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Interactions between a new class of eukaryotic antimicrobial agents and isolated rat liver mitochondria

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Members of a newly discovered class of eukaryotic antimicrobial peptides are shown to release respiratory control in isolated rat-liver mitochondria. They also dissipate the membrane potential and inhibit respiration. The uncoupling activity of the peptides decreases with time probably due to the presence of proteases in the mitochondrial preparation. Quinine and Mg^{2+} reduce the activity of the peptides. The nature of the dependence of the respiratory rate on the concentration of added peptides suggests that they are active in a multimeric form, consistent with the formation of a channel across the inner mitochondrial membrane. The channel allows passage of sucrose.

Introduction

Interest in the biological activity of oligopeptides has greatly increased over the past few years [1]. One reason is the appreciation of the role that peptide hormones play in the regulation of metabolism [2]. A second is the identification of oligopeptides as neurotransmitters. A third factor of importance has been the realization that in the structure of many enzymes, several domains can be identified each of which can be identified with part of the function of the enzyme [3]. In some enzymes, these domains consist of only part of a single polypeptide chain.

Two types of oligopeptide segments are of particular interest for the present paper. One is the prepeptide of many polypeptide chains, which directs the transport of the rest of the polypeptide chain to the appropriate cellular compartment [4,5]. The other is the transmembrane oligopeptide segments found in some ion channels and ion pumps [6]. Either type of domain may fold into an amphiphilic α-helix spanning a biological membrane [7,6]. Progress in the methodology of peptide synthesis now allows the design of oligopeptides in view of a specific function and the test of whether the designed peptides do indeed function as anticipated [5,8–13]. This may not only lead to biotechnically interesting compounds, but also to an increased understanding of the mechanism of prepeptide recognition, prepeptide translocation and ion transport.

It has long been known that certain microbes use oligopeptides as defense mechanisms [14]. Only recently has it become clear that vertebrates may also possess active defense mechanisms of this kind, directed against microbes [15–23]. Thus, *Xenopus laevis* secretes a broad spectrum of oligopeptides [24,25], three of which have been recently identified as strong antimicrobial agents [23,26]. Two of these have been called 'magainin-1' and 'magainin-2' [23], whereas a third already carried the name 'PGLa' [25]. We shall call these three peptides and their analogues collectively 'magainins'. At concentrations around 10 μM, these oligopeptides killed *Escherichia coli* [23,26,11]. In the presence of 5% trifluoroethanol, or when present at high concentrations in the presence of lipids, these compounds took on an α-helical conformation, as evidenced by 2D-NMR [27] and Raman [28] and CD [29] spectroscopy. They increased the permeability of a so-called black lipid membrane (which consisted of a quasi bilayer of phosphatidylserine, phosphatidylcholine and decane), through the formation of ion channels, which exhibited some specificity for anions over cations [30]. The latter phenomenon is not surprising in view of the positive charge of magainins of about +4, for a total length of 23 (21 for PGLa) amino acid residues [23].

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These structural and physical properties suggested that the magainins conform to the requirements for a channel-forming membrane-active peptide [2]. In turn, this suggested that the magainins may kill bacteria by permeabilizing their inner membranes. One of the prime effects of such a permeabilization would be a dissipation of the electrochemical potential difference for protons and a disruption of energy coupling in the target cell.

The organization of membrane-linked energy coupling in bacteria and isolated mitochondria is analogous. In mitochondria, however, it is more readily assayed. We, therefore, used rat-liver mitochondria to study effects of magainins on membrane-linked free-energy transduction. In this paper we describe the uncoupling activity that magainins exhibit towards rat-liver mitochondria. We report that the induced permeability is rather unspecific, that the uncoupling agent consists of a multimer of magainin peptides and that its effectiveness may be compromised by proteases. In addition to their uncoupling activity, the magainins also act as inhibitors of uncoupled respiration.

Materials and Methods

Materials. Rat-liver mitochondria were isolated from fasted (overnight), male Sprague-Dawley rats (between 250 and 275 g) following the procedure described by Pederson et al. [31]). The isolation, resuspension and storage medium was ice-cold 'medium B' (0.21 M mannitol, 70 mM sucrose, 0.5 g/l fatty-acid-free bovine serum albumin (BSA; from Calbiochem), 2.1 mM potassium-Hepes, (pH 7.4). During the homogenization of the liver an additional 1.0 mM EGTA was present. Respiratory rates were determined in 'medium C' (0.20 M sucrose, 50 mM KCl, 1.5 mM potassium-Hepes, pH 7.0) or 'medium D' (0.25 M sucrose, 2 mM Hepes, 0.5 mM potassium-EGTA, pH 7.15, at 298 K. In the latter medium, respiratory rates in the absence of peptides were time independent. In medium C respiratory rates tended to dwindle with time.

