Dimerization of truncated melittin analogues results in cytolytic peptides

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A synthetic peptide with the sequence of the first 20 residues of melittin and terminating with an additional cysteine amide was found to have cytolytic activity similar to that of melittin. It was apparent from MS data that the cysteine-terminating peptides had formed disulphide dimers. A peptide in which the thiol was blocked by iodoacetate showed no activity, whereas the same peptide blocked by acetamidomethyl showed activity marginally less haemolytic than that of melittin. Cytolytic activity of melittin analogues comprising the full 26 residues could be obtained with wide sequence permutations providing that a general amphipathic helical structure was preserved. In contrast, the activity of the dimers was dependent not only on retention of an amphipathic helix but also on certain individual residues and a free positive charge. A free N-terminus was essential for haemolytic activity. In addition, a lysine or arginine residue at position 7 and a proline at position 14 were found to be necessary for activity, although it was apparent that additional residues are important for retention of the full lytic potential.

INTRODUCTION

The haemolytic activity of melittin, the major component of bee venom, is well established [1]. Melittin is a 26-residue peptide in which residues 1–20 form an amphipathic helical segment and residues 21–26 constitute a highly basic region which has been reported to be essential for lytic activity [2,3].

Several theories exist as to the means by which melittin initiates cell lysis. It has been proposed that melittin forms ion channels which span membrane bilayers through the tetrameric association of melittin monomers [4]. An alternative proposal is that melittin forms wedge-like structures on partial penetration into the membrane, thus increasing membrane permeability [5]. More recent reports have suggested that binding to membrane proteins is involved [6,7].

Studies have shown that sequential removal of the C-terminal residues gradually reduces the lytic activity of melittin [6]. When the 6-amino-acid cationic segment is totally removed, the remaining peptide does not cause lysis, although it is capable of binding to erythrocytes [2]. The length of the amphipathic helical segment of melittin seems to be important for cell lysis, since analogues with shortened N-terminal sequences are very poor lytic agents [8]. These findings have led to the conclusion that the hydrophobic region is likely to be involved in binding, whereas the cationic region determines the lytic activity [9]. One report has suggested that melittin-(1–19) retains a small amount of haemolytic activity [10].

Replacement of lysine-21, lysine-23 or arginine-24 with leucine [11] or tryptophan [12] leads to an increase in haemolytic activity. A single deletion of any of the basic C-terminal residues [13] resulted in complete retention of haemolytic activity. These authors concluded that the C-terminal segment must only be sufficiently cationic to bind to the phospholipid head groups of the membrane for lysis to occur.

It has been well established that melittin exists as a tetramer in concentrated aqueous solution [14]. However, results obtained from cross-linking experiments suggest that the tetramer dissociates to the monomer in the immediate vicinity of the membrane surface and that in this environment the monomer retains a helical conformation, prior to insertion into the membrane [15]. Of interest was the observation that cross-linked tetramers were fully lytic and retained their lytic activity in high phosphate concentrations, in contrast to the native toxin where tetrameric melittin was demonstrated to lack such activity [15,16].

In our continuing study of lytic peptides suitable for the construction of immunotoxins [6], we synthesized a peptide consisting of the first 20 residues of melittin and with the addition of a C-terminal cysteine amide. The intention was to couple this peptide to a monoclonal antibody through the cysteine thiol group. Published reports [2] suggested that the 21-residue peptide would not be lytic in its own right but may assume activity on delivery to the cell membrane by the monoclonal antibody.

In this paper, we report that this truncated peptide is highly lytic, which we were able to show was the result of the formation of a dimer, from the oxidation of the cysteine thiol. Comparative analyses of related monomers and dimers containing various substitutions indicate that the mechanism of lysis of these dimeric peptides differs from that of native melittin and associated monomeric analogues.

MATERIALS AND METHODS

Materials

Native melittin, free of phospholipase, was purchased from Fluka Chemie AG, and was used without further treatment. Synthetic melittin was obtained from Auspep (Australia).

