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Shortened amoebapore analogs with enhanced antibacterial and cytolytic activity

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Abstract Amoebapores are cytolytic peptides of *Entamoeba histolytica* which function by the formation of ion channels in target cell membranes. Three isoforms (amoebapore A, B, and C) exist in amoebic cytoplasmic granules. They are composed of 77 amino acid residues arranged in four α-helical domains. In order to analyze the structure-function relationships, 15 synthetic peptides of 24–25 residues were constructed based on the assumption that the third helix is the membrane-penetrating domain and on the previous finding that positively charged residues are significant for activity. Activity of these short versions of amoebapores was determined towards artificial and natural targets, such as liposomes, bacteria, erythrocytes and a human tumor cell line. It was found that some of the novel peptides were highly active and showed a broader activity spectrum compared to the parent molecules.

Key words: Amoebapore; Antibacterial activity; Cytolysis; Pore formation; Synthetic peptide; *Entamoeba histolytica*

1. Introduction

Peptides with antibacterial and cytolytic activity are widespread in nature and have become the study subject of several biological disciplines [1,2]. Moreover, they are viewed as leads for the construction of improved bioactive molecules [2,3].

We are interested in the potential and in the structure-function relationships of amoebapores, pore-forming peptides of the protozoon *Entamoeba histolytica*, a parasite, which is the cause of severe destruction of tissues in the human host. These molecules exhibit cytolytic activity and are considered a major part of the cytotoxic machinery of *E. histolytica* (for review see [4]). Three isoforms, termed amoebapore A, B and C, exist in cytoplasmic granules of the amoeba, which were found to display antibacterial activity, and hence may be instrumental in preventing the growth of phagocytozed bacteria besides their extracellular killing activity [5]. They are 77-residue polypeptides stabilized by three disulfide bonds and predicted to be arranged in four mostly amphipathic α-helical domains [6]. Surprisingly, we have identified a structural and functional similarity between amoebapores and NK-Lysins [7], polypeptides that were recently discovered in pigs and localized to professional cytolytic effector cells of their immune system [8]. The first and third α-helical domains of amoebapore A are long enough to span a lipid bilayer and have been classified as membrane-seeking elements [9]. Synthetic peptides constructed according to these domains exhibited pore-forming activity. H3, a synthetic peptide derived from the third helix, was the most active peptide corresponding to the natural helices and exerts, like the parent molecule, cytolytic and antibacterial activities [10]. Besides the identification of the third helix of amoebapore A as a functional important domain, we found that positively charged amino acid residues are critical for the mode of action of the pore-forming peptide. Chemical modification of the amino acids lysine and histidine resulted in nearly complete loss of pore-forming activity. Whereas the modification of lysine residues inhibited the binding of amoebapore to membranes, the treatment of a sole histidine residue affected the oligomerization of the peptide upon membrane association [11]. Interestingly, the histidine residue is conserved in amoebapores, despite considerable sequence divergences among the three isoforms [5].

Following the information already obtained on the structure-activity relationships, we designed synthetic short versions of the naturally occuring peptides using the third helix of each isoform as leads. A variety of model peptides were constructed that are substantially reduced in size compared to the parent molecules and mimick to varying degrees the structural features of each isoform previously found to be essential for the activity of amoebapore. We monitored the membranolytic activities of the novel synthetic peptides against five different test bacteria and two cell types of human origin.

2. Materials and methods

2.1. Chemicals

Trifluoroacetic acid (TFA) and acetonitrile, both of HPLC grade, were from Applied Biosystems. Dithiobis(succinimidylpropionate) (DSP) was from Pierce. Crude phosphatidylcholine, type IIS from soy bean, dimethylsulfoxide, dithiothreitol, iodoacetamide, 2-(*N*-morpholino)ethanesulfonic acid (MES), and 2,2,2-trifluoroethanol were purchased from Sigma. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt.