Magainin-2 amide (H_2N-GlyIleGly LysPheLeu HisSerAla LysLysPhe GlyLysAla PheValGly GluIleMet AsnSer-$CONH_2$; in this paper also referred to as magainin-2 or mag-2) and PGLa (H_2N-GlyMetAla SerLysAla GlyAlaIle AlaGlyLys IleAlaLys ValAlaLeu LysAlaLeu-$CONH_2$) were the synthetic (and in the former case carboxyamidation) derivatives of the natural compounds [11,26]). Magainin* has a Tyr rather than a Phe in the 5 position but is otherwise identical to magainin-2 amide. We refer to these compounds collectively as 'magainins'. Z-peptide is the same as magainin-2 amide except that all the Lys and Phe have been replaced by their D isomers. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and valinomycin were obtained from Fluka, leupeptin, pepstatin, and aprotinin from Boehringer.

Membrane potential measurements. In experiments focussing on the electric potential developed across the membranes, we used a quartz vessel illuminated by a 100 W tungsten iodide lamp [50], equipped with a reference electrode, a Davies-type rapid response oxygen-electrode [33] plus reference electrode, a light pipe (at an angle of some 50° with the incident light) leading through a neutral density filter to a photomultiplier, a glass pH electrode, a TPP^+ electrode (modified from Refs. 34 and 35; see also Hendler, R.W., unpublished results). The inner chamber of the reaction vessel was surrounded by a water jacket maintained at 298 K by circulating water. Mixtures of O_2 and Ar could be blown over the surface of the suspension, typically at rates of 45 ml/min. Rat-liver mitochondria were incubated at 3 mg protein/ml, pH 7.4 and 298 K in 5.5 ml of medium C, 1.7 μM rotenone and 5 mM succinate. 'Membrane potentials' are apparent only; they were calculated by neglecting energization-dependent binding of TPP^+ [36,37].

In these experiments, light scattering was deduced from the light intensity emanating from the light guide. At the relatively high mitochondrial concentrations used in these experiments, light scattering was not proportional to the concentration of mitochondria (in contrast to the situation in the true 'light-scattering' experiments described below). Light scattering was calibrated in terms of mitochondrial 'matrix volume' (sucrose inaccessible, water accessible space) in a separate set of experiments. In these, mitochondria incubated at 3 mg protein/ml in medium C in the presence of 5 mM sodium succinate, and radiolabelled sucrose and water were spun through silicone oil [38]. Mitochondrial volume was followed as it varied with time after the addition of 3 nM of valinomycin. Subsequently added nigericin (at 33 nM) returned the volume and light scattering to their original value, leaving the relationship between the two properties intact.

Respiratory titrations. Respiratory rates were measured in a Gilson oxygraph vessel (Clark electrode, $T = 25$ °C, 1.8 ml volume). Unless otherwise indicated, mitochondria were incubated at 0.85 g protein/l, 298 K (pH 7.4) in air saturated medium D, in the presence of 1 μM rotenone and 5 mM succinate. In the usual 'serial titrations', the coupled respiratory rate ($J_O(0)$) was measured 2 min after the addition of succinate, and then the indicated amount of peptide was added. 2 min later the rate of respiration was reevaluated and more peptide was added. This sequence was repeated as often as needed to cover a particular range of peptide concentrations. At the end of most runs, 0.2 μM FCCP was added and the resulting respiratory rate determined. In 'parallel titrations', a new run was set up for every concentration of peptide. All rates were normalized to the rate in the absence of peptide and FCCP (i.e., coupled respiration).

Light scattering. Unless indicated otherwise, rat liver mitochondria were incubated at 0.3 mg protein/ml in 2 ml of 0.25 M sucrose, 2.5 mM Hepes-KOH, 1 μM rotenone (pH 7.2) in a Cary 16K dual-beam spectrophotometer. The initial apparent absorbance at 540 nm was 1.2. Peptide was added as aliquots from a solution of 10 (PGLa) or 30 (magainin-2 amide, Z-peptide) mg protein/ml pipetted onto a Teflon stick which was subsequently used to stir (the method of addition was not without implication for the results). This experimental set-up was more sensitive to changes in light scattering than the one described under membrane potential measurements.

Results

A drop in respiration-generated membrane potential

To investigate if the supposed ability of magainins to permeabilize biological membranes extends to the inner mitochondrial membrane, we incubated rat-liver mitochondria in a medium containing KCl, and added respiratory substrate and aliquots of PGLa. The uptake of TPP^+ into the mitochondria was used as a measure of the ('apparent') electric potential across the inner mitochondrial membrane [36]. Fig. 1 shows that additions of PGLa repeatedly caused a rather rapid partial dissipation of the membrane potential, just like the K^+-ionophore valinomycin ('val') and the protonophore FCCP did. It may be noted that the effect of PGLa on the membrane potential was transient; after a rapid initial decrease, the membrane potential tended to creep back up again.

