty acids and lysos compounds, suggesting that unlike in other mycoplasmas tested [2,3], this organism does not contain a potent endogenus lysophospholipase activity. This was further established by determining lysophospholipase activity in *M. capricolum* membranes and comparing it to that of *M. gallisepticum*. For such determinations it was necessary to obtain lysophospholipid-containing membranes in which the activity of the endogenous lysophospholipase could be controlled. This was obtained by treating [^{3}H]oleate-labeled membrane preparations with 10 μg/ml of the pancreatic phospholipase A_2 at pH 5.0. At this pH the pancreatic phospholipase is hydrolyzing membrane phospholipids at a rate close to its maximal rate whereas activities of lysophospholipases are very low [11]. Subsequent adjustment of the pH to 7.4 permits measuring the lysophospholipase activity of the membrane preparation. This activity was followed by determining the radioactive fatty acid released. Fig. 1 shows the lysophospholipase activity of isolated membranes of *M. capricolum* and *M. gallisepticum*. Whereas in *M. gallisepticum* lysophospholipase activity was pronounced reaching maximal level after 60 min of incubation at 37°C, membrane preparations of *M. capricolum* showed very little lysophospholipase activity.

Preservation of intact and viable M. capricolum cells

Fig. 2 shows the effect of bovine serum albumin on the preservation of *M. capricolum* cell intactness after phospholipase A_2 treatment. Without bovin serum albumin, phospholipid hydrolysis was accompanied by cell lysis as shown by the decrease in the absorbance of the cell suspension. In the presence of bovine serum albumin ($\geq 1\%$) the cells

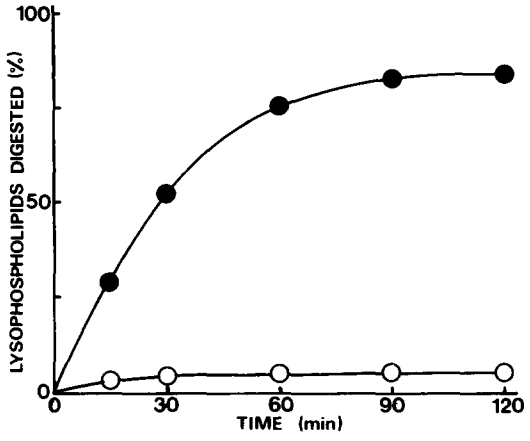

Fig. 1. Hydrolysis of membranous lysophospholipids by mycoplasma membrane preparations. Lysophospholipids were generated in [^{3}H]oleate-labeled membrane preparations of *M. capricolum* (open symbols) and *M. gallisepticum* (closed symbols), as described in Materials and Methods. The endogenous lysophospholipase activities were determined after incubation for the times specified in the figure by measuring radioactivity in the free fatty acid fraction [12].

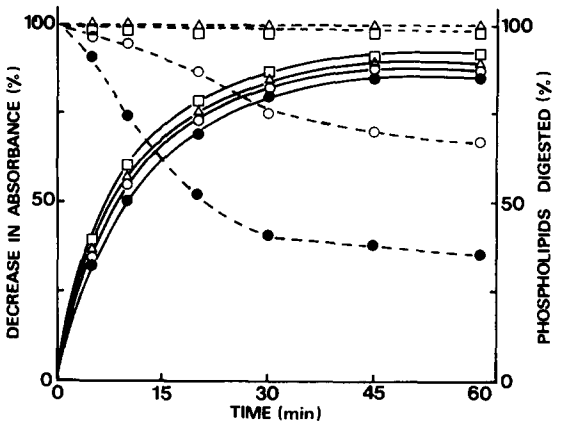

Fig. 2. The effect of albumin on hydrolysis of phospholipids and preservation of intactness of *M. capricolum* cells. [^{14}C]Palmitate labeled cells were incubated with 50 μg/ml phospholipase A_2 at 37 °C in a reaction mixture containing no albumin (●), 0.25% albumin (○), 1.0% albumin (△) and 4% albumin (□). Hydrolysis of phospholipids (solid lines) was determined according to radioactivity in the free fatty acid fraction. Cell intactness (broken lines) was expressed as the decrease in absorbance at 500 nm of the cell suspensions.

remained intact as indicated by a constant absorbance at 500 nm and by the retention of [^{3}H]thymidine-labeled components and the soluble NADH dehydrogenase activity within the cells (not shown). Lipid analyses of the phospholipid treated cells revealed that whereas in the absence of bovine serum albumin 65–75% of the free fatty acids and 90–95% of the lyso compounds were retained within the cells, in the presence of 1% bovine serum albumin only 2–5% of the free fatty acids with 90% of the lyso compounds were retained. Bovine serum albumin (1%) was thus in-