2.2. Synthetic peptides

All peptides were synthesized by solid-phase techniques utilising an Fmoc-based protecting group strategy. Peptides H3, A2, B2, and C2 were obtained in purity grade > 70% from Affiniti Research Products Ltd., UK. The other peptides used were obtained from Abimed Analysen-Technik GmbH, Langenfeld, as crude products. All peptides were dissolved in 0.1% TFA and purified by reversed-phase chromatography. Whereas peptides obtained from Affiniti Research were purified by HPLC only, peptides obtained from Abimed were purified in a two-step procedure. The initial purification step was performed on an FPLC system (Pharmacia LKB) at 20°C using a Resource-RPC column (3 ml, Pharmacia LKB). Elution was carried out with 30 ml of a linear gradient from 0 to 80% acetonitrile in water acidified with 0.1% TFA. Flow rate was 2 ml/min, and the effluent was monitored by the absorbance at 226 nm. Fractions corresponding to the main peak were collected, lyophilized and solubilized in 0.1% TFA. Final purification was achieved by RP-HPLC at 30°C using a HAIsil C8 column (250×2.1 mm, Higgins Analytical) connected with a 130 A separation system (Applied biosystems). Elution was performed with a linear gradient of 45–70% acetonitrile in water, acidified with 0.1% TFA, for 25 min. Flow rate was 0.25 ml/min, and the effluent was monitored by absorbance at 214 nm. Fractions represented by a single peak were collected. The purified peptides were lyophilized and stored at −20°C.

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2.3. Other peptides

Amoebapores were highly purified from *E. histolytica* HM-1:IMSS as previously described [5]. Melittin from the venom of *Apis mellifera* (in sequencing grade) was purchased from Sigma. MultiMark molecular weight marker was purchased from Novex.

2.4. Peptide analysis

Homogeneity of all synthetic peptides was confirmed by analytical RP-HPLC (HAIsil C18 column, 250×2.1 mm, Higgins Analytical). Theoretical amino acid compositions of the peptides were confirmed by amino acid analysis performed as previously described [11] and by ion spray mass spectrometry (performed by the manufacturers of the peptides). Peptide concentrations were determined by measuring the absorbance at 214 nm; the extinction coefficients were calculated [12] using the respective sequence information. Tricine-SDS/PAGE [13] was performed in 13% separation gels.

2.5. Bacteria

The bacterial strains used were *Bacillus subtilis* (strain 60015) and *Escherichia coli* K-12 (ATCC 23716) from the Botanical Institute, University of Hamburg; *E. coli* K-12 strain D31 [14]; *Yersinia enterocolitica* (96-C, no plasmid, serotype 0:9) from the Institute for Hygiene and Microbiology, University of Würzburg; and a clinical isolate of *Staphylococcus aureus* obtained at our Institute. Bacteria were usually grown overnight in Luria-Bertani (LB) medium with constant shaking at 37°C and subsequently inoculated in the same medium to reach the mid-logarithmic phase. *B. subtilis* was dispersed on LB-agar plates containing 1% glucose, grown overnight and subsequently inoculated in LB medium.

2.6. Assay for pore-forming activity

Determination of pore-forming activity of samples by monitoring the dissipation of a valinomycin-induced diffusion potential in liposomes has been described previously in detail [15]. For this assay, peptides were dissolved in 0.1% TFA. For reduction of peptides, dithiothreitol was added to a final concentration of 50 mM and samples were boiled for 10 min. Reduced samples were alkylated by incubation with 100 mM iodoacetamide for 30 min at 20°C.

2.7. Liposome association and crosslinking

Peptides (10 μM) were allowed to bind to liposomes (1 mg phospholipids/ml) of the type that were routinely used in the assay for pore-forming activity and, subsequently, chemical crosslinking was performed for 20 min using the homobifunctional crosslinking reagent DSP as detailed before [11]. In some experiments, peptides were reduced prior to liposome association.