Peptide synthesis

The peptides (Figure 1) were synthesized on an Applied Biosystems 430A Peptide Synthesizer, using the FastMoc strategy. Norleucine (designated as X) was inserted into peptides 1 and 2 to aid analysis.

Abbreviation used: $DiSC_3(5)$, 3,3′-di-isopropylthiadicarbocyanine iodide.

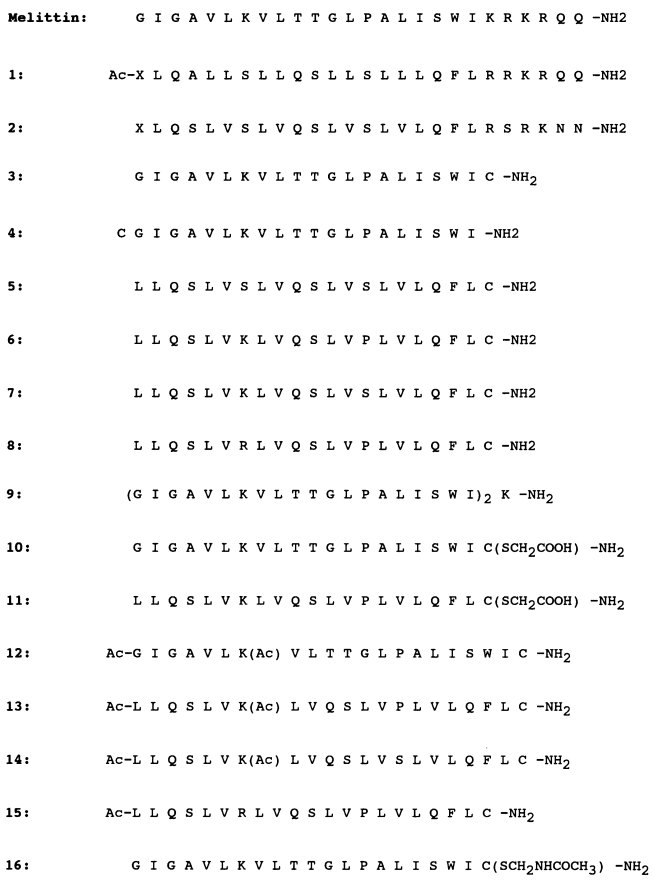

Figure 1 Sequences of the peptides used in this study

Ac, acetyl; X, norleucine.

RINK resin [17] was used as the support for the assembly of all peptides, yielding the peptides as the amides upon cleavage with trifluoroacetic acid. The cysteine side chain was protected by trityl, serine and threonine by t-butyl and lysine by t-butyloxycarbonyl groups. The peptides were purified by reverse-phase HPLC, using a VYDAC C18 column. Separation was achieved using a 20 min gradient from 5 to 60 % acetonitrile. The integrity of the peptides was established by amino acid analysis and matrix-assisted laser desorption MS or electrospray MS. The cysteine-containing peptides were oxidized when dissolved in DMSO in the presence of air to yield the disulphide dimer.

The lysine branched dimer, peptide 9, was synthesized by the technique described above, but FmocLys(Fmoc)OH was used at the C-terminus (where Fmoc is fluoren-9-ylmethoxycarbonyl). This yielded a crude product which consisted of a mixture of deletion peptides along with the desired peptide, which was purified by C18 reverse-phase HPLC. The monomeric cysteine peptides 10 and 11 were prepared by alkylating peptides 3 and 6 with iodoacetic acid in dimethylformamide, after prior reduction with tributylphosphine.

The acetylated peptides 12–15 were obtained by treating the parent peptides with an excess of acetic anhydride in dimethylformamide containing a trace of triethylamine.