TABLE I

THE EFFECT OF PHOSPHOLIPASE A_2 TREATMENT ON THE LEAKINESS AND VIABILITY OF *M. CAPRICOLUM* CELLS

M. capricolum cells wer grown in a medium [7] containing 0.5% bovine serum albumin, oleic and palmitic acids (10 μg/ml of each), 20 μg/ml of cholesterol with or without sphingomyelin (25 μg/ml). The cells were treated with 50 μg/ml of phospholipase A_2 at 37 °C for up to 60 min in a reaction mixture containing 1% bovin serum albumin. The hydrolysis of phospholipids was determined according to lipid phosphorus in residual lipids. Lipid analyses, determination of intracellular ion concentrations, cell viability and NADH dehydrogenase activity were determined as described in Materials and Methods. Results of NADH dehydrogenase activity were expressed as decrease in absorbance at 340 nm per min per mg cell protein.

Cells	Phospholipase A_2 treatment (min)	Absorbance at 500 nm	Phospholipids hydrolyzed (% of total)	NADH dehydrogenase (ΔA_{340}/min per mg protein)	Intracellular ions (mM)		Viability (c.f.u./ml) ($\times 10^{-11}$)
					Na^+	K^+	
Grown without sphingomyelin	0	0.30	6	20	20	250	2.4
	10	0.35	65	20	20	240	70
	60	0.32	80	18	18	140	20
Grown with sphingomyelin	0	0.29	4	19	20	250	3.0
	10	0.28	35	19	20	240	2.2
	60	0.29	55	17	20	190	2.2

cluded in the standard reaction mixtures. Only a partial removal of lyso compounds (up to 25%) was obtained by increasing the bovine serum albumin concentration in the reaction mixture up to 10%. Table I shows that although *M. capricolum* cells treated with phospholipase A_2 in the presence of 1% bovine serum albumin remained intact, as indicated by the constant absorbance at 500 nm and by the retention of NADH dehydrogenase activity within the cells, the phospholipid hydrolysis resulted in a 10-fold decrease in cell viability and a 40% decrease in K^+ content. Viability and K^+ content were, however, almost fully preserved when *M. capricolum* cells grown with sphingomyelin were subjected to phospholipase A_2 treatment. By growing *M. capricolum* cells with sphingomyelin the cells incorporated sphingomyelin (not degradable by phospholipase A_2) into their cell membrane to levels of 25% of total membrane lipids.

Effect of alterations in cholesterol and lysophospholipid content on cell intactness

M. capricolum cells can be adapted to grow in a cholesterol-poor medium (2 μg cholesterol per ml medium). The cholesterol to phospholipid molar ratio in the adapted cells was about four times lower than in native cells grown with 20 μg/ml cholesterol (0.2–0.3 mol cholesterol per mol phospholipid in the adapted cells compared to a ratio of 1.1–1.2 in the native cells). Fig. 3 shows that the rate and extent of phospholipid hydrolysis by phospholipase A_2 in cholesterol-poor adapted cells were similar to that observed in native cells. The hydrolysis of the adapted cells was, however, accompanied by a rapid decrease in the absorbance of the cell suspension whereas the intactness of the cholesterol containing native strain was preserved as indicated from the constant absorbance of the cell suspension, the retention of NADH dehydrogenase activity (ΔA_{340} of 19 ± 2 per min per mg cell protein), and of ^{3}H-labeled components within the cells (not shown). The levels of lysophospholipids and fatty acids retained within phospholipase A_2 treated cholesterol-poor adapted cells were similiar to those retained within treated native cells. Fig. 4 shows that cell lysis could be induced by adding *M. gallisepticum* membrane preparations (containing lysophospholipase) to phospholipase A_2 treated *M. capricolum* cells (containing lysophospholipids). The lysophospholipids in the cells were hydrolyzed apparently by an intermembrane interaction [20].

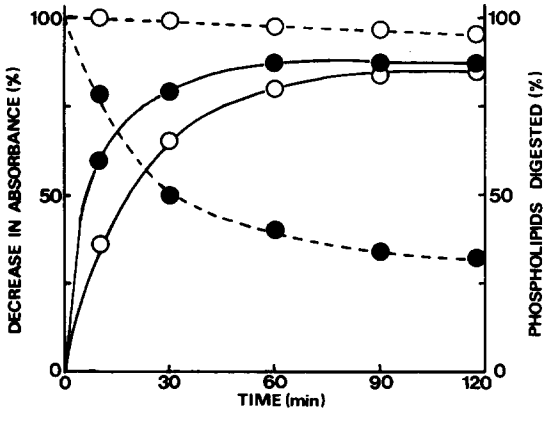

Fig. 3. The effect of cholesterol on the intactness of *M. capricolum* cells treated with phospholipase A_2. [^{14}C]Palmitated labeled cells were grown with either 2 μg per ml (closed symbols) or 20 μg per ml (open symbols) of cholesterol. The percent phospholipids digested (solid lines) was determined according to radioactivity in the free fatty acid fraction. Cell intactness was expressed as the percent decrease in absorbance at 500 nm of the cells suspensions (broken lines).

