ELSEVIER

Biochimica et Biophysica Acta 1235 (1995) 369–377

BBA Biochimica et Biophysica Acta

The cell membrane of *Mycoplasma penetrans*: lipid composition and phospholipase A_1 activity

Michael Salman, Shlomo Rottem *

Department of Membrane and Ultrastructure Research, The Hebrew University-Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel

Received 10 October 1994; revised 19 December 1994; accepted 6 January 1995

Abstract

Analysis of *Mycoplasma penetrans* membrane lipids revealed that, in addition to large amounts of unesterified cholesterol, *M. penetrans* incorporated exogenous phospholipids, preferentially sphingomyelin, from the growth medium. The major phospholipids synthesized de novo by *M. penetrans* were phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). In vivo labeling of PG and DPG by growing the cells with radioactive palmitate or oleate, followed by snake venom phospholipase A_2 treatment, enabled us to assess the positional distribution of fatty acids in these lipids. Saturated fatty acids were found preferentially in position 2 of the glycerol backbone, and not in position 1 as found elsewhere in nature, while unsaturated fatty acids prefer position 1. *M. penetrans* membranes contain phospholipase activity of the A_1 type, removing a fatty acid from the *sn*-1 ester bond of phospholipids. The activity was neither stimulated by Ca^{2+} nor inhibited by EGTA and had a broad pH spectrum. The substrate specificity of the enzyme was investigated with various natural lipids and with a fluorescent analog of the phosphatidylcholine. The enzyme was equally active toward phosphatidylcholine and phosphatidylglycerol, but did not hydrolyze diphosphatidylglycerol. The enzyme did not act on triacylglycerol, diacylglycerol or cholesteryl ester, but low activity was detected toward monoacylglycerol. The enzyme was heat-sensitive and detergent-sensitive, and was almost completely inhibited by *p*-bromophenacylbromide (50 μM), but was not affected by SH reagents. This study is the first one reporting phospholipase A_1 activity in *Mollicutes*. A possible role of this enzyme in forming lipid mediators upon the interaction of *M. penetrans* cells with eukaryotic cells is suggested.

Keywords: Phospholipase A_1; Membrane lipid; AIDS; (Molliicutes); (*Mycoplasma penetrans*)

1. Introduction

An unidentified mycoplasma was recently isolated from the urogenital tracts of AIDS patients [1]. On the basis of biochemical, metabolic and serological characteristics, this mycoplasma was identified as a newly discovered species and named *Mycoplasma penetrans* due to its invasive properties and intracellular location in eukaryotic host cells [2,3]. It has been demonstrated with other intracellular pathogens that, during invasion, the pathogen causes significant rearrangement of the host cytoskeleton, indicating that signals are transduced between the bacteria and host cell cytoplasm, across the eukaryotic cell membrane [4,5]. However, the nature of these signals and the mechanisms used to transduce them remain unknown.

Phospholipases seem to be involved in a variety of cellular processes in eukaryotic cells [6]. These enzymes play a fundamental role because they serve to generate an array of metabolites with distinct biological functions. Cleavage of phosphatidylinositol by phospholipase C releases inositol 1,4,5-triphosphate and diacylglycerol. Both products are second messengers. The former initiates the mobilization of calcium from intracellular stores, while the latter activates protein kinase [4,6]. Hydrolysis of the fatty acid present in the glycerol backbone of membrane phospholipids by phospholipase A releases precursors for the generation of eicosanoids and lysophospholipids that can either have a direct role in membrane perturbation or can

Abbreviations: SPM, sphingomyelin; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; C6-NBD-PC, 1-acyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminocaproyl)phosphatidylcholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)diacylphosphatidylethanolamine; C6-NBD-DAG, C6-NBD-diacylglycerol; C6-NBD-PA, C6-NBD-phosphatidic acid; C6-NBD-FA, NBD-caproic acid; C6-NBD-LPC, C6-NBD-lyso-PC; DTNB, dithiobis(2-nitrobenzoic acid); PBP, *p*-bromophenacylbromide; DTT, dithiothreitol; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; NEM, *N*-ethylmaleimide; FFA, free fatty acid.

* Corresponding author. Fax: +972 2 757413.

SSDI 0005-2736(95)00014-3

serve to generate additional active metabolites [6,7]. The present study revealed that the membranes of *M. penetrans* possess a potent phospholipase A_1 activity that has been partially characterized. It is suggested that this activity may trigger specific signal cascades in the host cell, influencing HIV replication.

2. Materials and methods

2.1. Organisms and culture conditions

Mycoplasma penetrans, *Mycoplasma capricolum* or *Mycoplasma gallisepticum* were grown in modified Channock medium [8], supplemented with 10% (v/v) inactivated horse serum (56° C for 30 min). Membrane lipids were labeled by growing the cells with [^{3}H]palmitic acid (0.02–0.2 μCi/ml, 55.8 Ci/mmol), [^{3}H]oleic acid (0.02–0.2 μCi/ml, 15 Ci/mmol), [U-^{14}C]palmitic acid (0.02–0.2 μCi/ml, 928 Ci/mol) or [U-^{14}C]oleic acid 0.02–0.2 μCi/ml, 57.4 Ci/mol). All the radioactive labels were the products of the Radiochemical Centre (Amersham, UK). The organisms were grown for approx. 24 h and harvested at the mid-exponential phase of growth ($A_{640} = 0.15$, pH 6.5) by centrifugation at $12\,000 \times g$ for 10 min. The cells were washed twice in a cold medium containing 0.25 M NaCl, 10 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$.