Haemolytic assay

Peptides were dissolved in DMSO at 5 mg/ml and serially titrated by 2-fold dilutions in PBS. The final concentration in the 96-well U-bottomed microtitre plates (Nunc) ranged from 200 μg/ml to 0.7 μg/ml. A 0.6 % (w/v) suspension of washed human red blood cells (100 μl) was added for 1 h. Plates were centrifuged at 150 ***g*** for 5 min, and 100 μl aliquots were transferred to a 96-well polyvinyl chloride plate (Dynatech Laboratories, Alexandria, VA, U.S.A.). Haemolysis was assessed by measurement of absorbance at 405 nm with an automatic EAR 400 SF ELISA plate reader (SLT Lab Instruments, Groedig/Salzburg, Austria). The percentage haemolysis was calculated by comparison with absorbances from a buffer blank ('no lysis' control) and a sample treated with 0.1 % Triton X-100 ('maximum lysis' control). Lytic activity could be calculated from the linear portion of the haemolytic titration curve for each peptide, where 1 lytic unit was defined as the concentration of peptide required to produce a given percentage haemolysis. The relative activities of peptides were assessed directly from the linear portion of the titration curves.

Flow cytometry

Flow cytometry, using a modification of the method of Weston et al. [18], was used in parallel with the haemolytic assays to give a more detailed analysis of the effects of the peptides. Light scattering in the direction of the laser beam (forward light scatter) was used as a measure of cell size. As an index of peptide cytolysis, forward light scatter was measured and plotted against cell membrane integrity, which was assessed by exclusion of the dye propidium iodide [19]. CEM T cell lymphoma cells (250 μl) were used for the cytolytic assay, at a concentration of 10^6 cells/ml in PBS. Cells were incubated at room temperature in the presence of peptides and 4 μg/ml propidium iodide (Sigma Chemical Co.). The concentration of peptide (100 μg/ml) was chosen from the haemolytic titration curves. Flow cytometry was carried out on a Coulter EPICS® Elite flow cytometer with illumination at 488 nm. Forward light scatter was measured at 488 nm and propidium iodide fluorescence was detected at $>$ 600 nm.

The fluorimetric measurement of cell membrane potential was also carried out. Some carbocyanine dyes dissolve in cell membranes and give a fluorescent signal which is dependent on the orientation of the dye molecules within the membranes [20]. This orientation is dependent on the voltage difference across the cell membrane, and therefore the intensity of the fluorescent signal is proportional to the voltage drop. Cells that are affected by cytolytic peptides develop ion channels that decrease the cell membrane potential, resulting in a rapid decrease in dye fluorescence. We have used the carbocyanine dye $DiSC_3(5)$ (3,3′-diisopropylthiadicarbocyanine iodide) (Molecular Probes, Eugene, OR, U.S.A.) to measure cell membrane potential and to indicate the formation of ion channels after addition of peptides (100 μg/ml) for 5 min. For membrane potential measurements, cells (in PBS) were pre-loaded with 0.05 μM $DiSC_3(5)$ in 0.9 % NaCl for 15 min at room temperature. Flow cytometry was carried out as above with illumination at 488 nm for light scatter and at 633 nm for excitation of $DiSC_3(5)$ fluorescence. The fluorescence of $DiSC_3(5)$ was measured at $>$ 633 nm. Because the fluorescence emission spectra of propidium iodide and $DiSC_3(5)$ overlap [19], the two dyes could not be used simultaneously.

RESULTS

Haemolytic assay

Table 1 summarizes the haemolytic effects of the peptides after 1 h at various concentrations ranging from 200 μg/ml to

Table 1 Haemolytic activity of melittin, melittin analogues and truncated monomeric and dimeric peptides

Percentage haemolysis was measured at various concentrations of the peptides, as shown.

