Membrane lipid composition protects *Entamoeba histolytica* from self-destruction by its pore-forming toxins

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Abstract The protozoan parasite and human pathogen *Entamoeba histolytica* is protected against killing by its own lytic effector proteins. Amoebae withstand doses of amoebapores, their pore-forming polypeptides, that readily kill human Jurkat T cells. Moreover, the polypeptides do not bind to the amoebic surface membrane as evidenced by using fluorescently labelled amoebapores and confocal laser microscopy. Experiments employing liposomes as a minimalistic membrane system and the major isoform amoebapore A revealed that the lipid composition of amoebic membranes prevents binding of the cytolytic molecule and that both the phospholipid ingredients and the high content of cholesterol contributes to the protection of the toxin-producing cell.

Key words: Amoebapore; Antimicrobial peptide; Self-protection; Cholesterol; Pore-forming protein; Lipid raft; *Entamoeba histolytica*

1. Introduction

Entamoeba histolytica is an amoeboid protozoon and the causative agent of human amoebiasis, one of the most life-threatening parasitic infections [1]. The amoeba is a remarkably potent 'killer cell' which destroys host tissues and lyses almost every target cell it encounters in a contact-dependent reaction [2]. Accumulated evidence including from experiments with gene-silenced amoebae revealed that a family of pore-forming polypeptides termed amoebapores are key components in cytolysis and pathogenicity [3–6]. Amoebapores permeabilize membranes of prokaryotic and eukaryotic target cells suggesting a function as a broad spectrum effector molecule [7–10]. The principle of killing other cells by secreting proteins that permeabilize target cell membranes is widespread in nature. Pore-forming toxins of bacteria, hemolytic proteins of invertebrates, the mammalian complement system and antimicrobial peptides of various origin are well-known examples of effector molecules acting according to this principle. However, most often the mechanism which confers resistance to the cells or epithelia that produce these lytic agents are not well understood.

Here, we analyzed the molecular basis of resistance of pathogenic amoebae to their membrane-active effector proteins by using viable amoebic trophozoites, target cells of human origin and lipid vesicles of various composition to monitor amoebapore-induced cell death and the selectivity of binding of the proteins to membranes. The data provide evidence that the distinct lipid composition of amoebic membranes, particularly its high cholesterol content, contributes significantly to the protection of amoebae against their own granule-derived toxic products discharged upon the cytolytic reaction towards host cells.

2. Materials and methods

2.1. Lipids

Cholesterol and soybean lipids (crude phosphatidylcholine type II-S, azolectin) were from Sigma (Deisenhofen, Germany). 3-*sn*-Phosphatidylcholine (PC) and phosphatidylinositol (PI) from bovine liver were purchased from Fluka (Germany). Ceramide, 3-*sn*-phosphatidylethanolamine (PE), 3-*sn*-phosphatidyl-L-serine (PS) from bovine brain and sphingomyelin from chicken egg yolk were obtained from Avanti Polar Lipids (Alabaster, AL, USA) or Fluka. *N*-Acyl-sphingosylphosphorylethanolamine (CAEphosphate) was from Matreya (Pleasant Gap, PA, USA).

2.2. Cultivation and harvesting of amoebae

Trophozoites of the pathogenic *E. histolytica* strain HM-1:IMSS were cultured axenically in TYI-S-33 medium containing 20 g/l trypticase, 10 g/l yeast extract, 10 g/l D-glucose, 2 g/l NaCl, 1 g/l K_2HPO_4, 0.6 g/l KH_2PO_4, 1 g/l cysteine, 0.2 g/l ascorbic acid, 22.8 mg/l Fe-ammonium citrate, 3% Tween 80, 2 U/ml penicillin, 0.2 g/l streptomycin, and 15% heat-inactivated bovine serum [11] in plastic tissue culture flasks. Trophozoites from cultures in late-logarithmic phase were harvested after being chilled on ice for 10 min, sedimented at 430×*g* at 4°C for 3 min and washed three times in ice-cold PBS-A (4.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 147 mM NaCl, 2.5 mM KCl, pH 7.4, 320 mosmol/kg).

2.3. Cell culture

Human leukemic Jurkat T cells (ATCC, Rockville, MD, USA) were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and amphothericin (0.25 μg/ml). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere and passaged three times a week.

