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Magainins and the disruption of membrane-linked free-energy transduction

(membrane-active cationic peptides/antibiotics/membrane potential/antimicrobial/oxidative phosphorylation)

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ABSTRACT **Magainins, a family of positively charged peptides, are partly if not wholly responsible for antimicrobial activity in skin extracts of *Xenopus laevis*. We report here that members of the magainin family—i.e., the 21-amino acid peptide PGLa and the 23-amino acid peptide magainin 2 amide (PGSa)—dissipate the electric potential across various energy-transducing membranes and thus uncouple respiration from other free-energy-requiring processes. We propose that this is a likely mechanism for the antimicrobial effects of these compounds.**

The greater part of the transduction of free-energy from catabolism to anabolism (e.g., ATP synthesis) requires redox-linked proton pumps to generate an electric potential difference across a membrane (1, 2). As protons flow back through the H^+-ATPase, the electric driving force (2) is applied to the synthesis of ATP from ADP and inorganic phosphate. Other membrane enzymes allow other thermodynamically uphill reactions (such as the uptake of substrates for metabolism) to be driven by such proton backflux.

Agents that make membranes leaky towards protons or other ions have been shown to disrupt energy coupling (3). Among these are small synthetic molecules such as carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which can shuttle protons across membranes, and mixed peptides such as the gramicidins, which form ion channels in biological membranes. The latter compounds are produced and excreted by prokaryotes as weapons against neighboring organisms. A third class of such weapons consists of "true" oligopeptides (in the sense that they consist only of the 20 "standard" amino acids in their L configuration). The colicins (4) are among these. It should be noted that not all of these compounds act primarily by depolarizing the membrane potential of their target cells; some seem to compromise the integrity of the outer rather than the inner membrane (4, 5).

Lower eukaryotes also use such oligopeptide weapons. As a response to injection of bacteria, insects produce cecropins (6), also called sarcotoxins (7, 8). This raised the question of whether vertebrates, and perhaps even mammals, use an oligopeptide-based defense mechanism in addition to their highly effective cellular and antibody immune responses. One such mechanism (presumably auxiliary to phagocytic and oxidative killing mechanisms), residing in rabbit and human leukocytes, consists of the so-called "defensins." These are cysteine-rich, lysine-poor oligopeptides of about 3.5 kDa in molecular mass (9, 10).

Recently, one of us (11, 12) purified antimicrobial activity from the skin of the amphibia *Xenopus laevis* as two 23-amino-acid-long positively charged peptides that lack cysteine but are rich in lysine. These peptides were called "magainins." Based on its primary structure (13) and activity (14), a similarly active 21-amino-acid-long oligopeptide called PGLa should also be considered a magainin. The primary structures of magainins (11) differ considerably from those of the peptides belonging to the other classes of amphibian peptides defined by Nakajima (15). The magainins are present in the complex mixture of peptides that had been shown to be secreted upon administration of noradrenaline to the frogs (16, 17).

In theory, the magainins and their active analogues (18) are able to form an amphiphilic α-helix that can span a biological membrane; as such, they fulfill the specifications (19, 20) for a peptide forming a model channel. In decane-containing phospholipid membranes, magainins induced channels with a slight specificity for anions (21). This suggested that the magainins may be the functional analogues of the membrane-deenergizing weapons of invertebrates mentioned above.

In this paper we report that the magainins can disrupt free-energy metabolism (1, 2) of *Escherichia coli*, and its functional analogue, the mitochondrion, by dissipating the electric potential across its membrane.

MATERIALS AND METHODS

Respiratory Titrations. Respiratory rates were measured in an oxygraph (Clark electrode, t = 25°C, 1.8-ml volume). Mitochondria at 0.8 g of protein per liter, 298 K, and pH 7.4 were incubated in 0.25 M sucrose/2 mM Hepes/0.5 mM EGTA/1 μM rotenone/5 mM succinate. One minute after the addition of succinate, the coupled respiratory rate [$J_0(0)$] was measured, the indicated amount of magainin 2 amide was added, and the average rate of respiration over the subsequent 5 min was measured with a Clark electrode. Then 0.2 μM FCCP was added, and the resulting respiratory rate was measured. All rates were normalized to the rate in the absence of magainin 2 amide and the presence of FCCP.