2.2. Membrane preparation

Membranes were isolated from the washed cells by ultrasonic treatment [9]. Washed cell pellets were suspended in 10 mM NaCl solution containing 0.5 mM β-mercaptoethanol and treated for 3 min at 4° C in W-350 Heat Systems sonicator operated at 50% duty cycles at 200 W. Unbroken cells were removed by centrifugation at $5000 \times g$ for 5 min and membranes were then collected by centrifugation at $34\,000 \times g$ for 40 min, washed once and resuspended in 10 mM NaCl solution.

2.3. Lipid analyses

Lipids were extracted from washed cells or isolated membranes by the method of Bligh and Dyer [10]. Total lipid phosphorus was determined after digestion of the sample in an ethanolic magnesium nitrate solution (10% $MgNO_3$ in ethanol; Ref. [11]). The total lipid fraction was chromatographed on silica gel G plates using the two-step developing system [12] to resolve neutral lipids. Polar lipids were separated on silica gel H plates (Kieselgel 60, Merck, Darmstadt, Germany) developed at 4° C in chloroform/methanol/water (65:25:4 by volume). Lipid spots were detected by iodine vapor, phospholipid spots by molybdate spray reagent, and glycolipids by the anthrone reagent [12]. In order to determine phosphorus in phospholipid spots resolved by thin-layer chromatography (TLC), the spots were scraped off the plate and digested with 0.2 ml ethanolic magnesium nitrate solution in the presence of silica gel. For determining radioactivity in the lipid spots, the spots were scraped off the plate into scintillation vials containing 5 ml of Lumax scintillation liquor and radioactivity was then measured in a Packard Tri-Carb scintillation spectrometer and expressed as disintegrations per min (dpm). Alternatively, the TLC plates were exposed to imaging plates (Fuji Photo Film, Japan) for 6 h, the imaging plates were then processed using the radiation image analysis system Fujix BAS 1000 (Fuji, Japan) and radioactivity in each lipid spot was determined. Free cholesterol was separated from cholesteryl esters by TLC [12]. Cholesterol and cholesteryl esters spots were scraped off the plate and extracted from the silica gel with chloroform. Total cholesterol, free cholesterol and cholesteryl esters were measured colorimetrically [13].

2.4. Measurement of phospholipase activity

Phospholipase activity of *M. penetrans* membranes was measured utilizing either fluorescent [14] or radioactive substrates. The fluorescent phospholipid substrate contained either 1-acyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminocaproyl)phosphatidylcholine (C6-NBD-PC) or *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)diacylphosphatidylethanolamine (NBD-PE), both products of Avanti Polar Lipids (Alabaster, AL). Equal volumes of the fluorescent substrate (1.2 mM in chloroform) and dipalmitoylphosphatidylcholine (DPPC, 1.8 mM in chloroform) were mixed, evaporated to dryness under a stream of nitrogen, and resuspended in a solution of 150 mM NaCl in 10 mM Tris-HCl (pH 7.4) to a final lipid phosphorus concentration of 300 μM. The lipid suspension was then sonicated for 30 s at 4° C in a W-350 Heat System sonicator operated at 200 W.

The radioactive phospholipid dispersions containing phosphatidylglycerol (PG) or diphosphatidylglycerol (DPG) were obtained from *M. gallisepticum* or *M. penetrans* cells grown in the presence of [^{14}C]palmitate or [^{14}C]oleate. The total cellular lipids were extracted and separated by TLC as described above and the PG and DPG lipid spots were scraped off the plates and eluted from the silica gel with chloroform/methanol (1:1 by vol.). The radioactive lipids were evaporated to dryness under a stream of nitrogen, resuspended in the reaction buffer to a final lipid phosphorus concentration of 650 μM (containing approx. $2 \cdot 10^5$ dpm/μmol), and dispersed by sonication as described above. In some experiments dispersions containing oleate-labeled PG were treated with snake venom PLA_2 (5 units per 20 μg lipids) for 1 h at 37° C to obtain radioactive 1-oleyl-lyso PG.

The standard reaction mixture (in a total volume of 100 μl) contained 25–500 μg cell or membrane protein, and either 18 nmoles of the fluorescent phospholipid mixture or 100 nmoles of the radiolabeled phospholipid dispersion

((2–5) · 10^4 dpm) in 150 mM NaCl and 10 mM Tris-HCl, pH 7.4. In control experiments the membrane preparations were replaced by 5 units of snake venom phospholipase A_2 (Sigma) and $CaCl_2$ was added to the reaction mixture to a final concentration of 5 mM. The reaction was carried out at 37° C for up to 1 h and was terminated by either heating at 60° C for 5 min, or by the addition of EGTA (5 mM) in the control experiment. The entire mixture was applied to Whatman LK6 plates (Whatman, Clifton, NJ), dried thoroughly and developed in chloroform/methanol/water (65:35:5 by vol.) for the separation of C_6-NBD-FA ($R_F = 0.87$), C_6-NBD-PC ($R_F = 0.45$) and C_6-NBD-lyso PC ($R_F = 0.13$). Fluorescent bands were scraped off the plates and C_6-NBD-FA and C_6-NBD-PC were eluted by a mixture of chloroform/methanol/acidic saline (0.9% NaCl in 0.12% HCl solution, 1:1:0.05 by vol.), whereas C_6-NBD-lyso PC was eluted by the mixture of chloroform/methanol/water (1:3:1 by vol.). The fluorescence of clear extracts was determined in a Perkin-Elmer spectrofluorimeter (excitation at 470 nm and emission at 525 nm) to evaluate fluorescence intensity in the reaction products. For the separation of radioactive products, plates were developed in chloroform/methanol/water (65:25:4 by vol.). Radioactivity in the lipid bands was measured as described above.

