

The plasma membrane of *Leishmania donovani* promastigotes is the main target for CA(1–8)M(1–18), a synthetic cecropin A–melittin hybrid peptide

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Reports on the lethal activity of animal antibiotic peptides have largely focused on bacterial rather than eukaryotic targets. In these, involvement of internal organelles as well as mechanisms different from those of prokaryotic cells have been described. CA(1–8)M(1–18) is a synthetic cecropin A–melittin hybrid peptide with leishmanicidal activity. Using *Leishmania donovani* promastigotes as a model system we have studied the mechanism of action of CA(1–8)M(1–18), its two parental peptides and two analogues. At micromolar concentration CA(1–8)M(1–18) induces a fast permeability to H⁺/OH[−], collapse of membrane potential and morphological damage to the plasma membrane. Effects on other organelles are related to the loss of internal

homeostasis of the parasite rather than to a direct effect of the peptide. Despite the fast kinetics of the process, the parasite is able to deactivate in part the effect of the peptide, as shown by the higher activity of the D-enantiomer of CA(1–8)M(1–18). Electrostatic interaction between the peptide and the promastigote membrane, the first event in the lethal sequence, is inhibited by polyanionic polysaccharides, including its own lipophosphoglycan. Thus, in common with bacteria, the action of CA(1–8)M(1–18) on *Leishmania* promastigotes has the same plasma membrane as target, but is unique in that different peptides show patterns of activity that resemble those observed on eukaryotic cells.

INTRODUCTION

The defensive role of a variety of antibiotic peptides in multicellular organisms is increasingly recognized (reviewed in [1–6]). The majority of these peptides promote membrane permeability, lack haemolytic activity and show preferential activity towards bacteria rather than towards mammalian cells (general review in [6]). The molecular basis of this cellular specificity has been only partially ascertained. For magainins, a class of membrane-active peptides from *Amphibia*, preferential activity on prokaryotes is mainly due to the higher acidic phospholipid content in the outer leaflet of the inner membrane, as well as to the absence of cholesterol [7]. These peptides promote channel formation in both eukaryotic and prokaryotic cells [9,10], but involvement of other internal organelles, such as mitochondria uncoupling has been described for them in hamster spermatozoa [11]. On the other hand, in eukaryotic cells the peptides often associate with other concurrent events for optimal activity. Thus, defensins from mammalian neutrophils need an endocytosis step and a lag of several hours to kill tumour cells, despite their fast bactericidal action [12,13]. The anti-tumour activity of synthetic cecropin analogues synergizes with the disruption of the cytoskeleton by antimicrotubular drugs [14]. In addition, activities against different cell types can differ broadly for a given peptide. Membrane-active fungicidal peptides, devoid of bactericidal activity, have been described [15]. NO production and apoptosis in the monocytic cell line Raw is induced by the cecropin A–melittin hybrid peptide CA(1–8)M(1–18), but not by other shorter analogues with similar bactericidal activity [16].

Leishmania is a protozoan parasite that affects humans as well as other mammalian hosts. The insect form of the parasite is the

flagellated promastigote, responsible for primary infection. Its plasma membrane displays some interesting peculiarities when compared with other eukaryotic cells [17,18]: (i) it is strongly negatively charged due to high levels of lipophosphoglycan (LPG), an anionic polysaccharide that covers more than 60 % of the whole surface [18]; (ii) it is endowed with a high proteolytic activity due to the presence of Gp63, a metalloproteinase with more than 5×10^5 copies/parasite [17]; (iii) its lipid composition is characterized by a slightly higher percentage of anionic phospholipids than the standard mammalian membranes, and has ergosterol instead of cholesterol [19]; (iv) endo- and exocytosis are restrained to a specialized area, known as flagellar pocket, due to membrane rigidity imposed by the highly stable subpellicular layer of microtubules [20].

Our groups have been involved in the study of the interaction of cecropin A–melittin hybrid peptides with both model [21] and biological systems [16,22–25]. These hybrid peptides, comprising the cationic N-terminal sequence of cecropin A followed by the hydrophobic N-terminal sequence of the bee venom toxin melittin, show much higher antibiotic activity than cecropin, and act on organisms otherwise resistant to cecropin A. At the same time they are devoid of the haemolytic activity associated with the C-terminal cationic sequence in melittin [23,24].

In this work we have detailed the mechanism involved in the killing of *L. donovani* promastigotes by CA(1–8)M(1–18) [25], a cecropin A–melittin hybrid active on some eukaryotic cells [16]. We have included the parental peptides in the study to compare differences with other membrane systems and to ascertain which components of the plasma membrane are involved in protection against antibiotic peptides, as a first step for a rational design of specific anti-*Leishmania* peptides.

Abbreviations used: BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein triacetoxymethyl ester; Bisoxonol, bis-(1,3-diethylthiobarbituric) trimethine oxonol; Boc, t-butyloxycarbonyl; CA(1–8)M(1–18), KWKLFFKIGIGAVLKVLTGLPALIS-NH₂; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; LPG, lipophosphoglycan; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight spectroscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMGCi, *N*-methylglucamide chloride; PG, phosphoglycan.