Peptide	Concn. (μg/ml) ...	Haemolysis (%) 200	100	50	25	12	6	3	1.5	0.7
Melittin		100	100	100	100	100	65	51	14	4
1		100	100	100	98	97	93	35	9	2
2		100	100	99	76	35	15	10	10	1
3		97	100	96	83	41	17	12	9	9
4		100	80	55	33	15	9	7	4	3
5		1	7	5	1	1	0	0	0	0
6		68	29	12	9	9	6	6	2	2
7		28	15	7	2	0	0	0	0	0
8		81	61	33	7	0	0	0	0	0
9		100	92	79	57	33	17	12	8	5
10		1	0	1	1	0	0	0	0	0
11		11	8	7	2	1	0	0	1	1
12		1	0	1	0	0	0	0	0	0
13		7	5	5	5	4	5	5	4	3
14		4	4	7	4	4	3	3	2	1
15		2	0	2	1	1	1	0	0	0
16		96	98	89	53	30	3	5	5	5

0.7 μg/ml. Peptide 1 is based on the sequence published by Degrado et al. [21], with the only variations being the replacement of the N-terminal leucine with *N*-acetylnorleucine, tryptophan-19 with phenylalanine and lysine-21 with arginine. Despite retaining only 31 % sequence identity with melittin, peptide 1 has similar lytic activity to melittin (Table 1). Likewise peptide 2, which has no sequence identity with melittin, has only a slightly decreased activity, i.e. around a 4-fold reduction.

Peptide 3, the truncated (C-terminus-deficient) analogue of melittin, was found to be highly active, causing significant haemolysis at concentrations as low as 12 μg/ml. The *N*-acetylated form of this peptide (peptide 12) and the monomer (peptide 10) were both completely inactive, even at the highest concentration of 200 μg/ml. Dimerization of this truncated peptide either by an N-terminal cysteine (peptide 4) or by a C-terminal lysine (peptide 9) also resulted in highly lytic peptides, which were slightly less toxic than peptide 3 (Table 1).

Peptide 5, the truncated (C-terminus-deficient) analogue of the lytic peptide 2, was completely inactive even at the highest concentration tested. Substitution of lysine and proline into this sequence at positions 7 and 14 respectively (peptide 6) resulted in significant lytic activity, albeit still around 4-fold less than that of peptide 2 on a molar basis. Both substitutions were necessary, since replacement only with lysine at position 7 (peptide 7) resulted in only mild lytic activity. Replacement at position 7 with arginine (in association with substitution of proline at position 14) produced a highly lytic peptide (peptide 8, Table 1) which was equally if not more active than peptide 6. In all these studies with amino acid substitutions, the haemolytic activity of the resulting peptides required a free unacetylated N-terminus (peptides 11–15, Table 1).

Flow cytometry

Cell viability

Results with untreated control cells are shown in Figure 2(A), and results with peptide 3 are shown in Figure 2(B). The figures plot forward light scatter (horizontal axis) against propidium iodide fluorescence (vertical axis). The dots on the diagram indicate measurements for individual cells, and their density indicates the number of cells in each area of the diagram. In the absence of peptide, most cells (93.6 %) lie in an area to the bottom right (quadrant 4). Dead or damaged cells have measurements in other quadrants, notably quadrants 1 (decreased forward light scatter and high propidium iodide fluorescence) and 2 (high propidium iodide fluorescence). An increase in the proportion of cells in the far right of quadrant 2 (increasing forward light scatter) is an indication of cell aggregation. Figure 2(B) shows an example of CEM cells after incubation with peptide 3 for 3 min at room temperature. Considerable cell death is apparent, as shown by the high propidium iodide fluorescence in a large proportion of the cells (quadrants 1 and 2). Some aggregation of the cells is also apparent.