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Abbreviations: CAEP, ceramide aminoethylphosphonate; CAEphosphate, *N*-acyl-sphingosylphosphorylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TRITC, tetramethylrhodamine isothiocyanate

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2.4. Purification of amoebapores

The purification of individual amoebapore isoforms from amoebic extracts using a Sep-Pak cartridge and two consecutive reversed-phase high performance liquid chromatographies (HPLC) has been described previously [7,10,12]. Purified peptides were lyophilized and stored at −20°C. The protein concentration was determined photometrically at 214 nm using the excitation coefficients calculated from the sequence information [13]. For the preparation of the amoebapore A, B and C mixture, all amoebapore fractions eluting from the first HPLC column were collected in a single tube instead of separating them. This mixture should consist of the amoebapores A, B and C in amounts found naturally in the amoebic granules in which the major isoform A represent approximately 70–75% of the amoebapores. For confocal fluorescence microscopy, the amoebapore A, B, C mixture was labelled with activated tetramethylrhodamine (TRITC) using FluoReporter Protein Labelling Kit (Molecular Probes Europe, Leiden, The Netherlands).

2.5. Determination of cellular ATP content

Jurkat cells (2×10^5) or amoebae (1×10^5) were incubated in the presence of the indicated concentration of amoebapores in 200 μl buffer (20 mM MES, 150 mM NaCl, pH 5.5) for 15 min at 37°C. Cells were sedimented by centrifugation and washed in buffer. Cell lysis was achieved by addition of 10 μl of 10% Triton X-100 to the cell pellet. Each lysate was transferred into a vial containing 190 μl luciferase reagent (CLSII, Boehringer, diluted 1:8 in ddH_2O) and chemiluminescence was measured in a luminometer (Bioluminat LB9500; Berthold, Wildbad, Germany). The percent of luminescence of amoebapore-treated cells relative to that of untreated cells was expressed as the cellular ATP content.

2.6. Fluorescence microscopy

Amoebae and Jurkat cells were fixed in 200 mM sodium cacodylate buffer, pH 7.4, 4% paraformaldehyde. Aliquots were incubated with TRITC-conjugated amoebapores (in MES buffer) and after three washes in PBS air-dried on slides, embedded with Mowiol (Calbiochem, Bad Soden, Germany) and analyzed by confocal microscopy using the Leica TCS NT confocal laser scanning system in combination with a Leica DMR microscope (Leica, Bensheim, Germany).

2.7. Preparation of plasma membrane and granular membrane fractions of E. histolytica

Freshly harvested and washed amoebae were resuspended in ice-cold 150 mM NaCl and lysed by nitrogen cavitation; after an incubation under nitrogen at 28 bar for 25 min, the suspension was released dropwise. The lysate was centrifuged at $430\times g$ at 4°C for 10 min, the supernatant was carefully removed and centrifuged at $27\,000\times g$ at 4°C for 25 min. The sediment represents a fraction enriched in cytoplasmic granules. The supernatant was carefully removed, centrifuged again at $150\,000\times g$ at 4°C for 40 min. This sediment represents the plasma membrane fraction. The granule fraction was resuspended in four volumes of 10% acetic acid and incubated overnight at 4°C under constant shaking. The acidic suspension was centrifuged at $150\,000\times g$ at 4°C for 40 min. This sediment represents the granular membrane fraction [14].

2.8. Extraction of lipids from E. histolytica trophozoites and membrane fraction

Extraction of lipids was performed according to the method of Folch et al. [15]. Briefly, sedimented trophozoites, plasma membrane, or granular membrane fractions of *E. histolytica* were treated with chloroform/methanol 2:1 (v/v), sonicated, and sedimented at $700\times g$ at 4°C for 5 min. The supernatant was removed and 1/4 volume of chloroform/methanol/water 8:4:3 (v/v) was added. The resulting two phases were separated and the lower phase, containing the extracted lipids, was concentrated to a fifth of the volume in a speed vacuum concentrator and subsequently dried to completeness in an exsiccator under vacuum overnight. The lipids fractions were stored at −20°C until use.

2.9. Preparation of liposomes

Liposomes were prepared from azolectin or dried amoebic lipid extracts. Additionally, a mixture (w/w) of defined phospholipids, i.e. PC (14%), PE (35%), PS (8.5%), PI (1%), sphingomyelin (1%), and ceramide (40.5%), was used to mimic the amoebic plasma membrane [14]. When indicated, various amounts of cholesterol were given supplementary to these mixtures prior to the preparation of the lipid films. The content of cholesterol was expressed in % of total lipids (w/w). Lipids were dissolved in chloroform, and the solvent was evaporated under a constant stream of nitrogen to give thin lipid films on a glass beaker surface. Residual chloroform was removed under vacuum overnight. The lipid films were suspended by shaking in liposome buffer (50 mM Tris maleate, 50 mM potassium sulfate, 0.5 mM EDTA, 0.02% sodium azide, pH 5.2) containing glass beads. The suspended lipids were analyzed by thin layer chromatography and the concentration of phospholipids was determined using a phosphorus assay [16]. In all experiments using liposomes, the phospholipid concentration was adjusted as indicated, and in some experiments cholesterol was added as supplement.