The light scattering by mitochondria decreases as their matrices swell [39,40]. The dashed line in Fig. 1 shows the result of light scattering measurements during the same experiment, translated into (apparent) changes in matrix volume, using an empirical curve based on sucrose/H_2O double isotope calibrations. PGLa and valinomycin both caused a decrease in light scattering

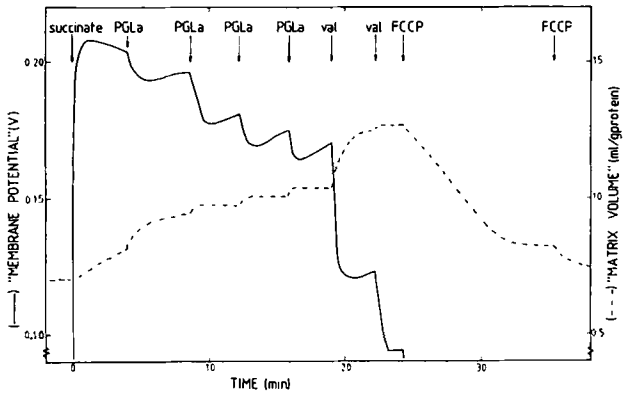

Fig. 1. The effects of PGLa on the membrane potential (———) of and (- - - - - -) apparent matrix volume of mitochondria. Aliquots of 50 μg of PGLa, 2 and 5 nmol of valinomycin and 0.5 and 1.0 nmol of FCCP were added as indicated.

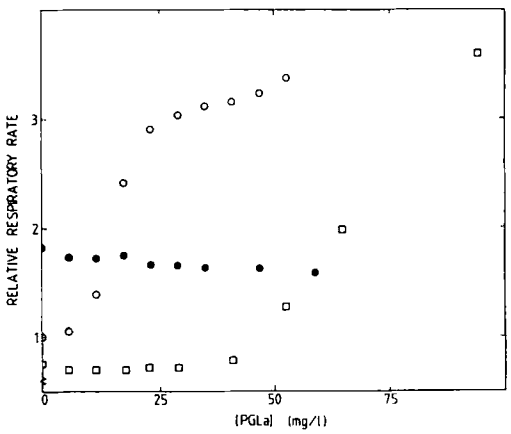

Fig. 2. The effect of PGLa on mitochondrial respiration in the absence (○), or in the presence of 5.5 mM quinine (closed circles) or 2 mM $MgCl_2$ (open squares). Mitochondria at 1.2 mg protein/ml were incubated in 0.23 M sucrose, 20 mM Hepes, 5 mM succinate, 2 μM rotenone (pH 7.2) at 298 K. At the end of the quinine and $MgCl_2$ experiments 0.2 nmol FCCP brought the relative respiratory rate up to 4.5 and 3.5, respectively (not shown).

(depicted in Fig. 1 as an apparent increase in matrix volume), which was largely, though not wholly, reversed by the added FCCP. PGLa and magainin-2 amide also decreased the membrane potential when chloride was absent, i.e., in 0.25 M sucrose, 2 mM Hepes, 1.7 mM potassium-phosphate, pH 7.15 (not shown). In the latter medium we observed (not shown; cf. Ref. 41) that the peptides also interfered with the phosphorylation of added ADP, much as FCCP does.

Release of respiratory control

Agents that permeabilize inner mitochondrial membranes are expected to increase the rate of otherwise coupled respiration [42,43]. The open circles in Fig. 2 confirm the ensuing expectation that PGLa increases the rate at which mitochondria oxidize succinate to fumarate. Since we could also show that the magainins inhibit the phosphorylation of ADP [41], we conclude that the magainins classify as uncoupling agents.

The magainins are highly positively charged [23]. Therefore, we wondered if we could reduce their activity by adding agents that are expected to screen the negative charge of the inner mitochondrial membrane. Indeed, quinine and Mg^{2+} reduced the uncoupling activity of PGLa (Fig. 2).

The first two lines of Table I reflect our additional findings that PGLa was somewhat more effective than magainin-2 amide and that Z-peptide (a stereoisomer of magainin-2 amide) was less effective than magainin-2 amide in releasing respiratory control.