2.8. Assay for antibacterial activity

Antibacterial activity was measured by a microdilution susceptibility test. Peptides were dissolved in 10 mM sodium phosphate, pH 5.2, and 2-fold serial diluted in the same buffer. Subsequently, a bacterial suspension (10 μl of LB medium containing 100 colony forming units) was added to the peptide solution (90 μl). The plates were incubated for 16–20 h at 37°C. The MIC (minimal inhibitory concentration) was defined here as the concentration of the highest dilution of peptides at which the growth of bacteria was clearly affected. Portions of the incubation mixtures were plated on LB-agar plates for counting the colony forming units. The MBC (minimal bactericidal concentration) was defined as the highest dilution of peptides at which no formation of bacterial colonies was observed. The values were expressed as the mean of at least two experiments with a divergence of not more than one dilution step.

2.9. Assays for cytotolytic activity

Hemolytic activity: Lysis of human erythrocytes induced by peptides in MES-buffered saline, pH 5.5, was determined by measuring the concentration of released hemoglobin [10]. Experiments were performed in duplicates.

Activity against tumor cells: Cytolysis of Jurkat cells induced by peptides in MES-buffered saline, pH 5.5, was determined by measuring the release of fluorescent dye [10]. Experiments were carried out in triplicate.

2.10. Hydrophobic moment plot

Mean hydrophobicities and mean hydrophobic moments of the synthetic peptides were calculated based on the normalized consensus scale proposed by Eisenberg et al. [16] using the PROSIS program (Pharmacia LKB). The values calculated were displayed graphically in a hydrophobic moment plot [16] as described [9]. Peptide sequences were represented as helical wheels according to Schiffer and Edmundson [17] using the same program.

3. Results and discussion

3.1. Design of synthetic peptides

Since among the four α-helical domains predicted for amoebapores, the third helix appeared to be the major functional domain, we constructed a variety of helix 3-based peptides (Fig. 1) and thereby reduced significantly the size of the pore-forming peptides. Nine of the 15 model peptides are related to amoebapore A and three peptides each to the other two isoforms. With the exception of H3, which refers to the initial study [10], all peptides were synthesized with an amidated C-terminus. The amide group probably mimick the natural environment of a residue within a protein more authentically than a negatively charged carboxyl group. Moreover, it is known from a number of naturally occurring membrane-active peptides that their carboxyl termini are amidated, e.g. the cecropins [18], antibacterial peptides from insect hemolymph, and melittin from the venom of bee species [19].

Peptides A1, B1, and C1 accurately match the predicted third helix of the respective isoform. In peptides A2, B2, and C2 the amino acid residue found adjacent in the sequences of the natural peptides is added to the C-terminus thereby mostly including a positive charge. The sole cysteine in these synthetic peptides theoretically possesses a free sulfhydryl group instead of being involved in a disulfide bond as

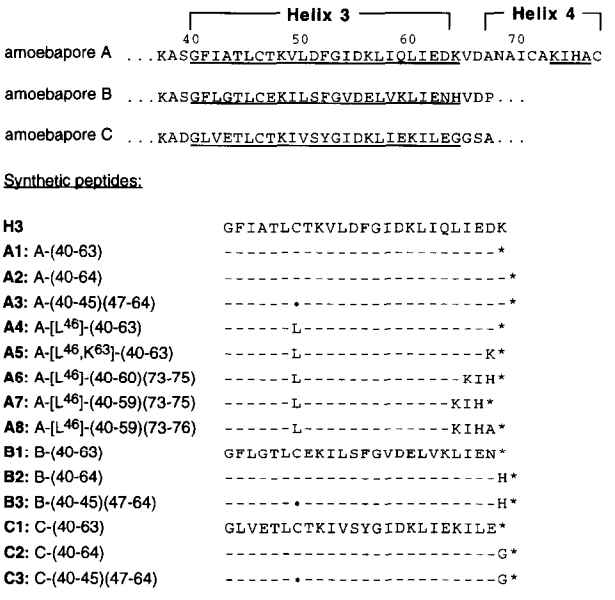

Fig. 1. Partial primary structures of the amoebapores and amino acid sequences of synthetic peptides derived from them. The one-letter notation of amino acid residues is used. Parts of the amoebapore sequences which were used for the construction of the synthetic peptides are underlined. Only residues differing from the respective top sequences are given, whereas identical residues are represented by a hyphen. A point marks the omission of amino acid residues. The asterisk indicates an amidated carboxy-terminus. Peptides designated as A-, B-, and C- are derived from amoebapore A, B, and C, respectively. The accurate designations of the peptides are preceded by the abbreviations used in the study (presented in bold).