2.10. Association of amoebapore A with liposomes

Amoebapore A (0.5 μM; $M_r = 8240$), diluted in liposome buffer, was incubated with various amounts of lipid vesicles in a total volume of 100 μl at 4°C under constant shaking for 1 h. After the incubation period, liposomes and liposome-associated peptides were separated from peptides in solution by centrifugation at 24 psi (approx. $100\,000\times g$) for 30 min using a Beckmann airfuge centrifuge. The supernatant was removed and analyzed for residual pore-forming activity and for reactivity to an anti-amoebapore A antiserum in an enzyme-linked immunosorbent assay (ELISA) assay.

2.11. Assay for pore-forming activity

Pore-forming activity of samples was determined by monitoring the dissipation of a valinomycin-induced diffusion potential in liposomes as described by Loew et al. [17]. Briefly, azolectin liposomes (40 mg/ml, Sigma) were prepared in 50 mM K_2SO_4, 0.5 mM EDTA, 50 mM Tris maleate, pH 5.2, and were diluted for the assay 1:4000 in a buffer with K^+ replaced by Na^+. Addition of valinomycin (1 nM, Sigma) resulted in a potassium diffusion potential that was monitored by the fluorescence quenching of 3,3′-diethylthiodicarbocyanine iodide (1 μM; Kodak) using a fluorescence spectrophotometer (LS50B; Perkin-Elmer) with excitation and emission wavelengths of 620 nm and 670 nm, respectively. Pore-forming activity was measured as the initial change of fluorescence intensity over time after adding the sample. One unit of activity was defined as a fluorescence increase to 5% of the pre-valinomycin intensity in 1 min at 25°C.

2.12. ELISA

The assay was performed in flat-bottom microtiter plates (Maxisorb, Nunc). The wells of the plates were coated with various dilutions of the supernatants (see Section 2.10) in 100 mM sodium carbonate

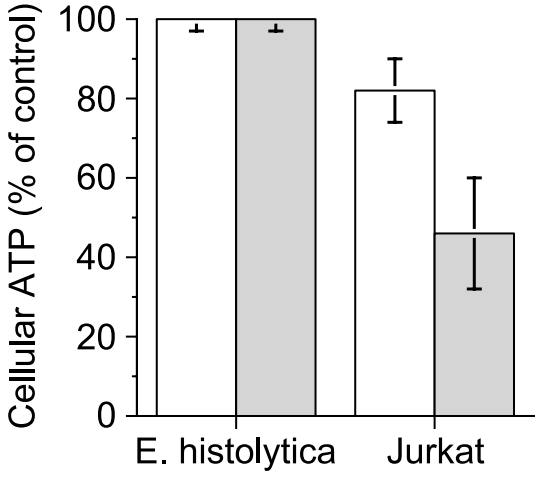

Fig. 1. ATP content of Jurkat T cells and *E. histolytica* trophozoites after incubation with amoebapores. Cells were incubated in the presence of amoebapores (2.5 μM, open bars; 5 μM, shaded bars). After 15 min, cells were sedimented and residual cellular ATP was determined by a luminometer using luciferase reagent. ATP content of cells was estimated as percent of control (cells in buffer alone). Experiments were done in triplicate. Error bars represent median ± range.

buffer, pH 9.5 at 4°C for 12 h followed by blocking with 5% milk powder in PBS at 20°C for 30 min. Subsequently, the plates were incubated with a rabbit antiserum to amoebapore A at a 1:100 dilution in 2.5% milk powder in PBS at 20°C for 2 h. After three washes with PBS containing 0.1% Tween 20, the plates were incubated with an anti-rabbit immunoglobulin antibody–peroxidase conjugate (Dako, Denmark) at 20°C for 2 h, washed with PBS/Tween, and the assay was developed using *o*-phenylenediamine and H_2O_2 as substrates. The resulting color reaction was stopped with 2 M sulfuric acid after 2–5 min and the absorbance at 490 nm was measured with an ELISA plate reader (Titertek Multiskan Plus/MK II; ICN Flow).