The hydrolysis of labeled triolein, mono- and diacylglycerols and cholesteryl ester by *M. penetrans* membrane preparations was determined in the reaction mixture described above, except that the substrates were sonicated in the presence of 0.2% sodium cholate. NBD-labeled diacylglycerol (NBD-DAG) was prepared by treating NBD-PC with *Bacillus cereus* phospholipase C (5 units/ml, Sigma) for 60 min at 37° C in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) and 5 mM $CaCl_2$. The NBD-DAG thus obtained was purified by TLC and eluted from the TLC plates as described above. [1-^{14}C]Monoolein was prepared from [1-^{14}C]triolein (45–55 Ci/mol, Centre d'Etudes Nucléaires de Saclay, France), digested with porcine pancreatic lipase (Sigma) as previously described [15]. TLC analysis revealed that the obtained monoolein (1300 dpm/nmol) contained 98% of the 1(3)-isomer and 2% of the 2-isomer. Cholesteryl [1-^{14}C]oleate (50–60 Ci/mol) was obtained from the Radiochemical Centre (Amersham, UK).

Enzyme activity was expressed as nmol substrate hydrolyzed/min/mg membrane protein and K_m in μM was calculated on the basis of a double reciprocal plot (Lineweaver-Burk). The results represented are the average of three or four experiments with different batches of the cells.

2.5. Analytical procedures

Protein was determined by the method of Bradford [16]. ATPase activity in the membrane preparation was determined using [γ-^{32}P]ATP as previously described [17]. Results were expressed as nmol P_i released per min per mg protein. NADH dehydrogenase activity was determined spectrophotometrically [18] in the presence of sodium deoxycholate (1 mg/ml). Results were expressed as nmol NADH oxidized per min per mg protein.

3. Results

3.1. Characterization of M. penetrans membranes

M. penetrans membranes were found to contain approx. 30% of the total cell protein and practically all of the cellular lipids. The membrane preparations had almost no NADH-dehydrogenase activity, which was found exclusively in the soluble fraction, but retained about 95% of the ATPase activity. The residual ATPase activity found in the soluble fraction may represent activity in the small membrane fragments obtained upon sonication not sedimentable at 34 000 × g.

3.2. Lipid analysis

Gross chemical analysis of *M. penetrans* lipids revealed a phospholipid-to-protein ratio considerably lower than in *Mycoplasma capricolum* grown under identical conditions (Table 1) and in other *Mycoplasma* species (160–230 nmol phospholipids/mg cell protein). The total

Table 1
Contents of phospholipids, free cholesterol and esterified cholesterol of various *Mycoplasma* species

Strain	Content (nmol/mg protein)				Total cholesterol/PL (molar ratio)	Reference
	PL	total cholesterol	free cholesterol	esterified cholesterol		
M. penetrans	125.5	179.4	157.1	22.3	1.43	–
M. capricolum	227.0	145.3	75.0	72.3	0.64	[33]
M. gallisepticum	167.0	81.0	76.0	4.7	0.48	[11]
M. pneumoniae	ND	143.0	87.5	58.0	ND	[23,24]
M. arginini	ND	87.5	58.2	27.5	ND	[23,24]

Lipids were extracted and analyzed for phosphorus, free cholesterol and esterified cholesterol as described in Materials and methods. The results are an average of three or four independent experiments utilizing different batches of cells.
PL, phospholipids; ND, not determined.

Table 2
Phospholipid composition of *M. penetrans* membranes

Fraction number	Tentative identification	R_F [a]	Lipid phosphorus (nmol/mg of membrane protein)	% of total	Radioactivity	
					[^{14}C]palmitate	[^{14}C]oleate
1	SPM	0.18	261.3	50.0	0.1	0.1
2	PC	0.30	32.3	6.2	0.3	0.2
3	PG	0.39	67.9	13.3	18.9	35.9
4	DPG	0.71	65.4	30.5	80.7	63.9

Membranes were isolated from cells grown in the presence of either [^{14}C]palmitate or [^{14}C]oleate. Lipid isolation and analyses were performed as described in Materials and methods. The results are the average of three or four independent experiments utilizing different batches of cells.
[a] R_F, the ratio of the spot distance to the solvent front distance when developed with chloroform/methanol/water (65:25:4).

cholesterol-to-phospholipid molar ratio was higher than in other representative mycoplasmas and, as *M. penetrans* membranes contained only insignificant amounts of cholesteryl esters, this ratio represents the free cholesterol-to-phospholipid ratio.

Thin-layer chromatography of the total lipid fraction on silica gel H plates developed by chloroform/methanol/water (65:25:4) revealed seven lipid spots. Four of the spots reacted with molybdate reagent representing phosphorus-containing lipids. None of the lipid spots reacted with anthrone reagent, which detects glycolipids, or with ninhydrin reagent, which detects amino lipids. The phospholipid spots were tentatively identified according to their comigration on the thin-layer plates with commercial standards and specific spraying agents as SPM ($R_F = 0.18$), PC ($R_F = 0.30$), PG ($R_F = 0.39$), and DPG ($R_F = 0.71$). The other three spots have high R_F values (R_F 0.85–1.00) and contained free fatty acids, cholesterol and a mixture of cholesteryl esters and di- and triglycerides.