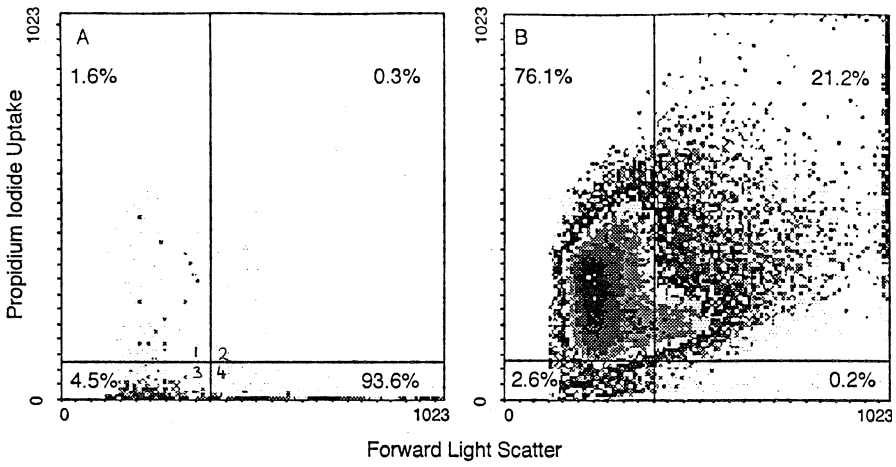

Figure 2 Flow cytometric measurement of the effects of cytolytic peptides

CEM lymphoma cells were incubated in the absence (**A**) or presence (**B**) of peptide 3 at a concentration of 100 μg/ml in the presence of propidium iodide. Flow cytometric measurements after 3 min of incubation are shown. The quadrants were set manually such that more than 90% of the control cells in (**A**) lie in quadrant 4. Quadrant 1, upper left; 2, upper right; 3, lower left; 4, lower right. Forward light scatter and fluorescence intensity are indicated in channel numbers.

Table 2 Quantitative analysis of cell cytolysis by flow cytometry

The cytolytic effects of the peptides were analysed from figures similar to Figure 2, and the percentage of cells remaining in quadrant 4 (undamaged cells) was estimated after 3 min. Control indicates absence of peptide.

Peptide	Cells in quadrant 4 (%)
Control	94.6
Melittin	0.1
1	3.6
2	0.2
3	7.1
4	12.6
5	67.4
6	4.4
7	55.0
8	20.6
9	7.4
10	58.7
11	81.7
12	65.9
13	84.4
14	85.4
15	83.7
16	10.4

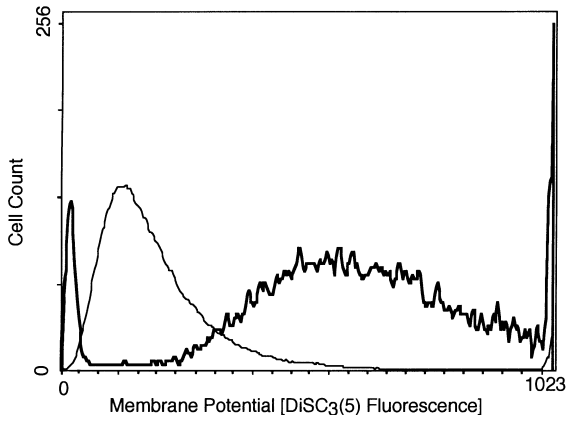

Figure 3 Measurement of cell membrane potential by $DiSC_3(5)$ fluorescence

Cells were loaded with $DiSC_3(5)$ and the fluorescence of the dye was measured by flow cytometry before (bold line) and 3 min after (thin line) the addition of peptide 3 at 100 μg/ml. Relative fluorescence intensity is indicated in channel numbers.

Table 2 summarizes the relative effects of the panel of peptides on CEM lymphoma cells, as indicated by the proportion of cells remaining undamaged after 3 min of incubation.

Membrane potential

The comparison of $DiSC_3(5)$ fluorescence in untreated cells and in cells treated with peptide 3 for 5 min is shown in Figure 3. The untreated cell population exhibited a broad distribution of red fluorescence, indicating the existence of a range of membrane potentials among the cells in the population, and a sharp peak of depolarized (dead) cells exhibiting low fluorescence. Upon treatment with the peptide, the distribution of fluorescence changed rapidly, with the peak shifting markedly towards decreased fluorescence, indicating a decrease in cell membrane potential affecting almost the entire population of cells. Similar results were obtained with the other lytic peptides, and the membrane potential changes observed (not shown) correlated well with cytotoxic activity as measured by the other flow cytometric parameters described above.

DISCUSSION

Peptides 1 and 2 were synthesized for the dual purpose of attachment to a carrier protein (thus only one available NH_2 group was included in the sequence of the peptides) and to investigate the extent of sequence identity required to retain lytic activity.