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MATERIALS AND METHODS

Reagents

Unless otherwise stated, reagents of the highest quality available were used and purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany). BCECF-AM [2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, triacetoxymethyl ester], bisoxonol [bis-(1,3-diethylthiobarbituric) trimethine oxonol], and rhodamine 123 were obtained from Molecular Probes (Leiden, Holland). Lipophosphoglycan (LPG) and phosphoglycan (PG) were a kind gift from Prof. S. Turco, Department of Biochemistry, Kentucky University, Lexington, KY, U.S.A.).

Parasites

Promastigotes of the R9 *Leishmania donovani* strain were grown in RPMI-1640 medium (Gibco, Paisley, U.K.) supplemented with 10% heat-inactivated bovine fetal serum, antibiotics, and 2 mM glutamine in a Belco roller device (Ace Glass, Vineland, NJ, U.S.A.) at 22 °C. For biochemical experiments, parasites were harvested at late exponential phase and washed twice with Hanks medium [26] to remove remains of serum components.

Peptides

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH₂) was from Sigma (St. Louis, MO), and devoid of contaminant phospholipase A₂ activity. Cecropin A (KWKLFFKKIEKVGQ-NIRDGIKAGPAVAVVGQATQIAK-NH₂) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). CA(1-8)M(1-18) (KWKLFFKKIGIGAVLKVLTTGLPALIS-NH₂) and its D-enantiomer (D-CA(1-8)M(1-18)) were synthesized by solid phase methods using Boc chemistry on *p*-methylbenzhydrylamine resin in a model 430A synthesizer (Applied Biosystems, Foster City, CA, U.S.A.) as described [24]. The *N*-acetyl analogue [NAc-CA(1-8)M(1-18)] was prepared by acetylation with acetic anhydride of the N-terminal-deprotected peptide resin. After HF cleavage the peptides were isolated and purified by C₁₈ reverse-phase chromatography and satisfactorily characterized for purity and identity by HPLC, amino acid analysis and MALDI-TOF mass spectrometry.

Assay of peptide activity on *Leishmania*

Standard assay of peptide activity was carried out by modification of the procedure described by Kinderlen and Kaye [27]. Parasites (2×10^7 promastigotes/ml, final concentration) were incubated with peptide at 25 °C for 15 min in Hanks medium [29] pH 7.0 supplemented with 1% (w/v) D-glucose (100 μ l, final volume); further activity of the peptide was stopped by addition of 1.5 ml of the same medium, and parasites were centrifuged in an Eppendorf centrifuge for 1 min at 4 °C, resuspended and washed twice in the same volume of Hanks, and finally in 100 μ l of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution in the same buffer (final concentration 0.5 mg/ml) and incubated in a 96-well culture microplate for 2 h at 25 °C. Precipitated formazan was solubilized by addition of an equal volume of 10% (w/v) SDS solution and absorbance was determined using a model 450 Bio-Rad Microplate reader equipped with a 600 nm filter. Minor variations in the incubation conditions of certain experiments are indicated when applicable.

Intracytoplasmic pH measurements

The pH of *Leishmania* promastigotes was measured after loading the parasites with BCECF-AM, as described [28]. Briefly, parasites (2×10^9 promastigotes/ml) were loaded with 6 μ M BCECF-

AM, pH 7.4, at 25 °C for 30 min in buffer A (136 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM D-glucose and 10 mM Tris/succinate, pH 7.0); an aliquot of this suspension was diluted with the same buffer, previously adjusted at different pHs (5.5–8.0) with succinate, to a final concentration of 2×10^7 promastigotes/ml. Fluorescence was recorded at 26 °C in an LS-50 Perkin–Elmer fluorimeter (excitation and emission wavelengths, 500 and 520 nm, respectively) with a slit width of 5 nm. A standard curve at different pHs was obtained by permeabilization of the BCECF-loaded promastigotes with either 10 μ M digitonin or 0.1% (w/v) Triton X-100 (final concentration).

Membrane potential

Membrane potential was estimated by the potential-sensitive dye bisoxonol, as described [29]. Parasites were resuspended in Cl⁻ medium at a final density of 2×10^7 parasites/ml (137 mM NaCl, 4 mM KCl, 1.5 mM KH₂PO₄, 8.5 mM Na₂HPO₄, 20 mM Hepes, 11 mM glucose, 1 mM CaCl₂, 0.8 mM MgSO₄, pH 7.4), or in *N*-methylglucamine-Cl (NMGCl) media (140 mM NMGCl, 11 mM glucose, 0.8 mM MgCl₂, 1 mM CaCl₂ and 10 mM Hepes, pH 7.4) for calibration. Bisoxonol was added from a 500 \times stock solution in DMSO (0.2 μ M, final concentration). Fluorescence (excitation 540 nm, emission 580 nm, 10 nm slit width both) were recorded continuously in a LS-50 Perkin–Elmer fluorimeter. Calibration was made at the same parasite density but with parasites resuspended in NMGCl medium, by successive additions of a KCl solution in the presence of 0.8 μ M gramicidin D. Potential values were calculated according to the Nernst equation assuming a 120 mM constant internal K⁺ concentration [29].

