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Magainin 2 amide and analogues

Antimicrobial activity, membrane depolarization and susceptibility to proteolysis

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We compared the abilities of synthetic magainin 2 amide and its analogues to inhibit the growth of *Escherichia coli* and to cause membrane depolarization in *E. coli* cells and cytochrome oxidase liposomes. The analogue, magainin A, was about 40-times more active than magainin 2 amide in inhibiting the growth of *E. coli* and had a much more sustained effect on the membrane potential. In the liposomal system, however, there was only approx. 20% difference between these two peptides in the reduction of membrane potential and uncoupling of respiration. Studies with pronase digestion suggested that the difference in potency may be due to differential susceptibility to proteolysis in the presence of membranes.

Magainin; Proteolysis; Membrane potential; Respiration; Cytochrome oxidase liposome; Uncoupling; (*E. coli*)

1. INTRODUCTION

The magainin [1,2] peptides (also designated PGS [3]), isolated from the skin of *Xenopus laevis*, exhibit broad-spectrum antimicrobial activity. More potent analogues of the 23-residue magainin 2 peptide were obtained by substitutions of alanine for glycine or serine, and an amidation at the C-terminus, which resulted in enhanced α-helical structure and antimicrobial activity [4]. Although natural magainin 2 has a free carboxyl-terminus [1–3], the amidated form has been used for many studies [5–9].

We have proposed that dissipation of membrane potential is the likely mechanism of action for magainins [6]. Magainins can induce anion-selective ion channels in black lipid membranes [7]. In mitochondria [8] and cytochrome oxidase liposomes [9], magainins decreased the membrane potential and released respiratory control. Subsequent proteolysis of the peptides reversed these effects [8,9].

Here, we compare representative magainin analogues with magainin 2 amide with respect to their action on cytochrome oxidase vesicles and *Escherichia coli* cells, as well as their susceptibility to proteolytic inactivation. Our results suggest that especially in the presence of membranes, the latter may be a factor of importance in the determination of the relative antimicrobial activities of the peptides.

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2. MATERIALS AND METHODS

Magainin 2 amide was synthesized by Dr J. Rivier under contract of the Contraceptive Development Branch, NICHD, NIH,

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Bethesda, and provided by Dr M. Zasloff. The magainin 2 amide analogues: [βAla[1],Ala[8,13,18]] (denoted Mag A), [βAla[1],Ala[13,18]] (Mag G) and [D-Ala[9,13,18]] (Mag H) were those described previously [4]. The purity of all peptides was greater than 93% as judged by HPLC monitored at 215 nm.

Pure cytochrome oxidase (10 nmol heme A/mg protein) was provided by Dr W. Caughey. Asolectin obtained from Fluka was further purified [10]. Cytochrome oxidase liposomes were prepared at a protein/lipid ratio of 1:50 (w/w) [11] and exhibited more than 10-fold respiratory stimulation by 0.56 μM valinomycin plus 0.56 μM FCCP. Pronase, valinomycin and FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone) were obtained from Fluka, TMPD (tetramethyl-*p*-phenylenediamine) from Eastman-Kodak, and DCC (*N,N'*-dicyclohexylcarbodiimide) from Sigma.

E. coli D31, grown overnight in Luria broth (LB), was diluted to a cell density of 0.6×10^8 cells/ml. The incubation at 37°C was continued in 5 ml LB in 12-ml glass tubes shaken at 300 rpm. The cell density was monitored using a Klett densitometer. When cell density attained a value of 10^8 cells/ml, various concentrations of peptide were added, the incubation was continued and growth rates were determined by monitoring the absorbance in the Klett densitometer for up to 24 h.

To measure membrane potential changes, cells grown as described above were harvested in the stationary phase, washed twice in ice-cold Tris buffer (0.12 M, pH 8), resuspended in the same buffer at 37°C, and after 1 min, EDTA was added to 1 mM [12]. At the end of the incubation period in EDTA (45 min at 37°C), cells were washed once and resuspended to an A_{600} of 30 in ice-cold medium D (250 mM sucrose, 2 mM Hepes, 0.5 mM EGTA; pH 7.4). Cells (0.4 ml) were added to 7 ml medium D in the presence of 40 μM tetraphenylphosphonium ion (TPP^+) and a TPP^+-selective electrode modified by one of us (R.W.H. from previous design [13]). A mixture of O_2 (12 ml/min) and Ar (70 ml/min) was passed over the surface of the stirred suspension at 25°C. Sequential addition of disodium succinate (1.4 mM), DCC (65 μM) and peptides (stepwise at 6.7 μg/ml [first aliquot] and 13.5 μg/ml [subsequent aliquots]) were made, ultimately followed by valinomycin (0.14 μM) and nigericin (0.07 μM). Electrode artifacts of peptide addition were controlled by adding peptides in the absence of energized membranes and by checking that the membrane depolarizing effect of peptides was reflected in a reduced response of the electrode to anaerobic-aerobic transitions.