Time dependence of the effects of magainins

Fig. 1 revealed that the effect of PGLa on the membrane potential is time dependent; after the initial drop, the membrane potential slowly recovered. The

TABLE I

Stereospecificity of the actions of magainins and interference of magainin-2 amide and PGLa with stimulation of respiration by the classical uncoupler FCCP

Rat-liver mitochondria were incubated at 0.9 g protein/l in medium C (without EDTA), in the presence of 1 μM of rotenone and 5 mM of succinate. Then, 25 mg/l of PGLa, or magainin-2 amide, 50 mg/l Z-peptide, or 50 mg/l followed by 500 mg/l of magainin-2 amide were added and the respiratory rate was recorded approx. 30 s later ('immediately'), as well as 3 min later ('relaxes to'). Then 0.1 μM of FCCP was added and the rate of respiration was recorded. Given are the respiratory rates normalized with respect to the rate observed in the presence of succinate alone (which varied by some 20% among the experiments). In the magainin-2 amide-excess case, the stimulation caused by the first 50 mg/l was 2.6. In the absence of added peptides, FCCP stimulated respiration by a factor of 9.

	PGLa	Mag-2	Z-peptide	Mag-2-excess
succinate	1	1	1	1
+ agent, immediately:	3.5	2.1	1.0	0.1
relaxes to:	2.6	1.7	1.0	0.1
+ FCCP	2.5	1.9	7.0	0.0

traces in Fig. 3 demonstrate that the stimulatory effect of the magainins on respiration also decreased with time. Upon addition of the magainin-2 amide, we noticed a 15 s lag phase. Subsequently, respiration increased, reached a maximum approx. 1 min after the addition of magainin-2 amide and then started to decline, although, in the upper two traces, it remained higher than before the addition of magainin-2 amide. Subsequent additions of FCCP, stimulated respiration, although at high magainin concentrations this stimulation was only minor (the bottom trace in Fig. 3; Table I). After addition, of FCCP alone, respiration was essentially constant in time (in medium D, in medium C there was a minor decrease with time; not shown).

The stimulation of mitochondrial respiration by PGLa also diminished with time after addition of the peptide (cf. the full line in Fig. 4), in this case with a half time of approx. 7 min. Leupeptin, a protease inhibitor, increased both that half-time and the maximum stimulatory effect of PGLa (cf., the dotted line in Fig. 4). Other protease inhibitors, i.e., 1.7 mM of PMSF, 56 mg/l of pepstatin A, and 56 mg/l aproprotinin, had little effect. By itself, leupeptin did not affect the rate of respiration of mitochondria with succinate as substrate in either medium (not shown).

We interpret these findings as indicative of substantial proteolysis of added PGLa by a protease that is relatively insensitive to PMSF, pepstatin and aprotinin, but sensitive to leupeptin. Such proteolysis was probably responsible for the time-dependent decrease of respiration after the addition of either magainin.

The bottom trace in Fig. 3 and Table I show that proteolysis of added peptide was not the only complication in these experiments: the addition of stimulatory

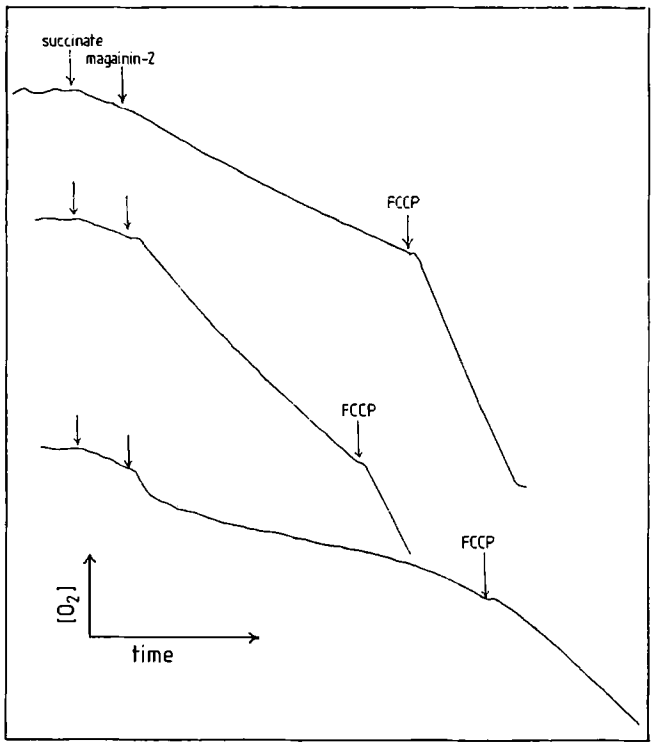

Fig. 3. The time dependence of the effect of magainin-2 amide on mitochondrial respiration. Mitochondria were suspended at 0.85 g protein/l in medium D plus 1 μM rotenone, and 5 mM succinate. At the indicated times 5 (upper trace), 10 (middle trace) μl out of a 3 g/l solution of magainin-2 amide in water was added. In the case of the bottom trace the addition was 5 μl out of a 30 g/l solution of magainin-2 amide. At the indicated times 0.2 μM FCCP was added. The time and $[O_2]$ arrows correspond to 5 min and 20% of air saturation, respectively.

amounts of magainin-2 amide (or PGLa) also had the effect that respiration could no longer be stimulated to the normal uncoupled rate by FCCP. Excess magainin-2 amide caused a complete inhibition of respiration.