Table 2 shows the major phospholipid of *M. penetrans* was SPM (up to 50% of total phosphorus). The ratio of SPM to PC (mol%) in *M. penetrans* membranes was approx. 8:1, much higher than the ratio found in the growth medium (0.25), suggesting that *M. penetrans* cells preferentially incorporate SPM from the medium. A preferential incorporation of SPM was observed in various *Spiroplasma* species, whereas some *Mycoplasma* species grown with serum incorporated more PC than SPM [19]. The table also shows that when the cells were grown with radioactive fatty acid, SPM as well as PC remained unlabeled, whereas PG and DPG were labeled. This suggests that SPM and PC are taken up unchanged from the growth medium.

When grown with radioactive fatty acids, PG and DPG were the only phospholipids labeled, suggesting that these lipids are synthesized de novo by the cell. The positional distribution of the fatty acyl chains in the PG and DPG of *M. penetrans* could be deduced from the results obtained following snake venom phospholipase A_2 (PLA_2) treatment, which catalyses the hydrolysis of the ester bond at position 2 of the glycerol. When grown with labeled palmitate, PLA_2 activity resulted almost exclusively in the release of a labeled FFA, whereas when grown with labeled oleate, most of the label was found in the lysophospholipid spots containing fatty acids linked to position 1. These results suggest that de novo synthesized lipids of *M. penetrans* show an unusual distribution of fatty acids with unsaturated fatty acids predominantly in position 1 and saturated fatty acids more abundant in position 2. Snake venom PLA_2 also catalyzed the hydrolysis of *M. penetrans* PC, incorporated unchanged from the growth medium, but did not affect the SPM fraction.

Most of the radioactivity in the neutral lipid fraction was recovered in the free fatty fraction. The amount of radioactivity in the FFA fraction depends on the radioactive fatty acid added to the growth medium. When the cells were grown with radioactive oleic acid, 24.5% of the total incorporated oleate was found as free fatty acid, whereas

Table 3
Hydrolysis of fluorescently labeled phosphatidylcholine by phospholipases

Enzyme preparation	Time of treatment	Fluorescence (arbitrary units) in:		
		C6-NBD-PC	C6-NBD-LPC	C6-NBD-FFA
M. penetrans membranes	0 min	1079	57	9
	60 min	43	993	109
Snake venom PLA_2	60 min	12	105	1018

Phospholipase activity was assayed with fluorescent phosphatidylcholine C6 NBD-PC as described in Materials and methods. The results are an average of three independent experiments utilizing different batches of cells.
PC, phosphatidylcholine; LPC, lysophosphatidylcholine; FFA, free fatty acid.

when grown with radioactive palmitic acid, only 4.1% of the label was found in the free fatty acid fraction.

3.3. Phospholipase activity of M. penetrans membranes

The low phospholipid content of *M. penetrans* membranes and the high amounts of FFA were the first indications of a phospholipase A activity. Indeed, incubating *M. penetrans* membranes at 37° C resulted in a rapid breakdown of a significant portion of the PG fraction (50% within 1 h of incubation) as judged by the decrease in the radioactivity of the PG band and the appearance of radioactivity in the free fatty acid fraction. The hydrolysis of the DPG fraction was, however, very low or nonexistent. To further confirm this conclusion, we used an exogenous fluorescent substrate C6-NBD-PC labeled with fluorescent NBD-caproic acid linked to position 2. When the reaction was completed, the fluorescent products were eluted from the gel and analyzed quantitatively. As shown in Table 3, the interaction of *M. penetrans* membranes with the fluorescent-PC yielded two fluorescent products: the major being C6-NBD-LPC (non-fluorescent fatty acid in the position 1 hydrolyzed); and the minor C6-NBD-FA (NBD-labeled FA). When snake venom PLA_2 was used as an enzyme source, most of the fluorescence was detected in the C6-NBD-FFA spot, whereas only a small amount was detected in the C6-NBD-LPC spot. Quantitative analysis of the fluorescence products revealed that the ratio of lysophospholipid to FFA after treatment of the substrate with *M. penetrans* membranes was approx. 10, whereas after treatment with snake venom PLA_2 the ratio was 0.1, suggesting that *M. penetrans* membranes posses an active phospholipase A_1. It seems, therefore, that the low amount of fluorescent FFA released following the hydrolysis of 2-C6-NBD-PC by *M. penetrans* membranes is due to the fact that the substrate is contaminated with 1-C6-NBD-PC (NBD-FA linked to position 1). Since no additional fluorescent products, such as C6-NBD-DAG (diacylglycerol) or C6-NBD-PA (phosphatidic acid), were detected and all the C6-NBD-LPC formed remained intact through prolonged incubation periods (up to 4 h), we can exclude the possibility of phospholipase C, D or lysophospholipase activities in *M. penetrans* membranes. The hydrolysis of the fluorescent C6-NBD-PC by *M. penetrans* membranes was markedly inhibited by lyso-PC. Thus, when substrate dispersions containing 20 or 40 mol% of 1-acyl lyso-PC (Sigma) were prepared, hydrolysis was inhibited by 30% and 70%, respectively.