2.13. Fluorescence resonance energy transfer (FRET)

The FRET assay was performed at 37°C and as described earlier in more detail [8]. Briefly, liposomes prepared from various phospholipids, doubly doped with fluorescently labelled PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidyl ethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE) (Molecular Probes, Eugene, OR, USA)) were prepared in buffer (8 mM sodium citrate, 100 mM KCl, 5 mM $MgCl_2$, pH 5.2) and filled into the cuvette (1 ml, final lipid concentration 10 μM) of a fluorescence spectrometer (SPEX F1T11, SPEX Instruments, Edison, NY, USA). NBD-PE was excited at 470 nm and the NBD donor and Rh acceptor emission was monitored at 531 nm and 593 nm, respectively. After 50 s, amoebapore A (0.1 μg, 0.3 μg or 1 μg, solubilized in 0.1% trifluoroacetic acid (TFA)) was added to the cuvette and the donor and acceptor fluorescence intensity was recorded over time. Intercalation of the peptide into the liposomes resulted in probe dilution and as a consequence in a lower FRET efficiency: the emission intensity of the donor increases and that of the acceptor decreases. The FRET signal is presented as the quotient of the donor and acceptor emission intensity ($I_{Donor}/I_{Acceptor}$). The addition of the peptide solvent (0.1% TFA) alone did not have a measurable effect on the FRET signal.

3. Results

3.1. Effect of amoebapores on the ATP content of target cells

Viable *E. histolytica* trophozoites and human Jurkat cells were incubated in the presence of amoebapores. After 15 min, the amount of cellular ATP was determined by the luciferase assay. Whereas the ATP content of amoeba was not affected by amoebapores, Jurkat cells lost approximately 50% of their ATP in the presence of 5 μM amoebapores (Fig. 1).