L-[³H]proline uptake

After standard peptide treatment, parasites were resuspended in PBS-Glc medium with 0.1 mM L-proline (specific radioactivity 100 Ci/mol, Amersham, Little Chalfont, Bucks., U.K.) and 25 μ M cycloheximide as described [25,30]. Proline incorporation in the presence of 1 μ M valinomycin was taken as negative control.

Respiratory activity

Oxygen consumption rate was determined as described [31], using a Clark-oxygen electrode (Hansatech, Norfolk, U.K.) thermostated at 25 °C. Promastigotes were washed twice in Hanks' medium and resuspended in respiration medium (125 mM sucrose, 65 mM KCl, 1 mM MgCl₂, 2.5 mM KH₂PO₄, 0.33 mM EGTA and 10 mM Tris/HCl pH 7.2) at a density of 2.5×10^9 parasites/ml and kept at 4 °C. Measurements were performed at a final parasite concentration of 10^8 parasites/ml after dilution in the same medium previously equilibrated with air.

Rhodamine 123 mitochondrial staining

Variation in the mitochondrial potential by peptide treatment was measured by Rhodamine 123 incorporation, as previously described [32]. Prior to the standard peptide assay, parasites were equilibrated with Rhodamine 123 (final concentration 0.3 μ g/ml) for 5 min at 32 °C, washed twice and resuspended in PBS-Glc medium. Dye incorporation was estimated in an EPICS-XL cytofluorimeter, with excitation at 488 nm and emission at 525 nm. Fluorescence measured in parasites incubated with 7.5 μ M FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was taken as negative control.

Measurements of ATP content

ATP content was measured by the luciferin–luciferase method [33]. After standard treatment of the parasites with CA(1–8)M(1–18), aliquots were taken at different intervals; ATP was extracted with HCl and measured on a LKB Bio-Orbit 1250 luminometer calibrated with standard ATP concentrations.

Electron microscopy

After peptide treatment, parasites were washed twice in PBS, fixed in 5% (w/v) glutaraldehyde in PBS, included with osmium tetroxide at 2.5% (w/v) for 1 h, gradually dehydrated in ethanol [30, 50, 70, 90 and 100% (v/v); 30 min each], and propylene oxide (1 h), embedded in Epon 812 resin, and observed in a Philips 2200 electron microscope.

Other methods

Hydrophobicity was calculated according to the normalized scale from Eisenberg [34]. IC_{50} (concentration of peptide required to inhibit half of the maximum MTT reduction) and its confidence limits (95% C.I.) were determined by the Litchfield and Wilcoxon procedure using the PHARM/PCS Version 4 software package for PCs. Number of experiments analysed is indicated in the legend of each figure. Statistical significance (* $P < 0.05$; ** $P < 0.01$) between mean values was analysed by Student's *t* test using SigmaPlot software version 2.0.

RESULTS

Comparison of different cecropin A–melittin hybrids on *Leishmania* killing

In Table 1, the peptide lethal concentration found against *Leishmania donovani* promastigotes are summarized and compared with those against other cell types. They differ slightly from those published previously [25]. The hybrid peptide CA(1–8)M(1–18) was much more active on *Leishmania* than cecropin A, which required concentrations higher than 50 μ M to induce only a poor killing effect [25]. CA(1–8)M(1–18) was less active than melittin, although the general cytolytic activity of this latter peptide is considered an obstacle for potential therapeutic use. The all-D-CA(1–8)M(1–18) hybrid showed higher leishmanicidal activity than its normal L-enantiomer; the LD_{50} ratio

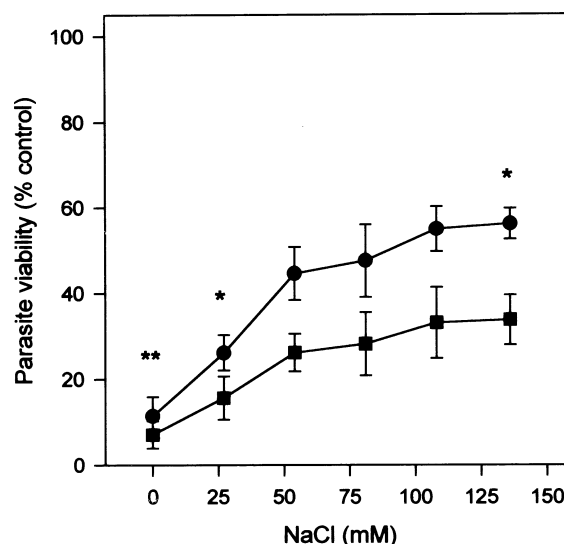


Figure 1 Inhibition of CA(1–8)M(1–18) lethal activity on *L. donovani* promastigotes by increasing ionic strength

CA(1–8)M(1–18): 1 μ M (solid square); 2 μ M (solid circle). Isosmolarity of the medium was maintained constant by supplementation with sorbitol. Data represent the mean of triplicate samples \pm S.D. from a single experiment, representative of three separate experiments.

between D and L forms was approx. 3 (Table 1), slightly higher than the average found on bacteria [35]. Loss of a positive charge, as in the *N*-acetyl analogue, decreased the leishmanicidal activity of CA(1–8)M(1–18) by half (Table 1).