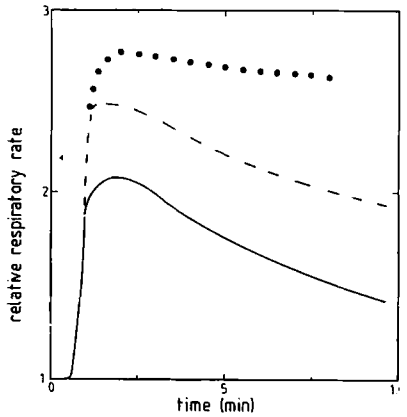

Fig. 4. Variation of the respiratory rate with time after the addition of PGLa in the presence and absence of leupeptin. Experimental conditions were as in Fig. 3, except that medium C was used and at time zero 13 mg/l of PGLa was added either in the absence (full line) or in the presence of 0.056 (dashed line), or 0.28 g/l (dotted line) leupeptin. Mitochondria had a respiratory control index (FCCP) of 7.6 in medium D.

Table I may also serve to demonstrate that not only the stimulatory effect, but also the inhibitory effect of magainin-2 amide on mitochondrial respiration is stereospecific: even when added at twice the concentration at which magainin-2 amide had strong effects, Z peptide, a stereoisomer of magainin-2 amide, did not stimulate or inhibit respiration.

Cooperativity

We confirmed that also under our present conditions (cf. Refs. 43 and 44) respiration varied linearly with added FCCP up to a sevenfold stimulation. This has been shown to be due to the high flux control coefficient of the proton leak with respect to state-4 mitochondrial respiration [43,44]. This justifies the use of respiratory rate as a linear measure of the permeability of the inner membrane (see also the Discussion section).

Many membrane active peptides act as oligomers [45,13,6] and it is of interest to see if the magainins behave similarly. If so, and if the majority of the added magainin is present as the monomeric (or less than hexameric) form, the induced permeability and hence the respiratory rate should depend sigmoidally on the added concentration of the magainins. A much finer titration with PGLa than the one shown in Fig. 2 confirmed that respiration varied sigmoidally with the added concentration of PGLa (data not shown).

The fact that the stimulation of respiration by magainins was time dependent (see above) complicates the interpretation of these 'serial' titrations (aliquots of the peptide were added sequentially to a single mitochondrial incubation). Most of the PGLa might be degraded between additions and the little that was left, may have built up until at some point it became sufficient to survive proteolysis. To test this alternative explanation of the observed sigmoidicity, we did a serial titration of respiration with PGLa both in the absence and in the presence of the protease inhibitor leupeptin. The protease inhibitor did not remove the sigmoidicity of the dependence of respiration on the added amount of PGLa (not shown).

That in the case of magainin-2 amide, proteolysis may affect the titration curve somewhat, is suggested by the observation (cf. the open to the closed circles in Fig. 5A) that when the time period between additions was increased from 60 to 90 s, more magainin-2 amide had to be added to obtain the same stimulation of respiration. To rule out the possibility that the sigmoidicity was caused by proteolysis, we also carried out a 'parallel' titration (i.e., starting a new incubation for every concentration of added magainin). Although this proce-