Membrane Potential Measurements. To probe the electric potential developed across the membranes, we used a quartz vessel equipped with a reference electrode, a Davies-type rapid-response oxygen electrode (22) plus reference electrode, a glass pH electrode, a TPP^+ (tetraphenyl phosphonium ion) electrode, and a salicylate electrode (modified from refs. 23 and 24; R.W.H., unpublished data). The inner chamber of the reaction vessel was surrounded by a water jacket maintained at 25°C by circulating water. Mixtures of

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Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenylphosphonium ion.

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O_2 and Ar could be blown over the surface of the suspension, typically at rates of 45 ml/min.

E. coli were suspended (6.3×10^9 cells per ml at 298 K) in 0.25 M sucrose/2 mM $MgSO_4$/5 mM potassium phosphate/10.7 μM TPP^+/43 μM sodium salicylate, pH 7.4. The salicylate was used as an indication of the pH gradient. The changes in the pH gradient in the experiments presented here were insignificant. Because the fractions of TPP^+ taken up by respiring bacteria and released upon subsequent addition of PGLa (see Fig. 3) were the same for total TPP^+ concentrations of 12.5 and 50 μM (not shown), and because the amount of TPP^+ disappearing was less than the amount of PGLa added, we interpret the induction of TPP^+ efflux by PGLa as being primarily due to a reduction in the transmembrane electric potential difference (see ref. 25). That the changes in extracellular concentration of TPP^+ reflect the membrane potential is supported further by the effects of the aerobic/anaerobic transitions and of the protonophore FCCP (10 in Fig. 3). Because of the impairment of TPP^+ permeability by the outer membrane, the response of the TPP^+ electrode to energization varied between preparations of bacterial cells (26). The response of the cells that had been pretreated with Tris/EDTA (27) was consistently like the response shown in Fig. 3. Because we prefer to demonstrate the effects of magainin on the membrane potential in cells that have not been pretreated, Fig. 3 presents results obtained in cells without such pretreatment.

Rat liver mitochondria were incubated at 3 mg of protein per ml, pH 7.2, and 298 K in 0.25 M sucrose/2 mM Hepes/0.5 mM EGTA/2 mM potassium phosphate/1.7 μM rotenone/25 μM TPPCl/5 mM succinate.

Preparations and Materials. *E. coli* strain D31 (28) grown in medium containing 10 g of Bacto-tryptone (Difco) and 5 g of yeast extract (Difco) per liter and 25 mM NaCl (pH 7.4) was washed twice and stored at 0°C in 0.25 M sucrose/2 mM $MgSO_4$/5 mM potassium phosphate, pH 7.4.

Rat-liver mitochondria were isolated from male fasted (overnight) Sprague–Dawley rats (between 250 and 275 g) as described by Pedersen *et al.* (29). The isolation and resuspension medium was ice-cold 0.21 M mannitol/70 mM sucrose/0.5 g of fatty-acid-free bovine serum albumin per liter/2.1 mM potassium Hepes, pH 7.4. During the homogenization of the liver, an additional 1.0 mM EGTA was present and bovine serum albumin was absent.

Magainin 2 amide (H_2N-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-$CONH_2$) and PGLa (H_2N-Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-$CONH_2$) were the synthetic carboxyamidated derivatives of the natural compounds (14). FCCP and valinomycin were obtained from Fluka.

RESULTS

Magainins Release Respiratory Control in Mitochondria. The respiratory rate of isolated rat liver mitochondria is subject to back pressure by the transmembrane electric potential and hence subject to control by the ion permeability of the inner mitochondrial membrane (1, 2). Consequently, if, as suspected, magainins induce ion permeability in biological membranes, then they should release respiratory control in mitochondria. Because bacteria tend to have little respiratory control, they are not suitable for this type of assay. The closed circles in Fig. 1 summarize the effect of magainin 2 amide on mitochondrial respiration. At low concentrations, magainin 2 amide was virtually without effect, but once a threshold amount had been added, respiration readily increased up to 3-fold with further additions of magainin 2 amide. By definition, release of respiratory control is the stimulation of respiration in the absence but not in the presence of a second uncoupler (or of ADP plus phosphate). When excess FCCP (a classical uncoupler) was present, respiratory rates did not increase with increasing concentrations of added magainin 2 amide (open circles in Fig. 1). Thus magainin 2 amide conforms to the definition of releasing respiratory control; it does not stimulate respiration by merely stimulating electron transfer in or toward the mitochondrial respiratory chain.