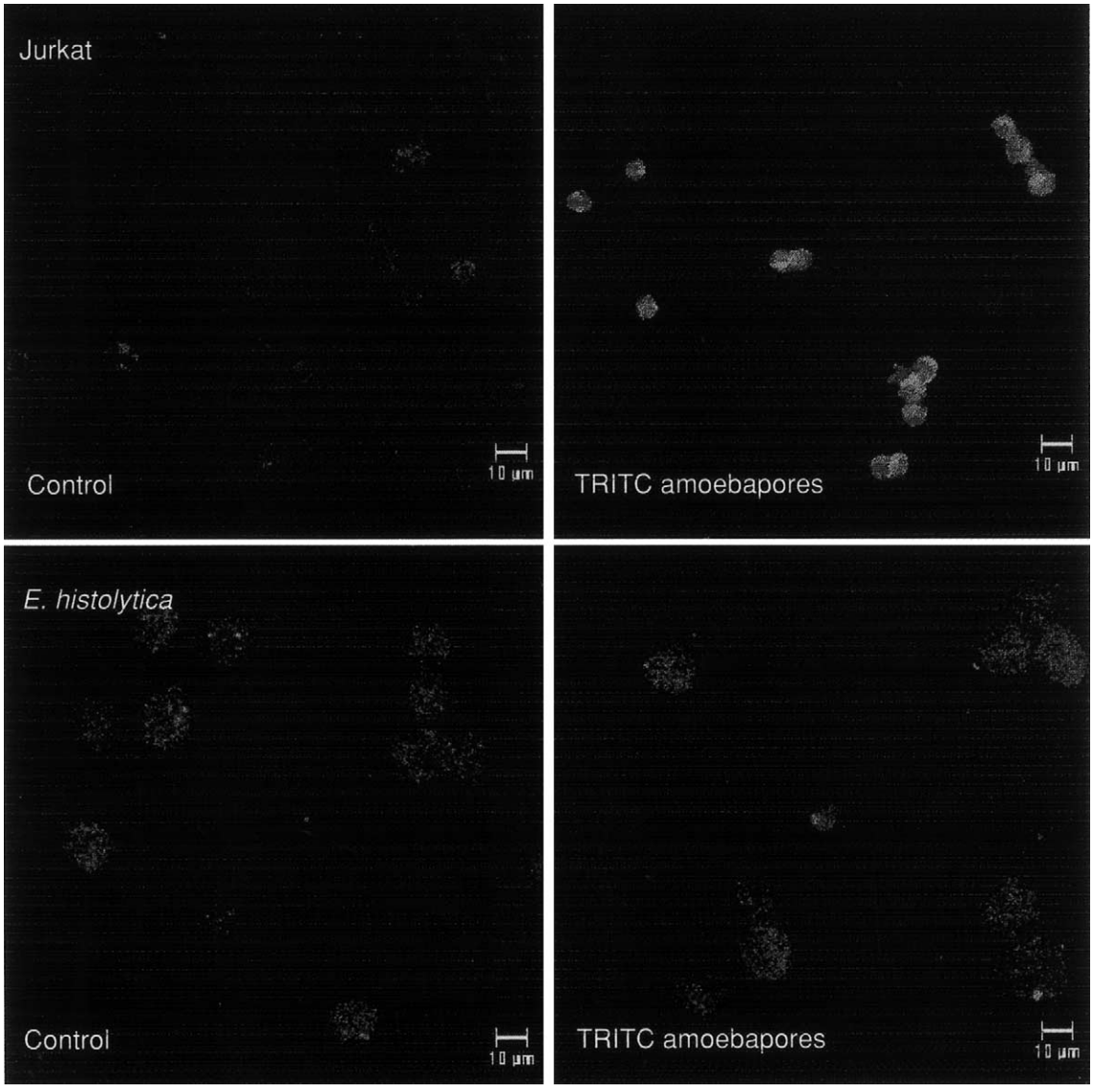

Fig. 2. Confocal microscopy of Jurkat T cells and *E. histolytica* trophozoites incubated with TRITC-labelled amoebapores. Jurkat cells (upper panels) or amoebae (lower panels), which showed a slight autofluorescence, were incubated with TRITC-conjugated amoebapores (total amount 30 μg) or as a control in buffer alone (MES, pH 5.5). Multiple focus planes (16) were accumulated to one image to gain optimal yield of fluorescence. Scale bar = 10 μm.

3.2. Target cell binding of amoebapores

Binding of TRITC-labelled amoebapores to target cells (human Jurkat T cells) and *E. histolytica* trophozoites was analyzed by confocal laser microscopy (Fig. 2). Cell fluorescence of Jurkat cells increased markedly in the presence of labelled amoebapores (upper panels). In contrast, an increase in fluorescence, i.e. binding of fluorescently labelled amoebapores, was not observed for amoebae. A slight autofluorescence of the trophozoites was detectable also in the control which is presumably due to endocytosed culture medium (lower panels).

3.3. Liposome binding of amoebapore A

To elucidate the molecular basis for the observed selectivity of amoebapore binding, we used an artificial membrane system with defined lipid compositions. In this set of experiments, amoebapore A was incubated in the presence of increasing amounts of various types of liposomes. After separating liposome-associated and unbound amoebapore by ultracentrifugation, we determined the concentration of residual amoebapore A in the supernatant (i.e. unbound amoebapore) by measuring the pore-forming activity. No reduction of activity in the supernatant was observed for amoebapore A incubated in the presence of liposomes composed of different *E. histolytica* lipid preparations (total membrane lipids, plasma membrane lipids and granular membrane lipids) even in a 1000-fold excess in weight of lipid vesicles indicating that binding to any of the amoebic membrane preparations had not occurred (Fig. 3A).

In order to dissect further the impact of the various components of amoebic membranes, we monitored the adsorption of amoebapore A to phospholipid vesicles and observed particularly the influence of cholesterol on the binding of the pore-forming peptide to membranes.

Amoebapore A readily binds to liposomes made of azolectin from soy bean, however, the binding capability was drastically reduced by adding cholesterol (Fig. 3B). The weight excess of azolectin vesicles necessary to result in 50% binding of amoebapore A increased from 15-fold in the absence of cholesterol to approximately 50-, 100-, and 300-fold when the cholesterol content in the membrane was 10%, 20%, and 30%, respectively.

To mimic to some extent the natural lipid composition of the amoebic plasma membrane, we prepared liposomes of a mixture of ceramide, PC, sphingomyelin, PE, PS, and PI. Here, we determined the concentration of residual amoebapore A in the supernatant (i.e. unbound amoebapore) by ELISA using an amoebapore A-specific antibody in addition to measurement of the residual pore-forming activity (Fig. 3C). The two assays gave matching results confirming that both methods are suitable to monitor the binding of amoebapore to membranes. The binding affinity of amoebapore A to these liposomes was at least an order of magnitude lower than to vesicles from azolectin and was substantially reduced further by addition of the high amount of cholesterol (46.5%) reported for amoebic membranes [14].

3.4. Intercalation of amoebapore A into liposomes composed of phospholipids and cholesterol

We used FRET spectroscopy as a sensitive marker for peptide/membrane interaction. In this assay, we included substitutes for ceramide aminoethylphosphonate (CAEP), the main