Factors involved in peptide–parasite interaction

To gain insight on the mechanism of action of CA(1–8)M(1–18) on *Leishmania* promastigotes, different modified assay conditions were used. At 1 μ M, CA(1–8)M(1–18) reached $95 \pm 5.6\%$ of its lethal activity in the first 2 min; the effect on parasite viability was irreversible, as washed parasites did not recover after 48 h (data not shown).

Ionic strength has been shown to be an important factor for the interaction of cationic peptides, such as defensins [12] or

Table 1 Primary structures, antibiotic activities and physical properties of the peptides used in this study

L. donovani promastigotes were incubated with the corresponding peptide concentrations, and viability evaluated by MTT reduction as detailed in the Materials and methods section. N.D., not determined.

Peptide	Lethal concentration (μ M)				Physical parameters		
	<i>L. donovani</i> *	SRC†	<i>E. coli</i> ‡	<i>S. aureus</i> ‡	α -helix§	<i>H</i>	$\langle \mu \rangle$ ¶
CA(1–8)M(1–18)	1.3 (0.77–2.08)	> 600	0.3	1.0	42	0.067	0.197
D-CA(1–8)M(1–18)	0.4 (0.32–0.45)	> 400	0.3	0.3	46	0.067	0.197
N-Ac-CA(1–8)M(1–18)	2.9 (1.26–6.72)	N.D.	N.D.	N.D.	N.D.	0.072	0.178
Cecropin A	> 50	> 200	0.3	> 300	25	–0.056	0.316
Melittin	0.3 (0.17–0.60)	4	0.8	0.2	75	–0.14	0.342

* Values expressed as LD_{50} calculated against promastigotes [25]. 95% confidence limits determined by the Litchfield and Wilcoxon procedure are included in parentheses.

† SRC, Sheep red cells. Data taken from [23,24].

‡ Taken from [23,24].

§ Percentage of α -helix in 12% hexafluoroisopropanol. Taken from [35].

|| Hydrophobicity per residue calculated according to normalized scale from Eisenberg [34].

¶ $\langle \mu \rangle$, Hydrophobic moment. Taken from [56].

Table 2 Inhibition of CA(1–8)M(1–18) leishmanicidal activity by polyanions

L. donovani promastigotes (2×10^7 parasites/ml) were incubated with $2 \mu\text{M}$ CA(1–8)M(1–18) in standard assay conditions in presence of either $200 \mu\text{g/ml}$ heparin or $300 \mu\text{g/ml}$ phosphoglycan under different orders of addition to the incubation medium. Parasite viability was evaluated by MTT reduction. Data represent the mean of triplicate samples \pm S.D. from a single experiment, representative of three separate experiments.

Assay conditions	Parasite viability (% of control)	
	Heparin	Phosphoglycan
Peptide activity (without polyanion)	20.1 ± 3.9	24.5 ± 2.2
Simultaneous addition (peptide + polyanion)	63.1 ± 4.2	36.2 ± 3.6
Previous incubation (peptide + polyanion)*	93.7 ± 4.9	67.9 ± 4.9
Previous incubation (parasite + polyanion)†	90.2 ± 4.7	58.4 ± 4.0

* Peptide and polyanion were mixed and incubated together for 15 min prior to the addition to the parasites.
† Parasites were incubated with polyanion for 15 min, then washed and peptide added.

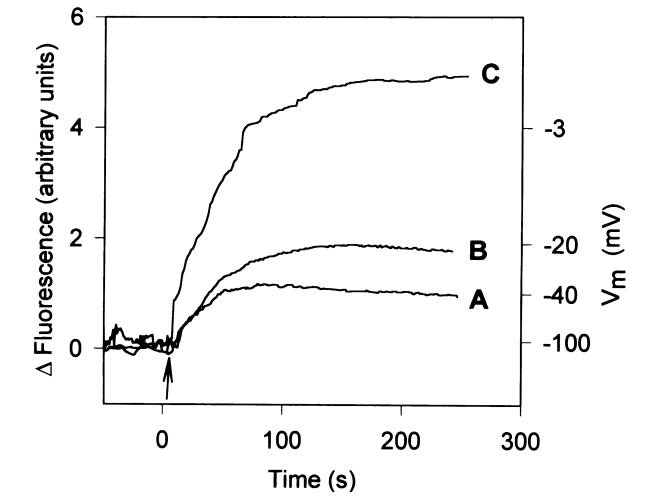


Figure 2 Collapse of plasma membrane potential by CA(1–8)M(1–18)

Parasites (2×10^7 promastigotes/ml) were equilibrated with $2 \mu\text{M}$ bisoxonol at 25°C . Fluorescence was recorded continuously (540 nm excitation, 580 nm emission), and background was subtracted from the values. Peptide addition is indicated by the arrow. Traces for CA(1–8)M(1–18): $0.5 \mu\text{M}$ (A); $1.0 \mu\text{M}$ (B); $2 \mu\text{M}$ (C). Potential values are calculated by the Nernst equation after calibration. Traces are representative of three separate experiments.

bactenecins [36] with acidic phospholipids, by quenching of their electrostatic interaction. In *Leishmania*, reduction of the ionic strength of the medium, while preserving isosmolarity by sorbitol, decreased parasite viability to a considerable extent (Figure 1). Heparin and PG, two polyanionic polysaccharides, inhibited the interaction of CA(1–8)M(1–18) with the parasite membrane (Table 2), quite likely by formation of a complex with charge neutralization [13,37]. The inhibitory effect was higher for heparin than for PG; the inhibition increased when interaction between peptide and polysaccharide was facilitated by preincubation prior to addition to the parasite suspension; in this case the effect was more pronounced for PG than for heparin. A similar effect was observed when parasite and polyanion were preincubated prior to peptide addition.