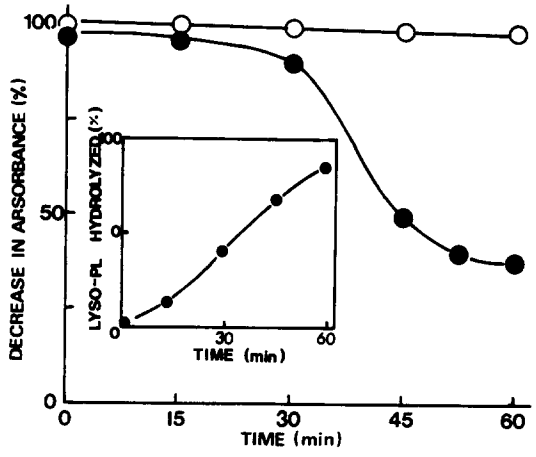

Fig. 4. The effect of hydrolysis of membranous lysophospholipids on the intactness of *M. capricolum* cells. Cells containing [^{3}H]oleate labeled lysophospholipids were obtained by incubating intact *M. capricolum* cells with 50 μg/ml phospholipase A_2 for 60 min at 37 °C. The cells were then incubated for 60 min without (open symbols), or with (closed symbols) *M. gallisepticum* membranes (100 μg/ml protein) and absorbance at 500 nm was measured at the times specified in the figure. Inset, lysophospholipids hydrolyzed.

Discussion

The data presented in this paper show that with the cholesterol-requiring *M. capricolum* cells, hydrolysis of membrane phospholipids was completed quite rapidly at a rate which was only little affected by the cholesterol concentration. In studies with *Mycoplasma myosides* subsp. *capri*, however, the presence of high amounts of cholesterol in the membrane prevented phospholipid hydrolysis by phospholipase A_2 apparently by affecting the packing of the phospholipids [21,22]. Although *M. capricolum* cells possessed very litte, if any, lysophospholipase activity, cells treated with phospholipase A_2 in the presence of bovine serum albumin were relatively intact though only 15% of their phospholipids remained undigested. The cell intactness was evidented by the unchanged absorbance of the cell suspension and by the retention of macromolecules within the cells. Viability, however, was tenfold lower in the phospholipase A_2 treated cells unless the cells were grown with sphingomyelin. The sphingomyelin was incorporated by *M. capricolum* cells to levels of 25–30% of total membrane lipids [6] bringing the total undigested lipid in the membrane of phospholipase A_2 treated cells to 40–45%.

The albumin present in the reaction mixture efficiently extracted the free fatty acids from the phospholipase A_2 treated cells but had little effect on the lysophospholipids that were accumulated within the cell membrane. As the hydrolysis of the accumulated lysophospholipids by a lysophospholipase preparation from *M. gallisepticum* resulted in a cell lysis it seems that the lysophospholipids are playing a role in maintaining cell intactness after phospholipase A_2 treatment. Yet, phospholipids do not form themselves stable bilayers but tend to form non-bilayer configurations mainly, hexagonal (H_1) phases [23]. Therefore, it seems that other membrane constituents have to exert a stabilizing effect on the bilayer of phospholipase A_2-treated *M. capricolum*. Our observation that cell stability was maintained in the cholesterol-rich native strain (1.1–1.2 cholesterol to phospholipid molar ratio) but not in the cholesterol-poor adapted strain suggests that cholesterol is the most suitable candidate for stabilizing the lysophospholipid-rich membrane. Studies on model membrane systems containing high amounts of lysophospholipids already show that the presence of cholesterol may affect the architecture [24] and permeability [25] of lipid vesicles. In the presence of 50 mol% cholesterol, lysolipids were found to form stable bilayers impermeable to glucose [25]. Furthermore, ^{31}P-NMR measurments showed that nonbilayer structures of lysophosphatidylcholine/ phosphatidylcholine mixtures are transformed to bilayer structures in the presence of cholesterol [4].

The data presented above suggest that phospholipase A_2 can be successfully utilized to study transbilayer distribution and movement of phospholipids in *M. capricolum* since the major prerequisite is met; the selective permeability properties of the cells are preserved even after extensive phospholipase A_2 treatment [26]. Our preliminary observation that the rapid rate and high extent of hydrolysis at 37 °C were the same in intact cells and isolated unsealed membranes is suggesting a rapid transbilayer movement of phospholipids in *M. capricolum* cells. Such movement of phospholipids from the inner to the outer leaflet of the membrane occurs at a fast rate relative to the time required for hydrolysis, thus accounting for the almost complete phospholipid hydrolysis upon treating intact *M. capricolum* cells with phospholipase A_2.

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