In cytochrome oxidase liposomes, background O_2 depletion rates (J_B) were measured polarographically [8,9] at 25°C in 1.8 ml of 50 mM potassium phosphate (pH 7.4), Na-EDTA (0.56 mM), sodium ascorbate (5 mM), TMPD (0.083 mM), and cytochrome *c* (0.035 mM). Liposomes were then added (0.19 mg/ml phospholipid in the oxygraph) and the coupled respiratory rate recorded (J_V) after 2–3 min. Respiratory rate (J_P) was again measured 1 min after the addition of each aliquot of peptide. Maximal rate (J_M) was reached during each titration when further additions of peptides or valinomycin and FCCP inhibited respiration [9]. The relative rate was then calculated as $\nu_1 = (J_P - J_B)/(J_V - J_B)$.

For titrations in the presence of pronase (0.056 mg/ml), the liposomal concentration was 0.17 mg/ml and each peptide was added (0.028 mg/ml) only twice, i.e. after pronase but before liposomes, and then again after liposomes. The decrease from the maximal (initial) respiratory rate after the second addition of peptide was calculated as the fraction remaining of the initial stimulation of respiration by the peptide.

3. RESULTS

3.1. *Relative antimicrobial activities*

Previously, the antimicrobial activity of Mag A was shown to be much higher than that of magainin 2 [4]. The carboxy-terminus of magainin 2 is not amidated [2], whereas that of Mag A and of all peptides tested in the present paper, is. We investigated whether Mag A was also much more active antimicrobially than magainin 2 amide. For this purpose, various concentrations of peptides were added to exponentially growing *E. coli* cells and light scattering was followed. Magainin 2 amide at 120 μg/ml and magainin A at 3 μg/ml inhibited growth equally. The peptides caused an initial decrease in absorbance followed by resumption of the absorbance increase with the original kinetics delayed by approx. 40 min. Indeed, at all concentrations of added peptide, growth eventually resumed the normal rate. This is in contrast to the effect of an uncoupler, such as 2,4-dinitrophenol, which increased the doubling time of the cell population (not shown).

3.2. *Mag A is more persistent than magainin 2 amide in decreasing the membrane potential of* E. coli *cells*

Fig.1 shows titrations of membrane potential with magainin 2 amide and Mag A. A decrease in membrane potential (inside negative) causes TPP^+ to leave the cells resulting in downward deflection of the traces shown in fig.1. Peptides were added after cells had developed maximal membrane potential in the presence of O_2 and respiratory substrate (succinate). Although both peptides decreased the membrane potential, the time dependence of their effects was rather different. Mag A caused slow but persistent drops in membrane potential, whereas upon addition of magainin 2 amide the membrane potential dropped rapidly initially, but largely recovered subsequently. At much higher cumulative concentrations, magainin 2 amide caused persistent drops in membrane potential.

3.3. *Uncoupling of respiration in cytochrome oxidase liposomes*

We have recently shown [9] that at concentra-

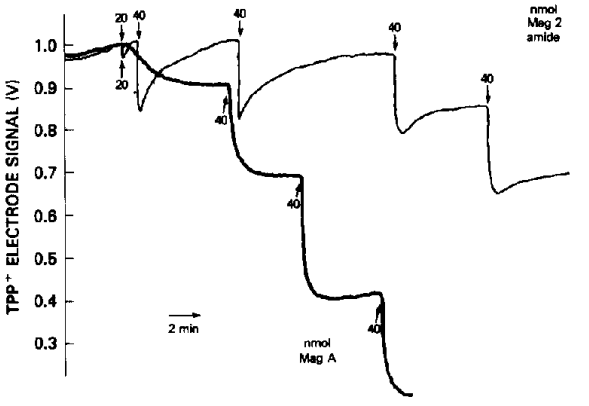

Fig.1. Decrease in apparent membrane potential of *E. coli* cells caused by magainin 2 amide (thin line), and Mag A (thick line). Additions in nmol are indicated.