In contrast with defensins [6] or cecropin A [38] which required a pre-existing membrane potential to permeabilize biological

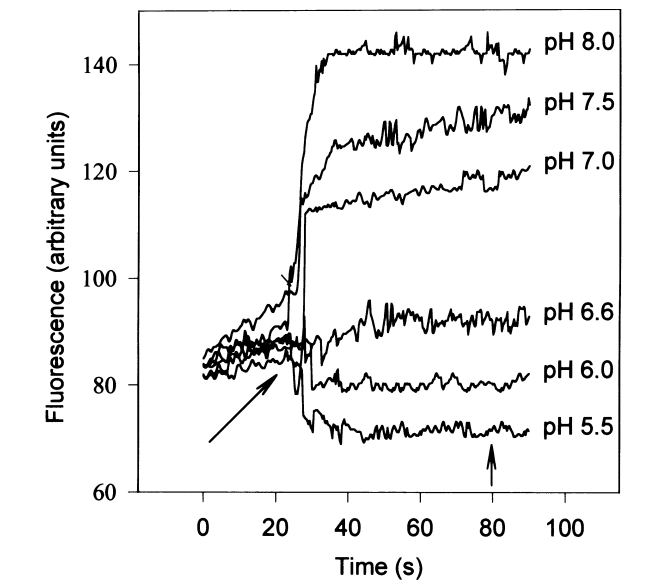


Figure 3 Equilibration between internal pH of *L. donovani* promastigotes with different external pHs after addition of $2.5 \mu\text{M}$ CA(1–8)M(1–18)

pH changes were monitored by the pH-dependent changes of BCECF loaded previously into the parasites as explained in the Materials and methods section. Traces at different external pHs are marked. Peptide addition is indicated by the first arrow. Final equilibration of both pHs is obtained after addition of digitonin ($10 \mu\text{M}$, final concentration) to parasite suspension (second arrow).

membranes, collapse of the promastigote membrane potential, induced by previous treatment with a sublethal dose of valinomycin only produced a partial reduction on promastigote susceptibility towards CA(1–8)M(1–18) [25].

Other parameters such as pH (range 5.0–8.0), calcium ions ($0.1\text{--}10 \text{ mM}$), EDTA (2 mM) or BSA ($1\text{--}100 \mu\text{g/ml}$) shown to be important for other antibiotic peptides such as defensins [12], counted in our case less than 10% variation in the peptide activity (data not shown). At 4°C , lethal effect is inhibited by $85 \pm 6.5\%$ and activity is maximal for temperatures in the $20\text{--}30^\circ\text{C}$ range.

Plasma membrane of the promastigotes is the direct target of the peptide

Since cecropin A, melittin and their analogues are able to permeabilize model and biological membranes, the effect of the peptide on the plasma membrane of the parasite was next investigated. Cecropin A permeabilizes planar model membranes to Na^+ and Cl^- , leading to a decrease in membrane potential [38]. To assess this effect on promastigotes, *Leishmania* membrane potential was measured after CA(1–8)M(1–18) addition by bisoxonol fluorescence. Collapse of membrane potential was reached at concentrations similar to lethal values (Figure 2). This result was confirmed by inhibition of proline uptake, a process directly linked to the protonmotive force [28], which was inhibited by $67.2 \pm 5.4\%$ at $1 \mu\text{M}$ peptide.

Leishmania maintains a strict control of the internal pH at 6.8 over an extensive pH range [28]. As measured by BCECF fluorescence loaded into the parasite, the addition of $2.5 \mu\text{M}$ CA(1–8)M(1–18) produced a fast equilibration between internal and external pH, suggesting H^+/OH^- permeabilization across the membrane (Figure 3).

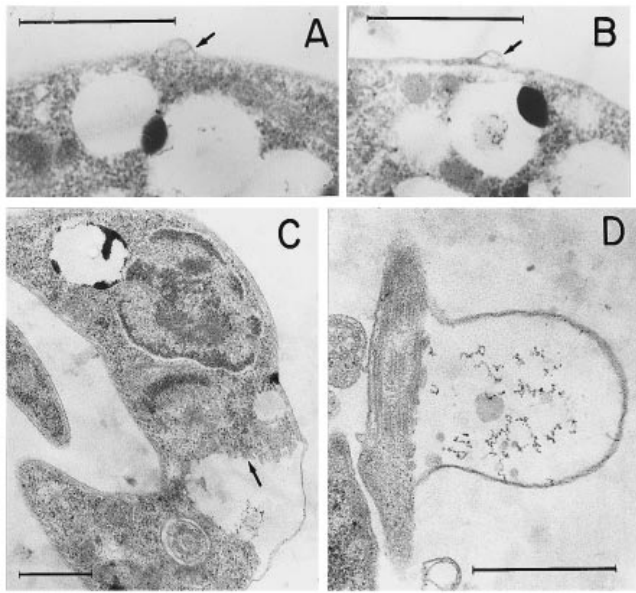


Figure 4 Electron microscopy of *L. donovani* promastigotes treated with different concentrations of CA(1–8)M(1–18)