3.4. Characterization of the phospholipase A_1 activity

The phospholipase A_1 activity of *M. penetrans* had no sharp pH optimum over the entire pH range tested (pH 4.5–9.5). The enzyme was sensitive to heating, losing about a third of its activity after exposure to 50° C for 5 min. The apparent K_m of the phospholipase A_1 of *M. penetrans* membrane preparation with NBD-PC as a substrate was calculated to be 36.4 ± 3.7 μM, with a V_{max} of 18 ± 4.6 nmol/min per mg membrane protein. As phospholipase A_1 protein represents only a fraction of the total membrane protein, the V_{max} determined may differ markedly from that of the purified enzyme. Likewise, the K_m may slightly differ with a purified enzyme preparation. The phospholipase A_1 activity of *M. penetrans* was neither stimulated by Ca^{2+} or Mg^{2+} (2.5–5.0 mM) nor inhibited by EDTA or EGTA (Table 4). A pronounced inhibition effect was observed with Zn^{2+}, Fe^{2+}, Fe^{3+} and Cu^{2+} (Fig. 1). Zn^{2+} at a concentration of 0.5 mM inhibited activity by 80%, whereas Fe^{3+} showed 50% inhibition at a concentration of 0.4 mM and a complete inhibition at 1.0 mM. Cu^{2+} (2.5 mM) inhibited enzyme activity by over 75% and Fe^{2+} (2.5 mM) by 45.9%. Co^{2+} or Mn^{2+} at concentrations of up to 2.5 mM had no effect on phospho-

Table 4
Effect of inhibitors on the phospholipase A_1 activity of *M. penetrans* membranes

Inhibitor		% Inhibition ± S.D. [a]
PBP	1 mM	95.4 ± 4.6
	50 μM	86.7 ± 5.9
DTT	1 mM	21.5 ± 3.2
	100 μM	15.6 ± 2.4
NEM	100 μM	16.6 ± 1.8
DTNB	1 mM	72.5 ± 5.3
	100 μM	36.5 ± 3.9
EDTA	5 mM	3.3 ± 1.8
EGTA	5 mM	19.9 ± 2.5

Membranes were incubated with the inhibitors for 30 min at 37° C, and phospholipase activity was assayed utilizing fluorescent phosphatidylcholine C6-NBD-PC as described in Materials and methods. The results are the average of three independent experiments.
[a] ± S.D. = ± standard deviation.

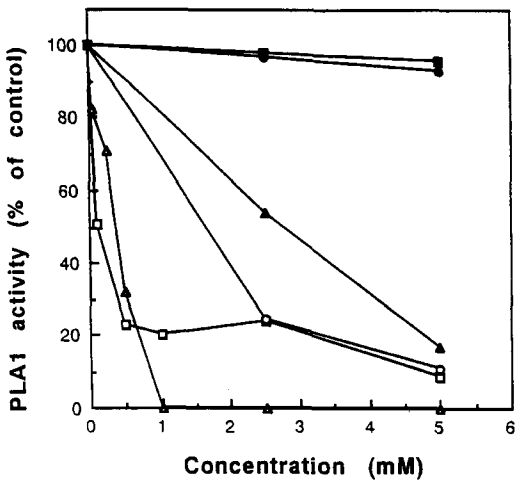

Fig. 1. Effect of cations on the phospholipase A_1 activity of *M. penetrans*. Various concentrations of cations were added to the reaction mixture and enzyme activity was determined as described in Materials and methods. □, Zn^{2+}; △, Fe^{3+}; ●, Mg^{2+}; ■, Ca^{2+}; ○, Cu^{2+}; ▲, Fe^{2+}.

Table 5
Hydrolysis of radioactively labeled mycoplasma phospholipids by phospholipase A preparations

Enzyme preparation	Phospholipid substrate	Time of treatment (min)	Radioactivity in lipid spots (dpm)					
			undigested PL		Lyso PL		FFA	
			dpm	%	dpm	%	dpm	%
M. penetrans membranes	PG	0	46650	93.7	2600	5.6	350	0.8
		60	29600	61.1	5400	11.1	13450	27.8
Snake venom PLA_2		60	6400	14.3	36900	82.6	1400	3.1
M. penetrans membranes	DPG	0	31000	86.3	2200	6.1	2750	7.6
		60	31500	93.5	1600	4.7	600	1.8
Snake venom PLA_2		60	2200	6.5	30600	90.0	1100	3.5

[a] Phospholipase activity was assayed utilizing [^{14}C]oleate-labeled PG from *M. gallisepticum* or [^{14}C]oleate labeled DPG from *M. penetrans* as described in Materials and methods. The results are the average of three independent experiments.
PL, phospholipid; Lyso PL, lysophospholipid; FFA, free fatty acids.

lipase A_1 activity. Table 4 also shows that bromophenacylbromide (PBP) completely inhibited the phospholipase A_1 at concentrations as low as 50 μM. This inhibitor was shown to alkylate histidine residues of mammalian PLA_2 active site [20]. Phospholipase A_1 activity was also inhibited by high concentrations (1 mM) of dithiobis(2-nitrobenzoic acid) (DTNB), whereas the activity was only slightly inhibited by *N*-ethylmaleimide (NEM) or dithiothreitol (DTT).