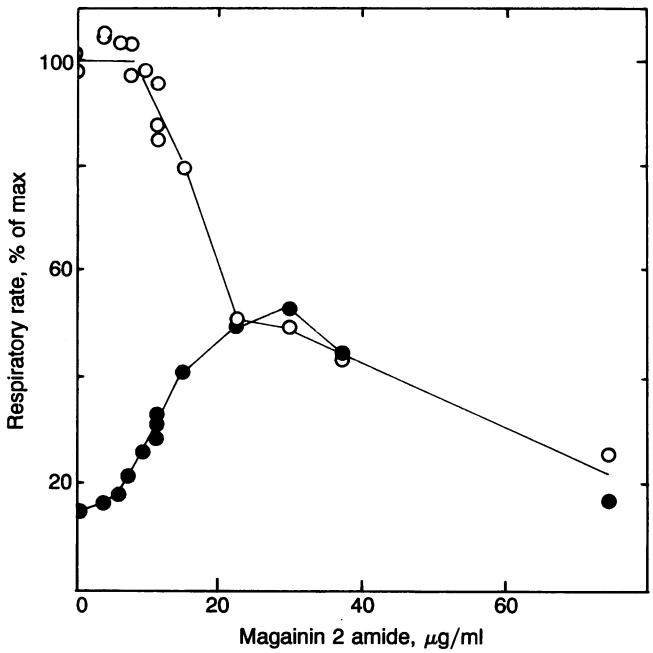

FIG. 1. Respiration of isolated rat liver mitochondria as a function of the concentration of magainin 2 amide, in the presence (○) or in the absence (●) of excess uncoupler (0.2 μM FCCP).

In fact, the effect of magainin 2 on electron transfer appears to be quite the opposite: in the presence of FCCP, magainin 2 amide inhibits respiration. Apparently, magainin 2 amide uncouples mitochondrial respiration and also inhibits it. This may also explain why the maximum (3-fold) stimulation of respiration by magainin 2 amide is less than the stimulation achieved in a titration with FCCP (6.5-fold).

Magainins Dissipate the Membrane Potential in Mitochondria and Thus Interfere with ATP Synthesis. If the magainins act by permeabilizing membranes, they should reduce the membrane potential developed in respiring mitochondria. The experiments shown in Fig. 2 demonstrate that they do. Upward deflection of the dashed line in Fig. 2 indicates release of TPP^+ and a decrease in electric potential across the inner mitochondrial membrane (25). By monitoring the pH of the medium (the full line in Fig. 2), we also followed ATP synthesis (a downward deflection of the full line is indicative of ATP synthesis; ref. 30).

Upon addition of oxygen to mitochondria in the presence of respiratory substrate (**1** in Fig. 2), a membrane potential was generated. When ADP was added (**2** in Fig. 2), the membrane potential dropped (because protons flowed inward via the H^+-ATPase; refs. 1 and 2) and ATP synthesis (steady alkalinization; see the full line in Fig. 2) set in. Addition of magainin 2 amide [or (not shown) PGLa] to a concentration of 4 μM caused a further drop in membrane potential and a reduction in the rate of ATP synthesis (**3** in Fig. 2). Upon addition of another two aliquots of magainin 2 amide, the membrane potential decreased greatly and ATP synthesis reverted to ATP hydrolysis (as witnessed by the acidification; **4** in Fig. 2). After this rapid decrease, the membrane potential had the tendency to recover (**5** in Fig. 2). Subsequent anaerobiosis (**6** in Fig. 2) caused only a small decrease in the membrane