(A, B) Blisters (arrow) and empty spaces appear after standard treatment with $0.5 \mu\text{M}$. At higher concentration ($2.5 \mu\text{M}$), detachment of plasma membrane from subpellicular layer of microtubules (C) or flagella (D) is observed. Bar magnification, $0.5 \mu\text{M}$.

Damage to plasma membrane was observed after peptide treatment; at low concentrations ($0.5 \mu\text{M}$) blisters and membrane blebs appeared as depicted in Figure 4(A, B). At higher concentration ($2.5 \mu\text{M}$), detachment of large areas of membranes from the subpellicular layer of microtubules and from the flagella (Figure 4C, D, respectively), as well as condensation of cytoplasmic areas inside the parasite, were spotted (Figure 4B). The degree of peptide damage in a population was irregular, indicating either non-homogeneous distribution due to initial peptide aggregation, or kinetic, rather than thermodynamic control of peptide binding to the membrane, as observed in other experimental models with this peptide [21,22].

Bioenergetic collapse of the parasite is a consequence of plasma membrane permeabilization

CA(1–8)M(1–18) inhibited *Leishmania* respiration in a dose-dependent manner (Table 3); the basal rate of oxygen consumption ($24.5 \text{ nmol}/10^8$ parasites) was inhibited by 70 % at $10 \mu\text{M}$. The higher (relative to IC_{50}) peptide concentration necessary for inhibition was due to the higher parasite density required for this technique and to the stoichiometrical mode of action of these peptides [39]. Intracellular ATP level dropped very fast after peptide addition (Figure 5). To test mitochondrial integrity, measurement of its membrane potential *in vivo* was performed. Figure 6 shows the maintenance of mitochondrial potential as measured by retention of Rhodamine 123. Even when there was a dose-dependent increase in the population of depolarized cells, its level did not reach the percentage of killed promastigotes at the corresponding peptide concentration; nevertheless, further damage subsequent to loss of cellular viability cannot be excluded. Cell depolarization is an all-or-none process, as no intermediate population between normal

Table 3 Inhibition of respiration in *Leishmania donovani* promastigotes by CA(1–8)M(1–18)

Oxygen consumption rates of a parasite suspension (10^8 parasites/ml) were measured in a Clark oxygen electrode. Experiments were expressed as the mean of three different experiments \pm S.D.

CA(1–8)M(1–18) (μM)	Oxygen consumption rate [nmol atomic oxygen (O)/mg protein \times min]
0	49 ± 2.4
0.25	36 ± 3.2
1	31 ± 2.7
2	26 ± 1.6
5	18 ± 2.1
10	13 ± 1.9

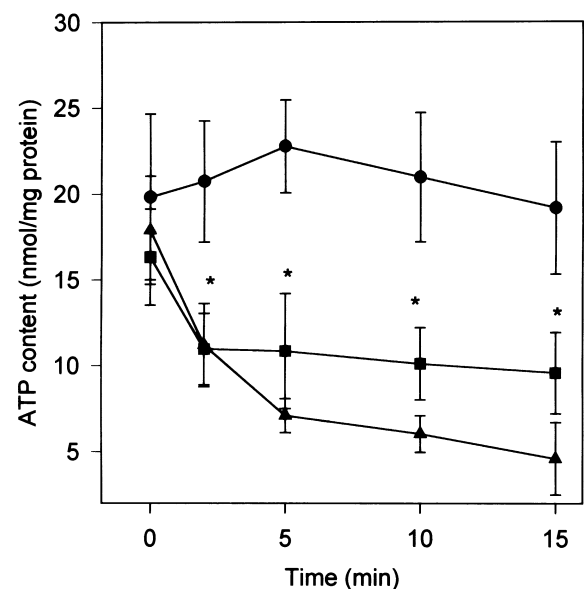


Figure 5 Decrease of ATP levels of *L. donovani* promastigotes after CA(1–8)M(1–18) addition

Promastigotes (20×10^6 parasites/ml) in Hanks medium supplemented with 10 mM D-glucose were treated with the peptide and aliquots of 1 ml were extracted at different times as indicated. ATP was extracted with HCl and measured by luciferin–luciferase method. Control parasites (solid circles). CA(1–8)M(1–18): $1 \mu\text{M}$ (solid squares); $5 \mu\text{M}$ (solid triangles). Data represent the mean for triplicate samples \pm S.D.

cells and the negative control in the presence of the uncoupling agent FCCP was observed.