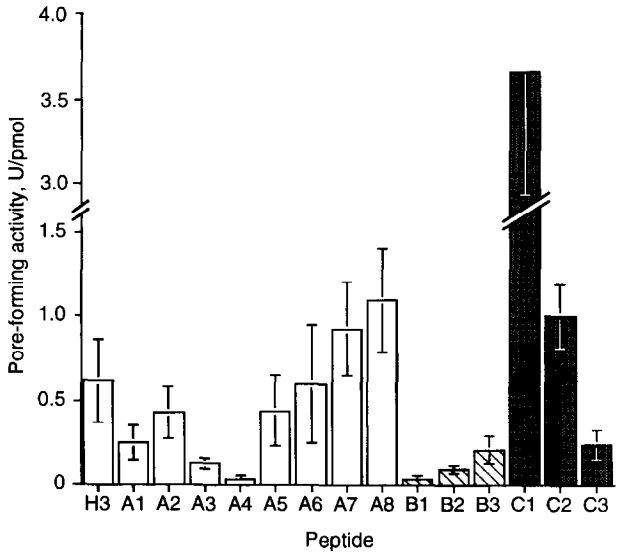

Fig. 2. Pore-forming activity of synthetic peptides derived from amoebapores, estimated by measuring the dissipation of a valinomycin-induced diffusion potential during the first minute after adding various amounts of peptides. Values expressed represent the mean and standard deviation of 7–19 independent experiments. For comparison, amocbapore A, B, C, and melittin have pore-forming activities of 4.1, 3.5, 7.0, and 0.1 U/pmol, respectively.

in the natural peptides. In order to estimate its influence on activity, we either replaced the cysteine residue by the hydrophobic and helix-stabilizing leucine or omitted the cysteine in the residual peptides. These alterations do not significantly affect the amphipathicity of the peptides, i.e. the segregation of hydrophobic and hydrophilic residues at opposite faces of a helix, suggested from helical wheel presentations (data not shown). In the peptide A5, additionally a negatively charged residue was replaced by a positively charged one. Here we expected enhanced binding to negatively charged phospholipids and hence an increase in activity. In the peptides A6, A7, and A8 the carboxy-terminal residues of helix 3 were replaced by 3 or 4 residues including a single histidine and residues found in its vicinity in amoebapore A. Since the latter peptides may be viewed as hybrids of helix 3 and helix 4, they represent a minimalistic approach to include critical features of amoebapore and are of particular interest. All of the constructed peptides are long enough to penetrate a membrane and fulfil the criteria of membrane-seeking and lytic peptides according to Eisenberg et al. [16], in that they possess a high hydrophobic moment, the measure of amphipathicity, and a moderate hydrophobicity (data not shown).

3.2. *Pore-forming activity*

All of the synthetic peptides exhibited pore-forming activity as measured by the dissipation of a valinomycin-induced diffusion potential in liposomes (Fig. 2). Although they were not as potent as their parent molecules, some of them were remarkably active. For amoebapore A and the reference peptide H3 substantially higher activity was measured compared to the initial study [10], although their activities relative to each other were constant. Most likely, the different treatment of peptides, i.e. lyophilization and direct solubilization in the buffer appropriate for the respective assay, is responsible for the observed differences in activity. In particular, stock solutions of H3 no longer became viscous because the formerly performed step of dialysing peptides against saline was omitted. Evidently, reversible self-aggregation already in solution was kept to a minimum by our current handling of the peptides resulting in enhanced activity.

C1 was the most active of the peptides studied, reaching a magnitude in the range of amoebapores. Notably, the substitution or omission of the sole cysteine residue mostly resulted in a significant loss of activity of the peptides. Likewise, reduction and subsequent alkylation of peptides containing a single cysteine residue (H3, A2, B2, and C2) resulted in a 30–40% loss of pore-forming activity (data not shown). In A5, the substitution of the C-terminal aspartic acid of A4 by a lysine residue led to a 10-fold enhancement of activity stressing the significance of positive charges. The helix 3/helix 4 hybrids (A6–A8) are the most active among the A-peptides seemingly confirming the minimalistic strategy mentioned above.