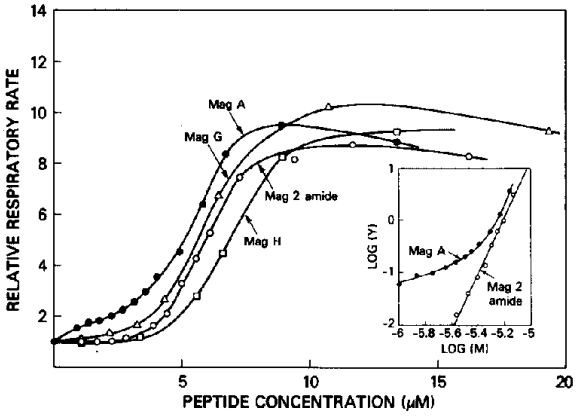

Fig.2. Increase in respiration rate of cytochrome oxidase liposomes due to uncoupling by the magainins: magainin 2 amide (○—○), Mag H (□—□), Mag G (△—△), and Mag A (●—●). (Inset) Double-logarithmic plot of $Y = (J_P - J_V)/(J_M - J_P)$ vs added concentration of peptide (section 2).

tions where it is antimicrobially active, magainin 2 amide uncouples the respiration of cytochrome oxidase liposomes. Fig.2 compares the abilities of various magainin analogues to uncouple respiration. The dose at which the peptides caused half-maximal stimulation of respiration did not differ by more than 20% among them. Mag A was the most potent and Mag H the least active of the four peptides tested. In terms of the effect on membrane potential in these liposomes (not shown), Mag A was approx. 20% more effective than magainin 2 amide. With either peptide the decrease in membrane potential was permanent.

A feature distinguishing the magainins from FCCP is the concentration dependence of their uncoupling activity. In the cases of magainin 2 amide, Mag G, or Mag H, the dependence is sigmoidal, whereas in the case of Mag A a linear concentration dependence at low concentrations precedes a sigmoidal dependence at higher concentrations. The cooperativity number calculated [9] from the data in fig.2 (slopes in the inset) for magainin 2 amide lies around 4–5. Mag A may exhibit the same cooperativity at high concentrations, but at low concentrations, its cooperativity parameter is less than 2. This parameter is indicative of the number of inactive units (monomers or aggregates) needed to form an actively uncoupling complex.

3.4. *Susceptibility to inactivation by pronase*

To study the relative sensitivities of the peptides to proteolytic inactivation, we preincubated pronase with 11 μM peptide for 2 min and then added cytochrome-*c* oxidase liposomes. Under these conditions we observed typical coupled respiratory rates, suggesting that, in the absence of liposomes, all peptides are sensitive to proteolytic cleavage by

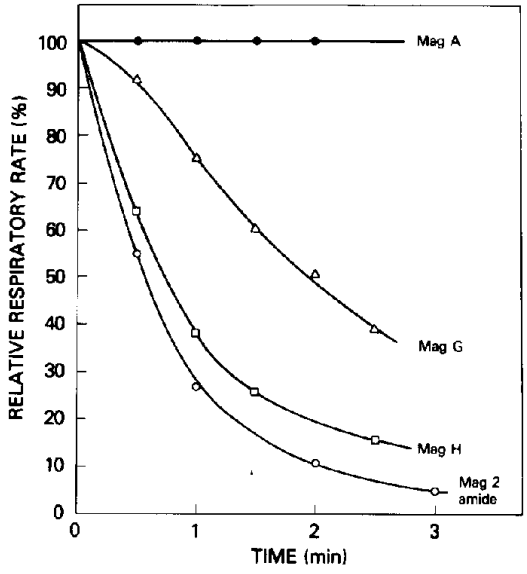

Fig.3. Ability of pronase to reverse the uncoupling activities of various magainins. Symbols as in fig.2. Zero time corresponds to 30 s after the second addition of peptide (section 3.4). For each peptide, the initial increase in relative rate (compared to the coupled rate) was set equal to 100%. The zero level on the *Y*-axis represents the coupled or reference rate of O_2 uptake.

pronase [9]. In contrast, in the presence of liposomes, the various peptides differed strongly in their susceptibility to pronase (fig.3). Pronase was able to reverse the uncoupling caused by magainin 2 amide, and to restore the coupled rate, but reversal was not observed with Mag A (fig.3). In all cases valinomycin plus FCCP remained capable of increasing O_2 consumption to the uncoupled rate (not shown), indicating that the cytochromes and membranes had not been irreversibly affected.