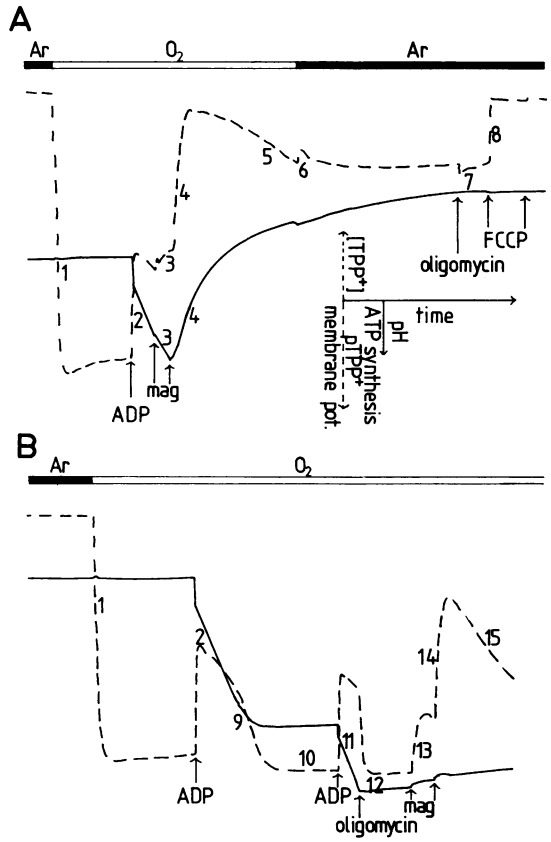

FIG. 2. The effect of magainin 2 amide on ATP synthesis (—) and membrane potential (– – –) in isolated rat liver mitochondria. (*A*) At the indicated times, 0.83 mM ADP, 8.3 and 16.6 mg of magainin 2 amide ("mag") per liter; 0.7 mg of oligomycin per liter, and 0.5 μM FCCP were added. The state of oxygenation is indicated by the bar at the top of the figure; white implies excess oxygen and black implies limited oxygen, as deduced from an oxygen electrode signal. The dashed line shows the activity of extramitochondrial TPP^+ as indicated by a TPP^+-selective electrode; downward deflection corresponds to an increase in membrane potential. Because of the differences in pKa between phosphate, ADP, and ATP, alkalinization [downward deflection of the signal of the pH electrode (solid line)] corresponds to ATP synthesis (30). The scales are indicated by the length of the arrows: 10 min for arrow labeled "time," (– – –) 0.5 unit for arrow labeled "$pTPP^+$" ($= -\log_{10}[TPP^+]$), and 0.1 unit for arrow labeled "pH." (*B*) As in *A* except for (*i*) the order of additions, (*ii*) the fact that the second addition of magainin 2 amide amounted to only 8.3 mg/liter, and (*iii*) the fact that after position 1, excess oxygen was present throughout.

potential, presumably because ongoing ATP hydrolysis sustained the remainder. Oligomycin (7 in Fig. 2), an inhibitor of the H^+-ATPase, caused an artifact on the electrode, followed by a slow decrease in membrane potential. Only the subsequent addition of FCCP (8 in Fig. 2) caused a rapid disappearance of that potential, suggesting that by this time, the membrane was again fairly impermeable to protons and hydroxyl ions.

Fig. 2*B* verifies that ATP synthesis would have continued had magainin 2 amide not been added (9 in Fig. 2*B*) and that the membrane potential would have returned to a high value after essentially all added ADP would have been phosphorylated (10 in Fig. 2*B*). After a subsequent addition of ADP (11 in Fig. 2*B*), addition of oligomycin abolished ATP synthesis and restored the membrane potential (12 in Fig. 2*B*) as expected. Also with the activated H^+-ATPase inactivated, magainin reduced the membrane potential (13 and 14 in Fig. 2*B*). Again [15 (compare with 5) in Fig. 2] the membrane potential tended to recover after each addition.

We conclude that magainin 2 amide (and PGLa) reduces the membrane potential in respiring rat liver mitochondria. The probable explanation for the recovery of the membrane potential at positions 5 and 15 in Fig. 2 is degradation of the added magainin 2 amide or PGLa by proteolytic enzymes present in the mitochondrial sample. We base this on the following observations. First, after centrifugation of mitochondria treated with magainin 2 amide or PGLa, the stimulatory activity of the peptide could not be recovered from the pellet or from the supernatant (whereas the mitochondria in the pellet and fresh mitochondria incubated in the supernatant could be uncoupled by added peptide; data not shown). Second, the stimulation of respiration by PGLa became independent of time when the protease inhibitor leupeptin was added (31). Third, in cytochrome oxidase liposomes, there was no recovery of the membrane potential and of respiratory control after a decrease had been induced by the addition of a magainin (unpublished work).

Magainins Dissipate the Membrane Potential of *E. coli*. Rat liver mitochondria lend themselves better to the study of proton-mediated free-energy transduction than do intact bacterial cells. This is because (*i*) their respiration is strongly controlled by the electric potential across their membrane (33, 2), (*ii*) they export synthesized ATP so that steady-state ATP synthesis can be assayed more readily, and (*iii*) the permeation of membrane-potential probes such as TPP^+ is more rapid. Since protonmotive free-energy transduction in *E. coli* and rat-liver mitochondria is otherwise rather analogous, the above results obtained with mitochondria suggested that the magainins also disrupt energy metabolism in *E. coli* by dissipating its membrane potential.