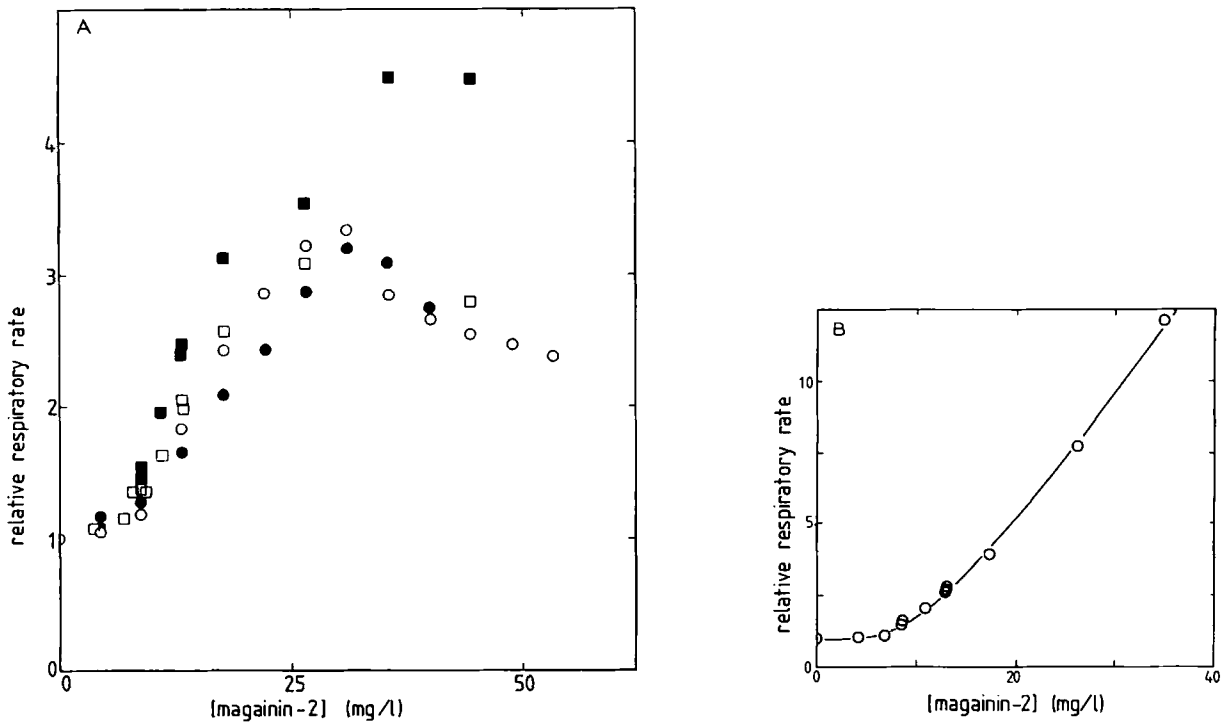

Fig. 5. Titrations of mitochondrial respiration with magainin-2 amide. Experiment carried out as described in the legend to Fig. 3. (A) Open and closed circles: serial titrations with 60 and 90 s, respectively between additions; squares: the results of parallel titrations; respiratory rate was evaluated either (closed squares) as the highest rate observed after the addition of magainin-2 amide, or (open squares) as the average respiratory rate during the first 5 min after the addition of magainin. The former method is less accurate, whereas the latter may still be biased by proteolysis. (B) Respiratory rates were corrected for the inhibitory effects of the magainins on respiration through division by the rate obtained when 0.2 μM FCCP was added at the end of the titration. The line is the nonlinear least square fit to Eqn. 1 on the assumption that C is proportional to the increase in (corrected) respiratory rate upon the addition of magainin-2 amide.

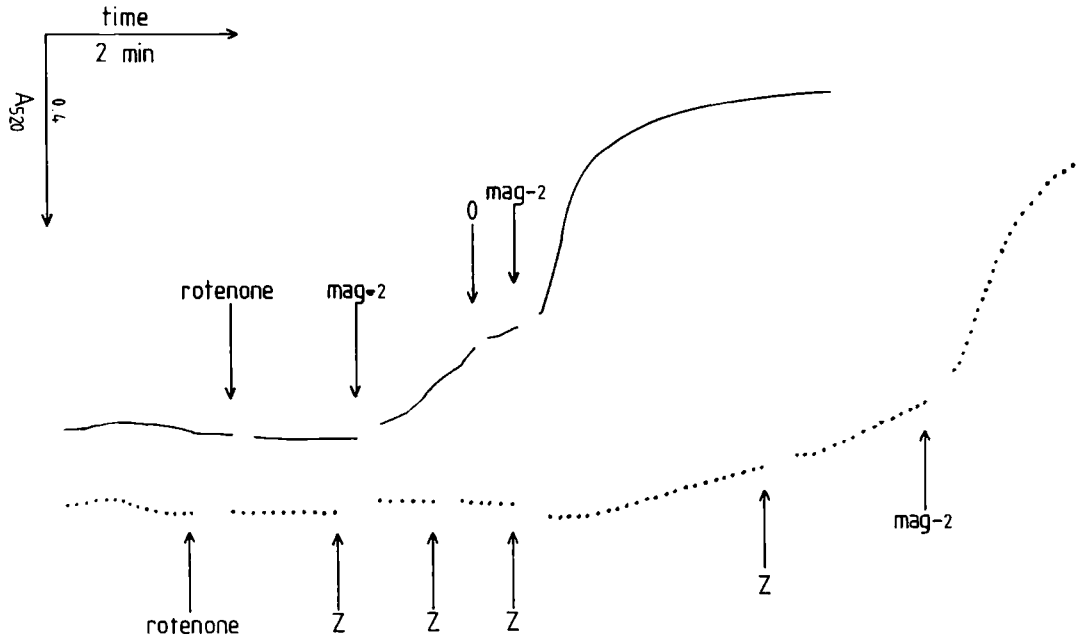

Fig. 6. The effects of magainins on light scattering by non respiring mitochondria incubated in sucrose medium (0.25 M sucrose, 2.5 mM Hepes, pH = 7.2). Upper trace (full line) additions were 1 μM rotenone, 7.5, 0 and 7.5 μg/ml of magainin-2 amide. Lower trace (dotted line) additions were 7.5, 7.5, 45, 45 μg/ml of Z peptide and 7.5 μg/ml of magainin-2 amide.

dure decreased the experimental accuracy somewhat, its results (Fig. 5A, closed squares, see also the open squares for a different way of evaluating the results) indicate that also in the parallel titration the dependence of respiration on added magainin concentrations was sigmoidal.