4. DISCUSSION

In this paper we address the question as to whether the vast differences in antimicrobial activity among a number of magainin 2 analogues [4] are due only to differences in their ability to disrupt membrane-mediated energy coupling. With this purpose in mind, we compared the abilities of these peptides to dissipate membrane potential in *E. coli* cells and cytochrome oxidase liposomes.

Because all our results point in the same direction, the explicit comparison between Mag A and magainin 2 amide may suffice. Whereas Mag A was 40-fold more active in inhibiting growth of *E. coli* than magainin 2 amide (section 3.1) and magainin 2 [4], it was at most 20% more active in stimulating respiration in cytochrome oxidase liposomes to half the uncoupled rate (fig.2) and only 20% more effective in decreasing the membrane potential in this system (not shown). These results suggest that the difference in antimicrobial activity of the magainin analogues A, G, H, and 2 amide, is not due simply to a substantial difference in their ability to permeabilize membranes. What then accounts for the differential antimicrobial activity?

The answer was suggested by the different time dependences of the effects of the peptides on the membrane potential in *E. coli*. At identical concentrations, both Mag A and magainin 2 amide [6] decreased the membrane potential of *E. coli* cells, but did this with different kinetics. Mag A resulted in the persistent decrease in membrane potential also caused by all peptides in cytochrome oxidase liposomes. Magainin 2 amide also caused an initial decrease in membrane potential, however, this was quickly followed by recovery of the potential, as if magainin were being degraded. The fact that this recovery of membrane potential was observed in *E. coli*, which contains various proteolytic enzymes [14], but not in cytochrome oxidase liposomes, suggested that proteolysis could be the cause of inactivation of magainins in the former system. The difference in effects between Mag A and magainin 2 amide might be due to the former being appreciably less susceptible to proteolytic degradation. Our finding (not shown) that cell populations treated with magainins ultimately resumed growth at their normal rate is consistent with the possibility that, due to proteolysis, the peptides are only active for a limited period of time.

To test the hypothesis that differences in susceptibility to proteolytic inactivation are, at least in part, responsible for the differences between the antimicrobial activities of magainin 2 amide and its analogues, magainins were added in the presence of pronase. It was observed that, while Mag A activity was protected from pronase during a 3 min incubation period with oxidase liposomes (as shown by undiminished uncoupled respiratory rate in fig.3), magainin 2 amide activity was decreased to near zero level during the same time (i.e. coupled respiration rate was restored by pronase), confirming our hypothesis. Of course, this confirmation does not constitute proof. We cannot rule out the possibility that Mag A and magainin 2 amide differ in their inherent membrane permeabilization properties as well. However, such a difference, if expressed in *E. coli*, would be obscured in cytochrome oxidase liposomes.

It is likely that Mag A partitions in the lipid phase much more readily than magainin 2 amide and is thereby better protected from proteolysis by the pronase. Compared with magainin 2 amide, Mag A has three glycines and one serine replaced with alanine, while Mag G has three glycines replaced with one β-alanine and two alanines. These changes enhanced the formation of an amphiphilic α-helix [4] which may increase the self-aggregation and affinity for membranes, hence decreasing the susceptibility to pronase cleavage.

Addition of pronase during the initial stage of membrane potential titration in oxidase liposomes with magainin 2 amide reversed the initial decrease of membrane potential (not shown). Of course, we cannot expect pronase perfectly to mimic the natural proteolytic defense system of *E. coli*. In-

deed, the reversal was not as fast or as complete as that observed in *E. coli* (fig.1). *E. coli* proteases are apparently more active against magainin 2 amide than pronase in the liposomal system. Our observations suggest that endogenous proteases may play an important role as a protection mechanism available to organisms against magainins. Thus, the propensity to form an amphiphilic α-helix, consequent interaction with membranes and shielding from proteases are important properties in guiding the future design of potent antimicrobial peptides.

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