To check this possibility, we again used the uptake of the lipophilic cation tetraphenyl phosphonium (TPP^+) as an indication of the electric potential (24, 34), now across the inner membrane of the bacterium. Fig. 3 shows the response of the (extracellular) TPP^+-specific electrode to the addition of TPP^+ and cells (positions 1 and 2, respectively, in Fig. 3; as in Fig. 2 downward deflection reflects an increase in membrane potential). The endogenous respiration of the cells sustained a membrane potential and caused uptake of TPP^+ (2 in Fig. 3). Addition of external substrate increased the membrane potential somewhat (3 in Fig. 3). Upon anaerobiosis, the membrane potential decreased (4 in Fig. 3); the supply of oxygen restored it (5 in Fig. 3). Most importantly,

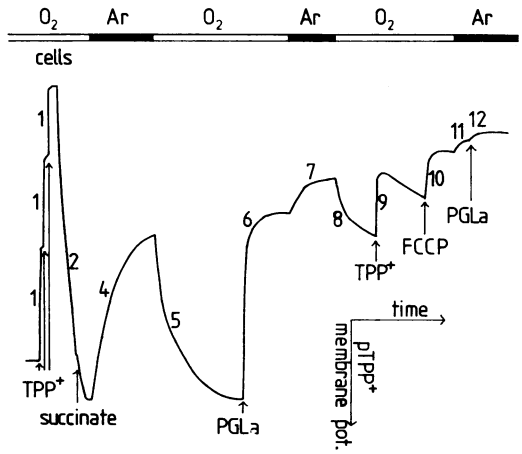

FIG. 3. Reduction by PGLa of the membrane potential of *E. coli*, as monitored through the effect of PGLa on TPP^+ uptake. At the indicated times, additional aliquots of 10.7 μM TPP^+, 2.1 mM sodium succinate, 5 μM (10 mg/liter) PGLa, 0.09 μM FCCP were added, or the gas phase over the suspension was replaced with Ar or O_2. Aeration status is indicated by the bar at the top as in Fig. 2. The length of the vertical arrow corresponds to 0.2 $pTPP^+$ unit, and that of the horizontal arrow corresponds to 20 min.

the addition of PGLa (or of magainin 2 amide, not shown) caused a sharp decrease in the membrane potential (**6** in Fig. 3), not quite to the level obtained in the subsequent anaerobic phase (**7** in Fig. 3). That the effect of PGLa on the signal given by the TPP^+ electrode was not due to a direct interaction between PGLa and the electrode was shown by the facts that (*i*) the subsequent supply of oxygen (**8** in Fig. 3) caused a much smaller change in TPP^+ concentration than before the addition of PGLa (**5** in Fig. 3), (*ii*) the electrode responded as before (**1** in Fig. 3) to the addition of TPP^+ (**9** in Fig. 3), and (*iii*) under deenergized conditions, the addition of PGLa hardly affected the electrode signal (**12** in Fig. 3).

The congruence of the effects of magainins on *E. coli* and rat liver mitochondria suggested to us that these compounds may act on free-energy-transducing membranes in general. Indeed, PGLa and magainin 2 amide also released respiratory control of and decreased TPP^+ uptake into small unilamellar cytochrome-oxidase liposomes (unpublished results).

DISCUSSION

How Do the Magainins Dissipate the Membrane Potential? Our experimental results give some clues as to how the magainins affect energy coupling. We consider it unlikely that the magainins act by solely causing "slip" (35, 2) in cytochrome oxidase or the H^+-ATPase or by solely causing the hydrolysis of intrabacterial or intramitochondrial ATP, since they stimulate the hydrolysis of ATP, reduce the membrane potential in the presence of oligomycin, induce passive swelling of mitochondria (31), and act on pure cytochrome oxidase liposomes (unpublished results). They may interfere with energy coupling in intricate ways, as do the so-called decouplers (36, 37). However, the fact that we observe a significant effect on the membrane potential suggests that the magainins also (if not solely) act as ionophores or ion channels—i.e., by increasing the ion permeability of the membrane. This is consistent with the observed tendency of magainin 2 amide to form an amphiphilic α-helix (38, 39). The effects we observed in rat liver mitochondria and liposomes persisted when the only anion present in significant amount was 2 mM Hepes (not shown). This phenomenon, the fact that magainins induce anion channels in lipid bilayers (21), and the positive charge of the magainins suggest OH^- efflux as the cause for the observed uncoupling. Our measurements of the pH gradient did not have sufficient resolution to decide on this possibility.