Assays of cytotoxic potential were carried out by measurement of haemolysis and flow cytometry. The results obtained by the different methods correlated well, with the exception of peptide 6, which gave greater apparent cell damage when measured by flow cytometry than when measured by haemolysis. The reason for this is unknown, but may reflect structural or compositional differences between the red cell and lymphocyte cell membranes.

The lytic ability of these peptides supports the published observations of Degrado et al. [3,21] regarding the prime requirements of an amphipathic helix and a basic C-terminal sequence. The results also support previous observations [22] that acetylation of the N-terminal residue or of the lysine residues has no effect. However, the importance of the lysine residues for lytic activity is the subject of some debate. Our results with the full melittin analogues, which show that lysine can be acetylated or even replaced by serine without affecting activity, support some previously published observations [3,6,21], but are in conflict with others which claim a special role for lysine-7 [8,11,13].

The major finding of the present study was that the disulphide dimer, peptide 3, had a lytic activity approaching that of melittin (on a molar basis). This is in contrast to reports [2,6] that shortened sequences of melittin lose activity, which reaches zero on the loss of the basic C-terminal sequence. This was further demonstrated by alkylating peptide 3 to give an inactive monomeric peptide (peptide 10). However, if the cysteine thiol was blocked by acetamidomethyl, an uncharged group, lytic activity was retained, and in fact only marginally reduced compared with that of melittin. These results suggest that the presence of the negative charge on the carboxymethyl group could be responsible for the loss of activity and that lytic activity may not be dependent on the formation of a dimer. If the position of the cross-link is placed at the N-terminus, instead of at the C-terminus, the activity is reduced but is still significant (peptide 4).

The sequence requirements of these dimers appear different to those of melittin and of the peptide analogues described earlier, since a dimer (peptide 5) based on the sequence of one of these analogues, peptide 2, had no significant activity. Various substitutions indicate that proline-14 and a basic side chain at position 7 are essential for haemolytic activity of the dimeric peptides. Furthermore, acetylation of any of these dimeric peptides destroys their activity (peptides 12–15). We have included an aromatic residue at position 19 in all of these peptides because of the reported important role that tryptophan plays in the activity of melittin [11,13,22]. Fluorescent studies of Trp-19 in various systems indicates that the tryptophan penetrates only superficially into the membrane [23,24], leaving the charged carboxy end exposed to the aqueous environment. However, as the dimers only have charges on residue 7 and the N-terminus, and as the cross-link can be on either end, it is difficult to envisage a similar lytic mechanism to that of melittin. In addition, it is equally

difficult to envisage a role for the free N-terminus and for lysine-7 unless they are involved in binding to the phospholipid head groups.

Thus it has been demonstrated that lysine-7 is not important for the lytic activity of melittin [3,6,18,22] and melittin-like peptides [3,6], despite reports to the contrary [8,11,13], but either lysine or arginine appears to be essential for the activity of the dimeric peptides. This is reinforced by the observation that acetylation of peptide 3 destroys its activity (peptide 12), whereas acetylation of melittin has no effect [22].

A similar observation is evident with proline-14, the inclusion of which enhances the activity of the dimer; however, removal of proline from melittin either has no effect or even positively enhances activity (peptides 1 and 2) [3,6].

Taken together, these results suggest that a lytic peptide with activity approaching that of melittin can be produced from an approximate 21-residue amphipathic peptide providing that the N-terminal amino group remains free and the peptide includes a lysine or arginine residue at position 7, a proline at position 14 and probably an aromatic residue at position 19. A cross-link at the N-terminus is less effective than one at the C-terminus. Despite these conclusions, it is noted that peptide 3 is the most lytic of the dimeric peptides produced, and that there is obviously some other sequence parameter involved other than those already discussed. Nonetheless, this study provides significant information for the generation of a unique class of toxic peptides from truncated analogues by selection of certain essential residues and dimerization.

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