Solubilizing the membranes with 0.1% of either Triton X-100, dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate (Zwittergent 3-12) or sodium dodecylsulfate completely inhibited the phospholipase A_1 activity. 3-((3-cholamidopropyl)dimethylammonio)-1-Propane sulfonate (CHAPS) 0.1% inhibited activity by 46%. Sodium deoxylcholate (0.1%) inhibited phospholipase A_1 activity by about 70%, whereas sodium cholate (0.1–2%) had no significant effect on the activity (92–84% of initial activity, respectively).

Treating *M. penetrans* membranes with trypsin or pronase (50 μg/mg membrane protein) for 30 min resulted in a 25% decrease in the phospholipase A_1 activity, whereas following proteinase K (50 μg/mg membrane protein) treatment, a decrease of over 60% in the activity was observed. Treating intact cells with either trypsin or proteinase K (10–50 μg/mg cell protein) for up to 60 min did not inhibit phospholipase A_1 activity. The protease-treated cells remained intact as judged from the unchanged absorbance (640 nm) of the cell suspension throughout the treatment and from the very low extent of [^{3}H]thymidine-labeled material released from the cells (data not shown).

3.5. Substrate specificity of phospholipase A_1

Since commercial substrates labeled with radioactive or fluorescent fatty acid at position 1 are unavailable, we prepared radiolabeled PG from *M. gallisepticum* cells grown with radioactive fatty acids. It was previously shown that PG synthesized by *M. gallisepticum* has a rather unusual positional distribution with unsaturated fatty acid being present in position 1 and saturated fatty acid in position 2 of the glycerol moiety [11,25]. Thus, by growing cells with [^{14}C]oleic acid, PG with radioactive oleate in position 1 was obtained.

As shown in Table 5, treatment of sonicated *M. gallisepticum* PG with *M. penetrans* membranes resulted in the release of free [^{14}C]oleic acid rather than of [^{14}C]oleyl-labeled lyso-PG. In control experiments with snake venom PLA_2, such treatment resulted in the release of labeled lyso-PG rather than of a free fatty acid. The

Table 6
Hydrolytic activity of *M. penetrans* phospholipase towards various lipids [a]

Substrate	Hydrolytic activity ± S.D. [a] (nmol/min per mg membrane protein)
Phosphatidylglycerol	16.4 ± 3.5
Phosphatidylcholine	18.0 ± 4.6
Triolein	< 0.01
Diolein (1,2-diacylglycerol)	< 0.01
Monoolein (1(3)-monoacylglycerol)	2.3 ± 0.4
Cholesteryl ester	< 0.01

Lipid hydrolysis by membrane preparation of *M. penetrans* was measured as described in Materials and methods. The substrate amounts in all cases was 40–100 nmol. Data are means of three independent determinations on different batches of the cells.
[a] ± S.D. = ± standard deviation.

lyso-PG thus released was not hydrolyzed following 2 h of incubation at 37° C with isolated *M. penetrans* membranes (data not shown), suggesting that *M. penetrans* phospholipase A cannot hydrolyze 1-acyl lyso-PG. The table also shows that [^{14}C]oleate-labeled DPG treated by *M. penetrans* membranes was not hydrolyzed, whereas snake venom PLA_2 hydrolyzed the radioactive DPG, releasing labeled lyso-DPG. Similar results were obtained with PG and DPG labeled with [^{14}C]palmitate. *M. penetrans* membranes hydrolyzed palmitate labeled PG, releasing labeled lyso-PG, but had no effect on radioactive DPG (data not shown). The lyso-PG released was not hydrolyzed, even after prolonged incubation periods (up to 3 h), suggesting that the enzyme cannot hydrolyze 2-acyl lyso-PG.

Table 6 shows the activity of *M. penetrans* membranes towards various neutral lipids as compared to phospholipids. It is apparent that whereas the triacylglycerol, diacylglycerol, and cholesteryl esters were not hydrolyzed, a low activity was detected with monoacylglycerol.

4. Discussion

Membranes of *M. penetrans* cells were obtained in the present study by ultrasonic treatment of the organisms. The membrane preparations thus obtained contained 28–35% of the total cell protein and essentially all of the cell lipids constituted mainly of phospholipids, cholesterol and free fatty acids. The high cholesterol content is to be expected from a cholesterol-requiring mycoplasma. Most of the cholesterol fraction was unesterified cholesterol, whereas the cholesterol ester levels observed were much lower than in other *Mycoplasma* species [21]. The high cholesterol to phospholipid molar ratio observed (1 · 43) implies that some cholesterol forms separate domains in the bilayer or is associated with membrane protein [22].

The phospholipid composition of *M. penetrans* is rather simple, comprising two de novo synthesized phospholipids, PG and DPG. The synthesis of PG and DPG, as the only de novo synthesized membrane lipids, appears to be a property shared by most *Mycoplasma* and *Spiroplasma* species tested so far [23,24]. As with other *Mycoplasma* species [11], the PG and DPG of *M. penetrans* show an unusual positional distribution of saturated and unsaturated fatty acids with saturated fatty acids localized in position 2 of the *sn*-glycerol phosphate and unsaturated fatty acids confined to position 1. Thus, the PG and DPG of *M. penetrans* can be labeled in positions 1 or 2 by growing the cells in the presence of oleic or palmitic acid, respectively. When grown in a medium which contains serum, the cell membrane of *M. penetrans* contained in addition to the de novo synthesized phospholipids, substantial amounts (over 50% of total phospholipids) of SPM and PC. Both lipids are incorporated unchanged from the growth medium, whereas in some other *Mycoplasma* species, the PC incorporated from the medium is modified by the deacylation-reacylation process to yield a disaturated PC [24,25]. Whereas most *Mycoplasma* species grown in a serum-containing medium incorporate more PC than SPM [21], *M. penetrans* resembles the *Spiroplasmas* species in incorporating preferentially SPM from the medium [26,27].