It was of particular interest to identify the property responsible for the dramatic discrepancy in activity between peptides

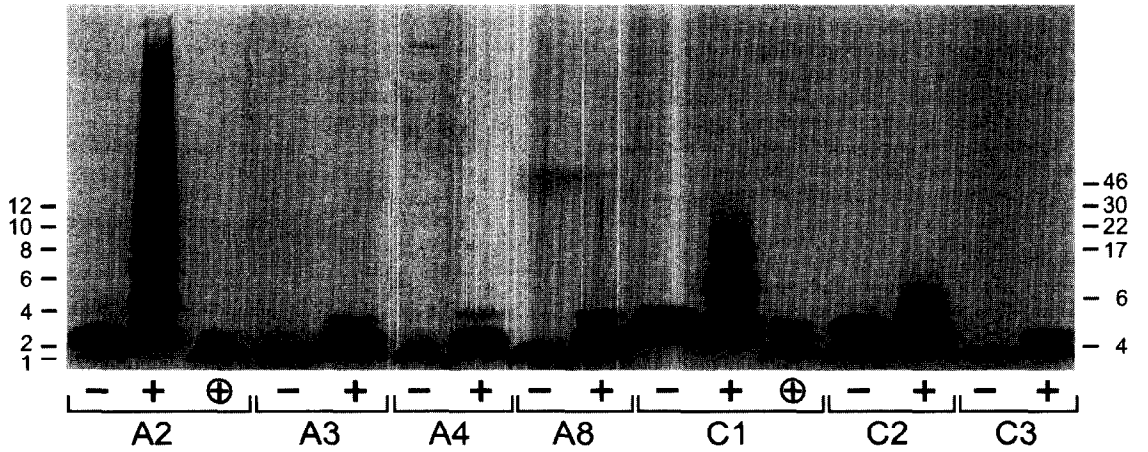

Fig. 3. Oligomerization of synthetic peptides upon association with liposomes. Peptides (10 μM) were allowed to interact with phospholipid vesicles at 4°C for 1 h; aliquots of the mixtures (1 μg peptide) were subjected with (+) or without (−) crosslinking by DSP to Tricine-SDS/PAGE. Additionally, some of the peptides were reduced prior to liposome association and crosslinking (⊕). The putative oligomerization state of the peptides is indicated at left. Molecular weight standards in kDa are indicated on the right.

Table 1. Antibacterial activity of amoebapore-based peptides

Peptide	*B. subtilis*	*S. aureus*	*E. coli* K-12	*E. coli* K-12 D31	*Y. enterocolitica*	Net charge
Amoebapore A	1–2 (2)	1–2(2)	>8 (>8)	4–8 (>8)	n.d.	0
H3	>40 (>40)	>40 (>40)	5–10 (20)	1–2 (5)	n.d.	−1
A2	5–10 (10)	>40 (>40)	5–10 (20)	2–5 (5)	n.d.	0
A3	>40 (>40)	>40 (>40)	2.5–5 (20)	1–2 (5)	n.d.	0
A4[a]	n.d.	n.d.	n.d.	1–2 (>2)	n.d.	−1
A5	0.2–0.3 (0.3)	>40 (>40)	1.3–2.5 (2.5)	0.3–0.6 (1.3)	1.3–2.5 (2.5)	+1
A6	0.2–0.3 (0.3)	2.5–5 (10)	0.3–0.6 (1.3)	0.3–0.6 (1.3)	0.6–1.3 (5)	+3
A7	0.1–0.3 (0.3)	2.5–5 (10)	0.3–0.6 (0.6)	0.3–0.6 (1.3)	0.3–0.6 (0.6)	+3
A8	0.2–0.3 (0.3)	0.6–1.3 (2.5)	0.3–0.6 (0.6)	0.3–0.6 (0.6)	0.3–0.6 (1.25)	+3
B2	5–10 (10)	>40 (>40)	5–10 (20)	1.5–3.1 (12.5)	n.d.	0
B3	10–20 (20)	>40 (>40)	>40 (>40)	0.7–1.5 (3)	n.d.	0
C1[a]	0.5–1 (>2)	>2 (>2)	>2 (>2)	>2 (>2)	n.d.	0
C2	>40 (>40)	>40 (>40)	5–10 (40)	1–2 (20)	n.d.	0
C3[a]	>2 (>2)	>2 (>2)	>2 (>2)	>2 (>2)	n.d.	0
Melittin	0.6–1.3 (1.3)	0.6–1.3 (2.5)	0.6–1.3 (2.5)	0.6–1.3 (2.5)	1.3–2.5 (2.5)	+6