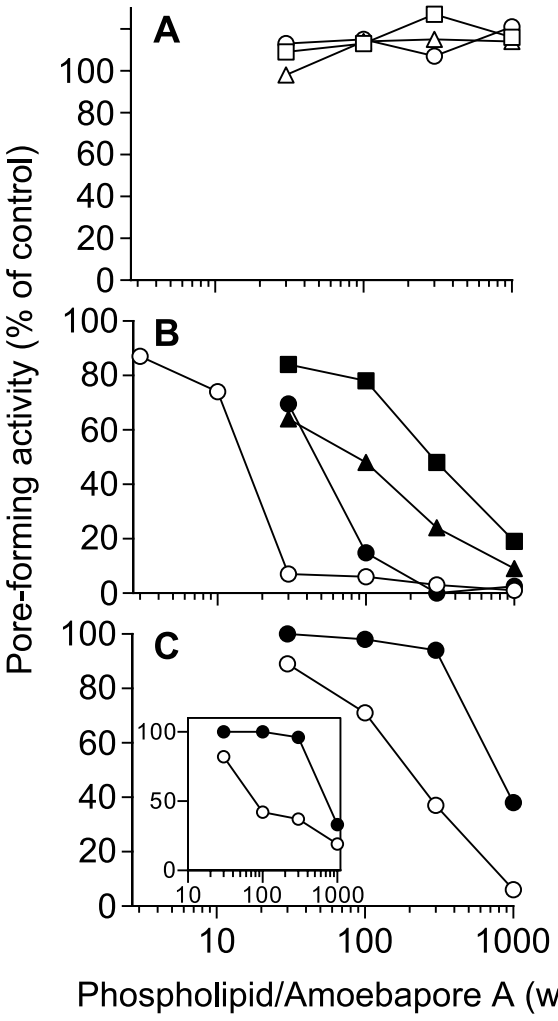

Fig. 3. Amoebapore A binding to lipid vesicles. After incubation of amoebapore A with liposomes of various compositions and subsequent sedimentation, the concentration of the peptide in the supernatant was determined by measuring the residual pore-forming activity. As control, amoebapore A was incubated in the absence of lipids. A: Lipid vesicles were prepared of trophozoite total lipids (open circles), granular membrane lipids (open triangles), or plasma membrane lipids (open squares). B: Lipid vesicles were prepared of azolectin (open circles), supplemented with increasing amounts of cholesterol (10%, filled circles; 20%, filled triangles; 30%, filled squares, w/w). C: Liposomes were made of a synthetic lipid mixture mimicking the natural lipid composition of the amoebic plasma membrane without (open circles) or supplemented with 46.5% (w/w) of cholesterol (filled circles). Inset: Binding of amoebapore A as measured in the same experiment by ELISA using specific antibodies. The *y*-axis shows the relative absorbance at 490 nm (given in % of control) and the *x*-axis again the phospholipid/amoebapore A ratio (w/w).

lipid component of the *E. histolytica* plasma membrane. CAEP is a unique sphingolipid with an ethanolamine headgroup which is linked to ceramide via a phosphono group (O-P-C) instead of the phosphate group (O-P-O) of ordinary phospholipids. This particular linkage mediates resistance to the action of phospholipases. However, it should not influence the overall physicochemical parameters which are responsible for membrane packing, fluidity, and the interaction with pore-forming proteins such as amoebapores. As mimetics for CAEP we investigated the interaction of amoebapore A