Light-scattering changes

When mitochondria swell, they scatter less incident light. Consequently, the uptake of osmotically active solutes can be monitored through the decrease in apparent absorbance of a mitochondrial suspension [40,39]. We used this assay to define the permeability induced by the magainins.

Fig. 6 shows that magainin-2 amide, but not Z-peptide, caused a decrease in light scattering when non-respiring mitochondria were incubated in a sucrose medium. Since PGLa also decreased light scattering (not shown), this suggests that the magainins cause an increase of the permeability of the inner mitochondrial membrane towards sucrose. As in the stimulation of respiratory rate, the effect depended sigmoidally on the concentration of each of the two peptides. Magainin-2 amide and PGLa began to reduce light scattering substantially at concentrations of approx. 3 μg/ml. In terms of μg peptide per mg protein (approx. 10) this is the same concentration as needed to cause release of respiratory control. Substitution of mannitol for sucrose in the medium did not significantly alter the effects of PGLa and magainin-2 amide, but the addition of 2 mM $MgCl_2$ abolished the effects of the peptides on light scattering even when the latter were added at up to 0.2 mg peptide per mg protein. 50 mM additional KCl and 2 mM quinine also reduced the effect of PGLa on light scattering, though to a lesser extent (not shown).

Discussion

Uncoupling and inhibition

In this paper, we showed that the magainins (i) dissipate the membrane potential across the inner mitochondrial membrane, (ii) (therefore) stimulate coupled respiration, (iii) inhibit uncoupled respiration, and (iv) cause a decrease in light scattering consistent with an increase in (sucrose) permeability of the inner mitochondrial membrane. The uncoupling effects of magainin-2 amide and PGLa on mitochondrial respiration are reminiscent of the effects reported for melittin [46], a positive peptide of 26 amino acids. At some 3 μM melittin and tetraacetyl melittin increased mitochondrial respiration more than 3-fold [46]. Prepeptides of proteins transported into the mitochondria were also shown to have these effects [5,9]. This supports the suggestion [6] that for peptides to increase the permeability of biological membranes it suffices to satisfy a limited number of criteria. One set of such criteria (there are various sets) is that they can form amphiphilic α-helices that can span a biological membrane.

It is considerably less obvious that such transmembrane α-helices should cause the inhibition of uncoupled respiration. How to explain this inhibitory effect? Some additional observations may be relevant here. First, magainins did not inhibit the oxidation of ascorbate quite as much as they inhibited the oxidation of

succinate (not shown). Second, they did not inhibit the oxidation of ascorbate by cytochrome-*c* oxidase not reconstituted into phospholipid vesicles. Finally, in cytochrome-oxidase liposomes the concentration at which the magainins started to inhibit respiration was much higher than the concentration at which they began to stimulate respiration [50]. Das and colleagues [46] attributed the inhibitory effect of alamethicin on respiration to an interference with a phosphate effect on interactions between cytochrome *c* and cytochrome oxidase. Since the inhibitory effects of the magainins were observed in the absence of phosphate, the same phenomenon can only be operative here if it were an effect of ionic strength rather than of phosphate itself.

Concentration dependence caused by cooperativity and affected by proteolysis

We showed that the stimulation of mitochondrial respiration by magainin-2 and PGLa decreased with time. Also the drop in membrane potential caused by PGLa was slowly reversed in time. Because the effect on respiratory rate could be reversed for PGLa in high KCl medium by the addition of leupeptin (a protease inhibitor), it seems likely that it is caused by proteolysis of magainin or PGLa. This suggestion was supported by the observation (not shown) that the stimulatory activity of PGLa and magainin-2 amide could not be recovered from supernatant or pellet after centrifugation of the mitochondria. The protease could be endogenous to rat-liver mitochondria, or a contaminant of our mitochondrial preparation. It is unlikely the latter possibility could be attributed to our preparation being particularly 'bad', as its respiratory control of 7.6 with succinate as the substrate is among the highest reported for rat-liver mitochondria.