DISCUSSION

Many animal antibiotic peptides act on the plasma membrane of pathogens, increasing their permeability to ions and other metabolites. Most studies on peptide–membrane interaction have been performed either on model membranes or on prokaryotes [7]. There is growing evidence of the activity of some animal peptides on specific eukaryotic cell types, but the molecular aspects of specific cell recognition by these peptides and their mechanism of action are not well understood.

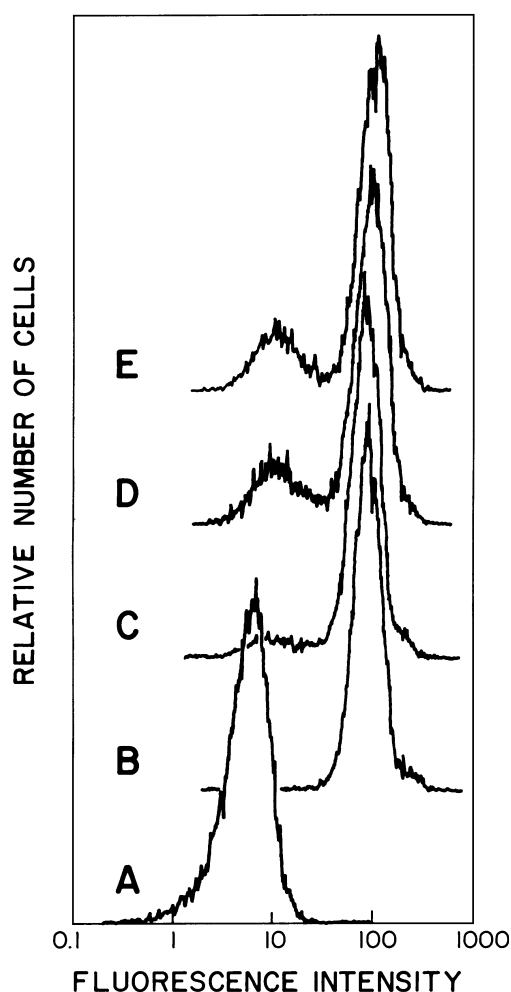


Figure 6 Mitochondrial membrane potential of *L. donovani* measured by Rhodamine 123 fluorescence after CA(1–8)M(1–18) addition

Parasites were loaded with rhodamine 123 as described in Methods and dye accumulation was measured by cytofluorimetry. (A) Parasites incubated with $7.5 \mu\text{M}$ FCCP. (B) Control parasites. (C–E) 0.5 , 2.5 , $5 \mu\text{M}$ CA(1–8)M(1–18), respectively.

The leishmanicidal activity of antibiotic peptides such as dermaseptin and SPYY has been described [40,41] but limited to observations such as inhibition of proliferation or morphological damage [40]. Definition of parameters involved in *Leishmania*–peptide interaction is needed to design anti-*Leishmania* peptides for potential pharmaceutical use or biological control of parasites, as recently described for *Trypanosoma cruzi* [42].

The activity of CA(1–8)M(1–18) on bacterial cells is confined to the plasma membrane and associated with acquisition of an amphipathic α -helix structure. This is confirmed by the lack of a chiral target, as evidenced by the higher activity of the all-D-enantiomer [35]. Among different proteins recognizing amphipathic α -helix peptide motifs such as ATPases, protein kinase C, phospholipase A_2 , G proteins or calmodulin [43], only the latter is able to interact with both melittin enantiomers [44]. However, *Leishmania* killing kinetics is too fast to be compatible with intracellular transport and perturbation of metabolism or signal transduction. In tune with this, the *Leishmania* plasma membrane is again the direct target of CA(1–8)M(1–18) with no involvement of other internal organelles. This mechanism

resembles that reported for bacteria, and differs from other animal antibiotic peptide–eukaryotic cell interactions, e.g. the slow killing process of tumoural cells by defensins that require an additional endocytosis step after membrane permeabilization [14]. Uncoupling of mitochondria and loss of its membrane potential on some eukaryotic cells has been described *in vivo* for magainins [11]. CA(1–8)M(1–18) acts also as an *in vitro* uncoupling agent in both plant [45] and rat liver mitochondria [22], but in *Leishmania* it produces the opposite effect, i.e. an impairment of the oxygen consumption rate. Furthermore, the majority of the promastigote population maintains a normal mitochondrial membrane potential *in vivo*, even at concentrations higher than lethal values. Accordingly, mitochondrial damage is the effect, but not the source, of irreversible deterioration of the organism after loss of internal homeostasis. Disruption of cytoskeleton is a requisite for the preferential killing of tumoural cells by cecropin-like peptides such as SB-37 and Shiva-1, which synergize with microtubule and microfilament depolymerizing drugs [14]. In *Leishmania*, despite a very stable cytoskeleton whose subpellicular and flagellar microtubules persist even after lysis by the peptide, damage to the plasma membrane by CA(1–8)M(1–18) is evident, as shown by electron micrographs. Blebbings induced by the peptide, with apparent expansion and segregation from the submembranal structures, are similar to those described for dermaseptin on *L. mexicana* [40] and for other cationic peptides on the outer membrane of Gram-negative bacteria, either by expansion due to massive peptide insertion [46], or by binding to cellular components released after bacterial death [47]. For *Leishmania*, the origin of membrane enlargement remains unexplained; though it is conceivable that constraints on membrane traffic and attachment of the plasma membrane to the very stable subpellicular layer of microtubules could be implied in the expansion process, hampering an initial accommodation of the outsized membrane, or modification of the hydrogen bonds among lipid components.