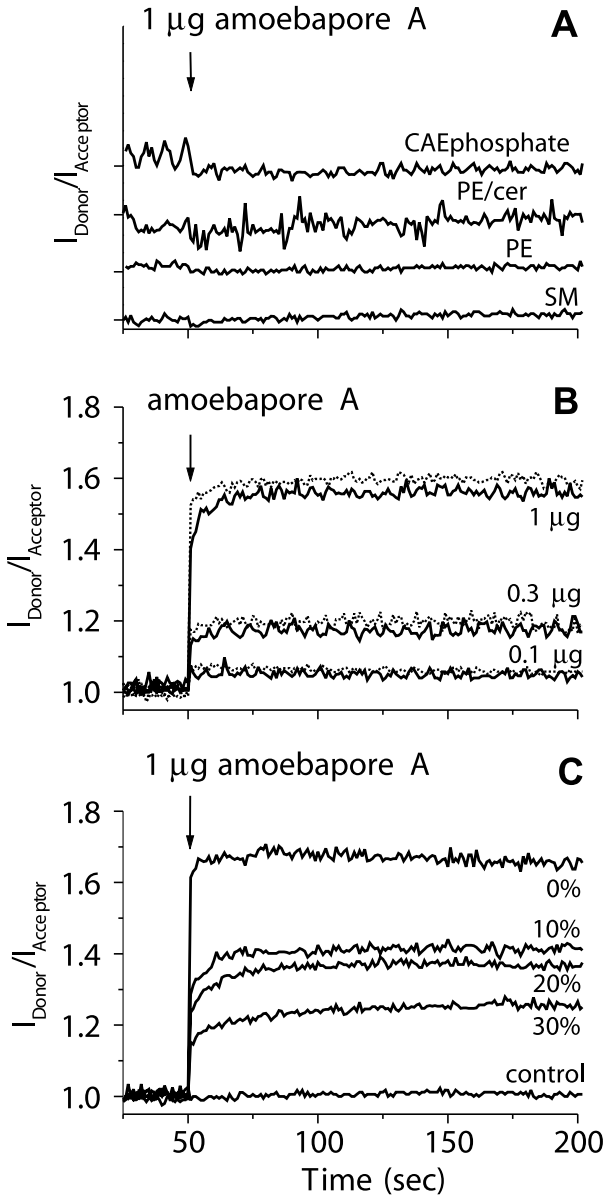

Fig. 4. Amoebapore A intercalation into lipid vesicles as determined by FRET spectroscopy. The peptide was added (arrow) in the indicated amounts to various liposomes double-labelled with NBD-PE and Rh-PE. A: Liposomes of sphingomyelin (SM), PE, a 1/1 molar mixture of PE/ceramide (PE/cer), or of CAEphosphate. Please note that in all cases no change of the FRET signal was observed. For better visualization, each curve is presented with different offset, accordingly, each dash at the *y*-axis represents a donor/acceptor ratio of 1.0. The axis scale is identical to B and C. B: Liposomes of PS (dotted line) or PG (solid line). C: Liposomes of PG supplemented with indicated amounts of cholesterol (0–30% w/w). An intercalation of the peptide leads to an increase of the FRET signal ($I_{Donor}/I_{Acceptor}$). Representative experiments each performed at least in duplicate are shown. The peptide solvent (0.1% TFA) served as a control.

with lipids sharing the ethanolamine headgroup (PE) or the ceramide lipid entity with CAEP (ceramide, sphingomyelin) or both (1/1 molar mixture of PE and ceramide). In addition we used CAEphosphate, a sphingolipid which is identical to CAEP except that the ethanolamine headgroup is linked via a phosphate instead of phosphonate to ceramide.

As a matter of fact, amoebapore A (1 µg) does not intercalate into any of the CAEP-mimetic membranes, i.e. ceramide (not shown), sphingomyelin, PE, PE/ceramide (1/1), and CAEphosphate (Fig. 4A). On the contrary, addition of amoebapore A to liposomes prepared from negatively charged phospholipids (PS or PG) resulted in a strong, dose-dependent increase of the FRET signal indicating that the peptide intercalates into the liposome membrane even at a low peptide/phospholipid ratio (Fig. 4B). Supplementing PG liposomes with cholesterol leads to a dose-dependent decrease of the FRET signal, suggesting that the sterol to some extent prevents membrane insertion of the peptide (Fig. 4C).

4. Discussion

Amoebapores, the pore-forming polypeptides of *E. histolytica*, are potent cytolytic effector molecules that are stored in and are released from cytotoxic granules. The killing of human host cells induced by amoebae takes place after cell–cell contact has been established [18]. More relevant for the amoebic life inside the colon, amoebapores act as antimicrobial agents to combat growth of ingested bacteria, the primary nutrient source of the amoeba, inside the digestive vacuoles (for review see [19,20]). Structurally, amoebapores belong to the family of saposin-like proteins, lipid-binding and membrane-interacting proteins. Granulysin and NK-lysin, membranolytic effector peptides from mammalian cytotoxic lymphocytes [21,22], are functionally similar members of that protein family. Besides this affiliation there are several lines of direct experimental evidence that amoebapores act by permeabilizing the membranes of their targets: depolarization of liposomes [7,9,23], oligomerization and pore formation in planar lipid membranes [8,24], influx of a DNA intercalating dye into amoebapore-treated bacteria [9,10], and fluorescent dye release from prelabelled metabolically active nucleated cells [4,9]. Recently, we demonstrated that amoebapores binds to the surface of phagocytosed bacteria inside the amoeba in vivo [10] and here we show that amoebapores readily bind to the surface of Jurkat cells.

Although the involvement of amoebapores in host cell destruction has been proposed decades ago and was confirmed by accumulated evidence, the issue remained unresolved how amoebae can kill several target cells within minutes coming out of the lytic attack unharmed and what the molecular basis is for the apparently inherent resistance of amoebae to their cytolytic mediators. Here, we observed that a loss of cellular ATP, a very sensitive marker of cell injury, does not occur after incubation of viable *Entamoeba* trophozoites with amoebapores in concentrations sufficient to reduce the ATP content of Jurkat cells by 50% in the same time period.

In general, resistance of eukaryotic cells towards pore-forming toxin attack may be due to various causes and has been investigated particularly for bacterial toxins. Absence of high affinity binding sites [25], inhibition of insertion of the pore-forming domain despite assembly of oligomers after binding to the target cell membrane [26], and the capacity to repair lesions in that the inserted pore is closed through constriction [27] are those mechanisms which are exemplified for various target cells that survive the assault of *Staphylococcus* α-toxin. Additional protection strategies have been reported for organisms encountering membranolytic toxins. They include the dependence of the sea anemone equinatoxin II on sphingomyelin [28], immunity proteins of Gram-positive bacteria, which bind

to bacteriocins and inhibit their activity [29], modifications of the protein receptor of colicin [30], and an ABC transporter, which expels the lantibiotic epidermin from the bacterial cytoplasmic membrane into the surrounding medium [31].