Antibacterial activity is expressed as MIC (MBC) in μM. > denotes no inhibition detected at the concentration indicated. n.d., not determined. Net charges were calculated by counting the N-terminus, lysine, arginine, and histidine as positive charges and a free C-terminus, aspartic acid, and glutamic acid as negative charges.[a]Peptides were not soluble in the buffer at concentrations higher than 2 μM.

which seemed to be quite similar in terms of their primary structure, e.g. C1 and C3. Natural pore-forming peptides tend to undergo self-association after insertion into a membrane, suggesting peptide-peptide interactions relevant for pore formation [20]. Since the capability of undergoing such self-assembly has also been found to be an inherent quality of amoebapores [11,14], we examined the molecular organization of the various peptides after association to liposomes. Upon covalent crosslinking of interacting peptides and subsequent electrophoretic analysis, it became apparent that the decrease in pore-forming activity within the two series of related peptides A2/A3/A4 and C1/C2/C3, parallels their reduced ability to form higher molecular-mass entities (Fig. 3). Without chemical crosslinking, the more active A2 and C1 peptides are found to be in the state of disulfide-bonded dimers since reduction and alkylation prior to membrane association resulted in monomeric behavior of the peptides. The finding that the monomeric state of the peptides is virtually preserved by the latter treatment, even after membrane insertion and chemical crosslinking, implies that the dimer constitutes a critical starting point for oligomerization and for enhanced activity. With regard to A8 (and also A6, A7; not shown), it is interesting to note that the incorporation of a histidine at the peptide's C-terminus apparently is not sufficient to allow an oligomerization process similar to that found with amoebapores.

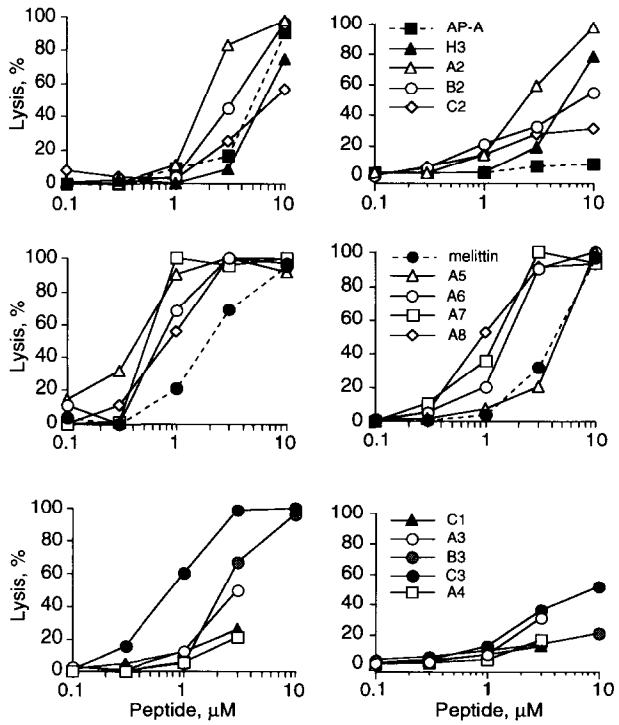

Fig. 4. Cytolysis induced by amoebapore-based peptides. Membrane damage of target cells after exposure to peptides was measured by release of a fluorescent dye from prelabeled Jurkat cells (left) and release of hemoglobin from human erythrocytes (right). The term AP-A here represents amoebapore A.