A fast equilibration between internal and external pH follows peptide addition, disrupting the internal pH homeostasis maintained along a broad range of external pHs [28]. This is accompanied by leakage of other ions across the membrane, with dissipation of membrane potential and, thereby, inhibition of proline active transport. The end result is the energetic collapse of the organism, with waste of ATP in a fruitless attempt to recover lost ionic gradients.

Leakage across the plasma membrane of the promastigote by CA(1–8)M(1–18) is not restricted to H^+/OH^- equilibration, as trichloroacetic acid-precipitable material absorbing at 280 nm increases its concentration in the external medium (data not shown). It has been previously shown in other biological models that this peptide can induce permeability not only to ions, but also to larger solutes such as 20 kDa fluoresceinated dextran trapped in liposomes [21] or sucrose and NADH in rat liver mitochondria [22]. These results fit better with a mechanism of destabilization of the lipid bilayer (reviewed in [48]), than with the formation of discrete and stoichiometric pores, as proposed for cecropins in planar membranes [38]. Furthermore, liposome permeability and bacteria killing are achieved by cecropin A–melittin analogues only 15 residues long, unable to cross the membrane as a monomer in an α -helix conformation [24]. Still, a mixed mechanism cannot be entirely ruled out; for instance, melittin interacts with membranes by different mechanisms, depending on its concentration [49].

A further aspect to be investigated is the role that the electrostatic interaction between the peptide and the membrane plays in the permeabilization process. For cryptidins [50] and cecropins [51], a direct correlation between cationic character

and antimicrobial activity has been established. In the present case, loss of one positive charge in the *N*-acetyl-CA(1–8)M(1–18) produced a sharp decrease (50%) in leishmanicidal activity. This is similar to previous findings for cecropin A on several bacterial cells [52]. On the other hand no obvious correlation could be found for either α -helix content, hydrophobicity or hydrophobic moment (Table 1) and biological activity, in contrast to observations with other peptides [53].

A membrane potential is essential to induce membrane permeabilization by defensins [13] and cecropins [35], although this last case is disputed [39]. CA(1–8)M(1–18) permeabilizes liposomes in the absence of potential [21], as well as depolarized mitochondria [22] or parasites [25]. For *Leishmania*, membrane potential is around -120 mV [28] and its abrogation by valinomycin impairs, but does not preclude, membrane permeabilization [25]. Membrane potential favours a deeper penetration of cationic peptides in the lipid bilayer, and for peptides with a hinge region, such as cecropins and melittin, it can drive the cationic region of the peptide across the membrane to form a channel [38,48]. CA(1–8)M(1–18) displays an intermediate situation, as the melittin hinge sequence -GLPA- is maintained in the peptide but much closer to the C-terminal end than in parental peptide.

Aside from lipid composition, other constituents of the membrane can play a role in membrane–peptide interaction. The main non-lipid components of the promastigote plasma membrane are the metalloproteinase Gp63 and the anionic oligosaccharide LPG. Given the abundance and proteolytic specificity of Gp63 [17], the relatively similar activities between proteinase susceptible (L-) and proteinase-resistant (D-) versions of CA(1–8)M(1–18) is quite remarkable. Presumably, the time required for the peptide to deliver the lethal hit to the cell would be short enough to allow only a small percentage of peptide inactivation by the proteinase. The inhibition of the lethal peptide activity by LPG is likely to be due to the formation of an anionic glycocalyx around the parasite [18]. The mutant strain R2D2, with much lower anionic character [39], is more susceptible to CA(1–8)M(1–18) than the wild strain ($46 \pm 6.2\%$ vs. $75 \pm 4.5\%$ viability respectively, at $0.5 \mu\text{M}$ peptide concentration). LPG prevents to some extent the peptide from reaching the membrane; a similar result is obtained with melittin and analogously in erythrocytes, where enzymatic removal of sialic acid residues increases haemolysis [54]. Heparin inhibition is higher than with PG, in correlation with their respective negative charge densities. Formation of peptide–polyanion or parasite–polyanion complexes, prior to the addition to parasite or peptide, respectively, increases *Leishmania* survival. Other LPG activities, such as the stabilizing effect of LPG on membranes [55], could as well restrain peptide insertion. The higher inhibition achieved by heparin suggests a possible role of the extracellular matrix of eukaryotic cells, a structure rich in sulphated oligosaccharides, as a self-protective factor in the activity of animal antibiotic peptides.

Strong differences in parasite–peptide interaction [25] have been observed for the vertebrate-infecting form of the parasite, the amastigote, which has a much lower content in Gp63, localized in lysosomes instead of the plasma membrane, and with 10^4 -fold down-regulation of LPG relative to the promastigote level. Surprisingly, this form is far more resistant to the peptide than promastigote. The molecular basis responsible for this different susceptibility of the amastigote is presently under investigation.

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