The extremely low phospholipid contents (125 nmol of lipid phosphorus per mg cell protein) and the high amounts of free fatty acids were the first indications of an endogenous phospholipase activity. Further experiments have shown that incubating isolated *M. penetrans* membranes at 37° C resulted in the breakdown of significant portions of membrane PG, releasing almost exclusively unsaturated fatty acid. As in *M. penetrans* unsaturated fatty acids are linked predominantly to carbon 1 of the *sn*-glycerol, it is apparent that the activity detected is that of phospholipase A_1. The presence of a phospholipase A_1 activity was further confirmed by analyzing the end products of reaction mixtures utilizing PG specifically labelled at position 1 or 2, as well as fluorescent PC labeled at position 2 by NBD-caproic acid. Analysis of the hydrolysis products of NBD-PC revealed 90% of lyso-NBD-PC and 10% of free NBD-caproic acid that may suggest a mixture of phospholipase A_1 and PLA_2 or a low specific phospholipase A_1. Nonetheless, our observation that upon applying snake venom phospholipase A_2, 10% of the fluorescently labeled products were lyso NBD-PC, and considering that snake venom phospholipase A_2 possesses the ability to hydrolyze only fatty acid linked to position 2, it seems that the commercial 2-C6-NBD-PC contains small amounts (~ 10%) of C6-NBD linked to carbon No. 1 [28].

Our study is the first one reporting a specific phospholipase A_1 activity in *Mollicutes*. The activity is a true mycoplasma activity, rather than a media contaminant, as we failed to demonstrate measurable phospholipase A_1 activity in our media lots. Furthermore, the horse serum utilized for media preparation is heat-inactivated under conditions (56° C for 30 min) that were shown to completely inhibit the phospholipase A_1 activity of the *M. penetrans*. Very little is known about phospholipase A_1 activity in bacteria, whereas it is more common in eukaryotes. Several proteins purified as phospholipase A_1 from *Mycobacterium phlei* and *Escherichia coli* [15,29] also display phospholipase A_2 and lysophospholiase activity. Most of the phospholipase A_1 activity of mammalian cells are of lysosomal origin and have broad substrate specificity, hydrolyzing various phospholipids, as well as acylglycerides and also catalyzing transacylation reactions [30]. Unlike the broad specificity of these phospholipases, the phospholipase A_1 of *M. penetrans* has a restricted substrate specificity. Of the various substrates tested in our study, the phospholipase A_1 of *M. penetrans* hydrolyzed PC and PG but not DPG and has a very low activity toward either 1-acyl-lyso-PG or 2-acyl-lyso-PG. Like the phospholipase A_1 activity of *Penicilium notatum* [31], the *M. penetrans* enzyme was unable to cleave triacylglycerol,

diacylglycerol or cholesteryl oleate, but hydrolyzed monoacylglycerol.

Many phospholipases seem to be regulated by intracellular levels of free calcium ions, cAMP or pH [7,20]. These regulatory principles, however, do not control the mycoplasmal phospholipase A_1, as this activity was neither stimulated by Ca^{2+} nor inhibited by EGTA. Furthermore, the phospholipase A_1 activity of *M. penetrans* has a broad pH spectrum and was not affected by cAMP. The high specificity of phospholipase A_1 of *M. penetrans* and the inhibition by lyso components may suggest that this enzyme is regulated by intracellular substrate and/or product levels.

The most investigated system in bacteria is that present in the outer membrane of *E. coli*. This enzyme was termed detergent-resistant phospholipase A_1, since it retains its activity in the presence of detergents [29]. As with the *E. coli* enzyme, the phospholipase A_1 of *M. penetrans* was not affected by SH reagents or by prolonged incubation with dithiothreitol and was substantially inhibited by either *p*-bromophenylbromide or by Zn^{2+} or Fe^{2+} ions. The *M. penetrans* enzyme, however, differs from the *E. coli* enzyme in being sensitive to heat treatment, irreversibly losing activity at temperatures of $\geq 60°$ C. It is sensitive to detergents, being inactivated at SDS concentrations as low as 0.1% and does not require free Ca^{2+} ions for maximal activity.

M. penetrans has a unique property of being able to invade mammalian cells [3] and to produce profound cytopathic effects that may lead to cell death [3]. During the process of invasion of many intracellular pathogens, significant rearrangement of the host cytoskeleton were noticed, indicating that signals are transduced between the bacteria and the host cell cytoplasm across the eukaryotic cell membrane [5]. A variety of lipidic molecules were shown to act as mediators in the signal transduction [6]. The formation of the lipid mediators in cells are regulated by several mechanisms, but the rate limiting step is believed to be the action of phospholipases on cellular phospholipids. The possibility that the high activity of phospholipase A_1 in the cell membrane of *M. penetrans* is playing a role in the pathogenesis of *M. penetrans* is therefore very intriguing. We have previously shown that the AIDS-associated *M. fermentans* cells are able to fuse with T-cell line cells as well as with peripheral blood lymphocytes [32] and recently the fusogenic activity of *M. penetrans* has been also demonstrated (M. Salman, unpublished data). It is likely that the mycoplasmal PLA_1 as well as other membrane components are inserted into the cytoplasmic membrane of a eukaryotic cells upon fusion. The PLA_1 may then trigger specific signal cascades by catalyzing the hydrolysis of *sn*-1 fatty acyl chain of different phospholipids in the eukaryotic cell membranes. The products of PLA_1 activity can be further metabolized as precursors in the production of specific proinflammatory lipid mediators or substances, influencing the HIV replication. Further studies on the consequences of mycoplasma-host cell interaction and the role of phospholipase A_1 should be undertaken.