The curvature of the dependence of respiration on added magainin concentration reveals the effective cooperativity of the magainin molecules. To evaluate this we plotted $\log\{v/(1-v)\}$ vs. the logaritm of the added amount of magainin (not shown). Here v is the respiratory rate relative to the highest rate obtained in the titration [50]. The slope in these plots tended to lie around 4 for magainin-2 amide and around 3 for PGLa, suggesting that the active unit for these two magainins contain four and three molecules, respectively. (It should be noted that the accuracy with which these numbers could be determined leads to an uncertainty of ± 1 in both cases).

However, our experiments suggested that the dependence of respiration on peptide concentration may be more complicated than just sigmoidal; after the initial sigmoidal dependence, respiration tended to vary linearly with added magainin concentration until it reached a maximum, after which inhibition set in. The sigmoidicity and linearity can be understood as follows: if we assume that there exists an equilibrium exclusively between monomeric magainin-2 amide molecules (at concentration M) and complexes (at concentration C) consisting of precisely n monomers, then:

$$KC = M^n = (T - nC)^n \qquad (1)$$

Here T is the total concentration of added magainin-2 amide and K is the dissociation constant of the complex. If the stimulation of respiration is proportional to the concentration of the magainin complexes, then this equation predicts that at low concentrations of magainin respiration should vary with the nth power of the added magainin concentration (T), whereas at higher concentrations it should vary linearly with the added magainin concentration. This is indeed what was observed in Figs. 2 and 5.

To establish which values for the cooperativity parameter of n are consistent with this model and our experimental results, we had to minimize the effects of the above-mentioned complications on the titration curve. To minimize the effects of proteolysis, we focused on the parallel titration with magainin-2 amide. To correct for the inhibitory effect of magainin on respiration, we added excess FCCP at the end of each run of Fig. 5 and determined the respiratory rate in the presence of magainin-2 amide and FCCP. For each concentration of magainin we then divided the rate in the presence of magainin-2 amide alone by the rate in the presence of both magainin-2 amide and excess FCCP. The open dots in Fig. 5B give the results of this correction for the inhibitory effect of magainin and the full line gives the best (nonlinear-least-squares) fit to Eqn. 1. The value of n for this fit was 3.9 (4.3 when average rates of respiration during the first 5 min after magainin addition were used). However, other magnitudes of n (allowing the other parameters to take also other values) were also compatible with the experimental results, for as long they exceeded 2.

Recently we observed that in stimulating respiration in cytochrome-oxidase liposomes, magainin-2 amide exhibited a cooperativity number of 5 (at room temperature). This value is not significantly different from the 4 found for the mitochondria.

Mechanism of action

The primary structure of magainins satisfies the requirements for ion-channel forming peptides [5,45,13]. Addition of magainins to black lipid membranes has been shown to induce the formation of ion channels, with some preference for the conduction of anions over cations [30]. The present paper adds that magainins uncouple mitochondria. That they do this while dissipating the membrane potential suggests that the magainins act as uncouplers rather than decouplers [47,48] and that they do not just cause slip in the electron-transfer linked proton pumps [48,43]. The concentration

dependence of the uncoupling effect suggests that a complex of some 4 magainin-2 molecules is the uncoupling agent. The uncoupling of mitochondrial respiration in our medium D (essentially sucrose with a little Hepes and EGTA) is best explained if the, positively charged, magainins caused permeability for OH^-, or of Hepes. The magainins also induced permeability of the inner mitochondrial membrane to sucrose.

At the concentrations at which they begin to release respiratory control, the magainins do not seem merely to cause 'lysis' of the mitochondria, as shown by a number of observations. Their effect on the membrane potential reverses with time (presumably due to proteolysis) (Fig. 1), their effect on light scattering under respiring conditions is largely reversible (Fig. 1), their effect on respiration and on apparent sucrose permeability is greatly reduced by Mg^{2+} or quinine (Fig. 2).

Rather, our results suggest that the magainins form channels in the inner mitochondrial membrane and that these channels have only limited selectivity. They may be anion selective [30], or rather cation antiselective (i.e., they are permeable for neutral as well as for negatively charged molecules). At least in this sense magainins differ from shuttling ionophores such as FCCP and valinomycin, which are much more selective and from other pore formers, such as gramicidins, which are less selective than valinomycin, but still much more cation selective than the magainins. Another difference is the cooperativity; whereas valinomycin acts as the monomer, the magainins appear to act as a complex of at least four monomers [49]. On paper, a magainin monomer is long enough to span a biological membrane when it is in the α helical form. In contrast, gramicidins are too short to span a lipid bilayer and appear to form head-to-head dimers, with one monomer in each lipid monolayer. Interestingly, in rat-liver mitochondria, gramicidin analogues have recently been shown to form pores as an antiparallel double stranded helix dimer, suggesting that in mitochondria a shorter distance has to be spanned by a channel to cause uncoupling [32].

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