Importantly, we failed to detect fluorescently labelled amoebapores on the surface of amoebae by confocal microscopy. This experiment indicates that the prevention of binding of the amoebic protein to the membrane is the main mechanism to confer protection of amoebae against their own toxins. The preference of amoebapores for negatively charged phospholipids described previously and shown also here by FRET spectroscopy certainly contributes to the inefficiency towards amoebic trophozoites: though the amoeba plasma membrane also contains significant amounts (ca. 10%) of anionic phospholipids (PS and PI) [14], it is likely that these lipids are located mainly at the inner membrane leaflet as found with other eukaryotic cells (see [32,33] and references therein) and hence are not accessible on the cell surface. Moreover, a non-phospholipid membrane compound appears to contribute substantially to the protection of amoebae as cholesterol reduces the binding affinity of amoebapore to azolectin liposomes by more than two orders of magnitude and it substantially reduces the intercalation of the peptide into negatively charged PG vesicles. Whereas bacteria lack cholesterol, it is an important component of eukaryotic cell membranes. For *E. histolytica*, the plasma membrane content of cholesterol is considerably high (46.5% per mol of phospholipids) [14]. The effects of cholesterol on membrane properties are diverse. It increases the membrane fluidity in the gel phase but essentially contributes to membrane rigidity in the physiologically relevant liquid crystalline phase by enhancing the ordering of the fatty acid acyl chains [34] and therefore has been dubbed a 'dynamic glue' [35]. Accordingly, the activity of many membrane-permeabilizing polypeptides, e.g. the antimicrobial peptides magainin 2 and gramicidin S, is substantially decreased when cholesterol has been introduced into artificial target membranes [36–38]. Thus it is likely, and consistent with our data, that cholesterol also contributes to some extent to the target selectivity of amoebapores. However, as the binding to and intercalation into the phospholipid bilayer is significantly reduced but not omitted when cholesterol was introduced as evidenced with liposomes composed of azolectin or PG, the finding that lipid vesicles made from natural amoebic membranes does not bind amoebapore at all cannot be explained entirely by the high content of cholesterol. Moreover, amoebapore binding affinity to lipid vesicles that mimic to some extent the plasma membrane of amoebic trophozoites in its composition of commercially available phospholipids and in its high cholesterol content was extremely low but still detectable. This may indicate that (i) membrane asymmetry, which is actively generated in vivo [33], is not represented in the model system; as a consequence, significant amounts of negatively charged lipids are presented to the liposome surface and thus enable amoebapore A binding to these vesicles, and/or (ii) a component not introduced into the artificial system contributes to amoebapore resistance. Interestingly, the unusual phosphonolipid CAEP is a major phospholipid constituent of the amoebic plasma membrane [14]. CAEP is particularly known for its stability against hydrolysis and may help to protect the amoebic membranes against several aggressive components. Notably, the secretory, lysosome-like granules of amoebae contain a full complement of hydrolytic enzymes in addition to amoebapores [39–42]. Although the mode of action of amoebapores has nothing to do with hydrolysis, CAEP would clearly help to prevent the intercalation of amoebapore A, as shown by FRET spectroscopy using a very similar, but commercially available, lipid (CAEphosphate) or lipids mimicking either the headgroup (PE) or the fatty acid chain (ceramide, sphingomyelin) properties of CAEP. It is suggestive to think that the large amount of sphingolipids (39% CAEP [14]) in the plasma membrane of *E. histolytica*, which is almost twice as abundant as the sphingolipid content of other eukaryotic cell membranes (20–25% sphingomyelin [33,43,44]), would, in combination with cholesterol, result in the formation of membrane-stabilizing lipid rafts in high density and would thereby contribute significantly to the self-protection of *E. histolytica* against amoebapores.

In conclusion, a particular membrane lipid composition that does not allow the binding of amoebapores provides a virtually satisfactory explanation for the resistance of amoebae to their own toxic proteins. The rapid and continuous turnover of the amoebic surface membrane by endo- and exocytotic events and the total coverage of trophozoites by glycosylphosphatidylinositol-anchored proteophosphoglycan molecules [45] may further contribute to protection against lethal damage. In its natural environment, the human colon, *E. histolytica* is surrounded by a variety of microorganisms that may produce toxic components to succeed against their competitors. The principle of possessing membranes almost non-vulnerable to amoebapores could not only spare amoebae from being lysed by their own effector proteins but may also provide protection against some membrane-active toxins of other colon-dwelling microbes.

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