Acknowledgements

This work was supported by The Israeli Ministry of Health. We thank M. Tarshis and S. Yedgar for their help and advice.

References

[1] Lo, S.-C., Hayes, M.M., Wang, R.Y.-H., Pierce, P.F., Kotani, H. and Shih, J.W.-K. (1991) Lancet 338, 1415–1418.
[2] Lo, S.-C., Hayes, M.M., Tully, J.G., Wang, R.Y.-H., Kotani, H., Pierce, P.F., Rose, D.L. and Shih J.W.-K. (1992) Int. J. Syst. Bacteriol. 42, 357–364.
[3] Lo, S.-C. (1992) in The Mycoplasmas (Maniloff, J., ed.), pp. 525–548, American Society of Microbiology, Washington.
[4] Ruschkowski, S., Rosenshine, I. and Finlay, B.B. (1992) FEMS Lett. 95, 121–126.
[5] Wick, M.J., Madara, J.L., Fields, B.N. and S.J. Normark. (1991) Cell 67, 651–659.
[6] Liscowitch, M. (1992) Trends Biochem Sci. 17, 393–398.
[7] Glaser, K.B., Mobilio, D., Chang, J.Y. and Senko, N. (1993) Trends Biochem. Sci. 14, 92–98.
[8] Razin, S. and Rottem, S. (1976) in Biochemical Methods in Membrane Studies (Maddy, A.H., ed.), pp. 3–26, Chapman and Hall, London.
[9] Razin, S. (1983) in Methods in Mycoplasmology (Razin, S. and Tully, J.G., eds.), Vol. 1, pp. 225–233, Academic Press, New York.
[10] Bligh, F.G. and Dyer, W.S. (1959) Can. J. Biochem. Biophysiol. 37, 911–917.
[11] Rottem, S. and Markowitz, O. (1979) Biochemistry 18, 2930–2935.
[12] Rottem, S. (1983) in Methods in Mycoplasmology (Razin, S. and Tully, J.G., eds.), Vol. I, pp. 269–275, Academic Press, New York.
[13] Rudel, L.L. and Morris, M.D. (1973) J. Lipid Res. 14, 364–366.
[14] Reisfeld, N., Lichtenberg, D., Dagan, A. and Yedgar, S. (1993) FEBS Lett. 315, 267–270.
[15] Gassama-Diagne, A., Rogalle, P., Fauvel, J., Willson, M., Klaébé, A. and Chap, H. (1992) J. Biol. Chem. 26, 13418–13424.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Shirvan, M.W., Shuldiner, S. and S. Rottem, S. (1989) J. Bacteriol. 171, 4417–4424.
[18] Ne'eman, Z. and Razin, S. (1975) Biochim. Biophys. Acta 375, 54–68.
[19] Rottem, S., Adar, L., Gross, Z., Ne'eman, Z. and Davis, P.J. (1986) J. Bacteriol. 167, 299–304.
[20] Mayer, R.J. and Marshall, L.A. (1993) FASEB J. 7, 339–348.
[21] Razin, S., Kutner, S., Efrati, H. and Rottem, S. (1980) Biochim. Biophys. Acta 598, 628–640.
[22] Le Grimellec, C., Zollinger, M. and Giocondi, M.C. (1982) Biochim. Biophys. Acta 689, 309–318.
[23] Bittman, R. (1993) in Subcellular Biochemistry (Rottem, S. and Kahane, I., eds.), Vol. 20, pp. 29–52, Plenum Press, New York.
[24] Rottem, S. (1980) Membrane lipids of mycoplasma, Biochim. Biophys. Acta 604, 65–90.
[25] Rottem, S. and Markowitz, O. (1979) FEBS Lett. 107, 379–382.
[26] Davis, P.J., Katzenell, A., Razin, S. and Rottem, S. (1985) J. Bacteriol. 161, 118–122.

[27] Hackett, K.J., Ginsberg, A.S., Rottem, S., Henegar, R.B. and Whitcomb, R.F. (1987) Science 237, 525–527.
[28] Martin, O.C. and Pagano, R.E. (1986) Anal. Bichem. 159, 101–108.
[29] Nishijima, M., Nakaike, S., Tamori, Y. and Nojima, S. (1977) Eur. J. Biochem. 73, 115–124.
[30] Kucera, G.L., Sisson, P.J., Thomas, M.J. and Waite, M. (1988) J. Biol. Chem. 263, 1920–1928.
[31] Saito, K., Sugatani, J. and Okumura, T. (1991) Methods Enzymol. 197, 446–453.
[32] Dimitrov, D.S., Franzoso, G., Salman, M., Blumenthal, R., Tarshis, M., Barile, M.F. and Rottem, S. (1993) Clin. Infect. Dis. 17, 305–308.
[33] Gross, Z., Rottem, S. and Bittman, R. (1982) Eur. J. Biochem. 122, 169–174.