3.3. Antibacterial activity

The growth inhibition (MIC) and the bactericidal (MBC) activities of the peptides against several Gram-positive and Gram-negative bacterial isolates were determined in a sensitive suspension assay (Table 1). Some of the model peptides were remarkably potent, in particular A5 and the hybrid peptides A6, A7, and A8, which lysed all bacteria tested very efficiently. The MBC values of antibacterial activity mostly were close to the MIC values, which indicates a bacteriolytic rather than a bacteriostatic effect of the peptides. Since the number of cationic residues of the synthetic peptides coincides with an increase of antibacterial activity a positive net charge appears to be important for activity. This may be due to binding by an electrostatic interaction of the positively charged peptides with the highly negatively charged bacterial surface. Such behavior may be expected particularly from the hybrid peptides A6, A7, and A8, which were active in the range of melittin, a potent cytolytic peptide used as a reference peptide since it is active against all bacteria tested. The target preference varied among the synthetic peptides as indicated by the different susceptibility of the bacterial isolates and does not reflect that of amoebapore A. *E. coli* K-12 D31 was found to be the most sensitive isolate; it was lysed by all of the peptides tested, even by amoebapore A, most likely because this mutant has a defect in its lipopolysaccharide structure [13]. The natural peptide was primarily active against the Gram-positives but ineffective against the wild type *E. coli* in the concentrations tested. Unfortunately, some of the novel synthetic peptides were not soluble in the

incubation buffer in the appropriate concentrations, even when they were solubilized in methanol, dimethylsulfoxide, or trifluoroethanol prior to dilution in that buffer. As a consequence, peptides A4, C1, and C3 could be assayed only up to 2 μM and A1 and B1 could not be tested at all, although all were readily soluble in 0.1% TFA.

3.4. Cytolytic activity

The cytolytic activities of amoebapore A and amoebapore-based peptides were assayed towards Jurkat cells and human erythrocytes in MES-buffered saline at pH 5.5 (Fig. 4). Again, some of the synthetic peptides were not soluble in that buffer, or at least not in a satisfactory concentration, and then activity was determined up to a concentration of 3 μM only. Whereas amoebapore A exhibited cytolytic activity against Jurkat cells and failed to lyse human erythrocytes, those peptides with a positive net charge, i.e. peptides A5–A8 were found to be considerably active against both types of cells.

Interestingly, peptides A3, B3, and C3, in which the sole cysteine has been omitted, were also quite effective against the tumor cell line, but were less active against erythrocytes. Two of the peptides exerted only minor cytolytic activity, i.e. peptides A4 and C1, and hence are probably more promising model peptides for antibiotic applications. C1 was the peptide with most pronounced pore-forming activity and it also displayed antibacterial activity against *B. subtilis*. Peptide A4 only exerted negligible pore-forming activity, but inhibited the growth of *E. coli* K-12 D31. Both of the peptides were of poor solubility in the buffers used; it could be a future goal to improve the solubility of these peptide by slightly altering their sequences.

The results presented here may have implications as to the structure-function relationships of amoebapores. Some of the helix 3-derived peptides are highly active against bacteria and eukaryotic cells; all of them depolarize lipid vesicles, indicating that they function – in analogy to amoebapores – by a direct action on the membranes of the target cells. Thus, the notion that helix 3 may be the membrane penetrating structural element is strengthened and include all of the three isoforms. Those of the synthetic peptides described here that are considerably potent may aid the design of improved antimicrobial and tumoricidal peptides. The lower activity of amoebapores against natural target cells combined with a markedly larger size compared to some model peptides needs to be interpreted. Amoebapores are peptides proofed by nature and are capable of creating stable ion channels with distinct characteristics [4], which should not be expected for the small model peptides. The seemingly lesser efficacy of the natural peptides may be a toll for an extraordinary stability against proteolytic degradation provided by a rigid disulfide-bonded structure and may additionally result from the necessity of protecting amoebae from their own toxic factors.

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