A second activity of the magainins was that they inhibited uncoupled respiration (open circles in Fig. 1). Although similar effects have been observed with other uncouplers (e.g., ref. 40), the inhibitory and uncoupling concentrations are closer for the case of the magainins in rat liver mitochondria. In mitochondria the inhibitory effect may contribute to the dissipation of membrane potential and to the interference with ATP synthesis. In cytochrome oxidase liposomes, inhibition was observed only at concentrations that are at least 10-fold higher than the uncoupling concentrations (unpublished results). In bacteria, with respiration depending more on endogenous substrate, membrane potential was dissipated at concentrations significantly below those necessary to inhibit respiration substantially (data not shown).

The increase in respiratory rate depended sigmoidally on the concentration of added magainin 2 amide (closed circles in Fig. 1). This may reflect positive cooperativity between magainin monomers. [Because of the additional inhibitory effect of magainin (open circles in Fig. 1), the actual cooperativity may even be stronger than is immediately apparent from the closed circles in Fig. 1.] A multimeric magainin complex may well be responsible for the induced membrane permeability (31, 41). An analysis of respiratory stimulation in cytochrome-oxidase liposomes was consistent with four to six magainin 2 amide molecules forming the active unit (unpublished results).

At the concentrations at which they stimulated respiration and decreased the membrane potential, the magainins did not irreversibly lyse or solubilize the mitochondrial membranes (not shown): (*i*) the respiratory activity could still be centrifuged down at 11,000 × *g* (3 min), (*ii*) the decrease in light scattering (indicative of swelling or lysis) caused by the addition of magainin 2 amide was partly reversible (upon anaerobiosis), (*iii*) the biologically inactive stereoisomer of magainin 2 amide that has all the lysine and phenylalanine analogues in the D rather than L configuration was without effect on bacterial membrane potential at 5 times higher concentrations, (*iv*) the membrane potential (see **5** and **15** in Fig. 2; compare **6** and **8** in Fig. 3) and controlled respiration recovered with time after the addition of the peptides (this recovery could be slowed down by the addition of protease inhibitors), and (*v*) the stimulation by magainin 2 amide of respiration in cytochrome oxidase liposomes could be completely reversed by the addition of a protease (unpublished results). Moreover, on the basis of their primary structure, magainins are expected (19, 20, 42) and have been shown (21) to form ion channels in lipid bilayers at a lower concentration than that at which they solubilize the latter.

Selectivity. Antibiotics must have two paradoxical properties: they should be lethal to microbes and they should be harmless to their hosts. Therefore, it may seem unlikely that an agent causing a generalized effect on membrane-linked free-energy transduction could function in and around *Xenopus laevis*. Indeed, our demonstration that magainins interfere with free-energy transduction by mitochondria generates the paradox that *Xenopus laevis* seems to secrete substances that could be lethal to itself. A number of considerations address this paradox. First, the magainins appear to be quite susceptible to proteolysis (41, 43) and *Xenopus* secretes proteases that are active on peptides of the magainin family (44). Second, the majority of the magainins are secreted only under certain conditions (16). Third, to act on the mitochondria, the magainin would first have to pass through the plasma membrane of the eukaryotic cells. Finally, the activity of the magainins may well be a subtle function of the lipid composition of the target membrane (e.g., all the membranes we assayed were poor in cholesterol), of the membrane potential itself (see ref. 45), and of synergism between different magainins (ref. 41 and unpublished observations). Interestingly, these points suggest that magainin analogues may differ considerably with respect to target specificity.

The Killing Mechanism of Magainins. The effects of PGLa and magainin 2 amide on membrane-linked free-energy transduction and the essential role of membrane-linked free-energy transduction in microbial metabolism suggest that these compounds kill bacteria by causing a decrease in their membrane potential. The effects on membrane potential (and ATP synthesis if we hold the mitochondria to be representative of microbes) are certainly strong enough to make this likely. Also, the effects we observed occurred at the concentrations (about 10 mg/liter) required for these agents to kill bacteria (12, 14). The energetic intermediate in membrane-linked free-energy transduction is the proton electrochemical potential difference—i.e., the electric potential difference plus the pH difference across the membrane—rather than the electric potential difference alone. However, in the systems investigated in this paper, the pH gradient is by far the smaller component of the two. Where appropriate, inspection of external pH changes and of salicylate uptake did not reveal changes in pH gradient that would compensate for the observed dissipation of membrane potential, suggesting that the dissipation of membrane potential by the magainins did invoke dissipation of the proton electrochemical potential difference. That the electric potential difference

between the internal and the external aqueous bulk phases may not be the (only) central intermediate in membrane-linked free-energy transduction (review in ref. 47) is not addressed here.

Sarcotoxin 1, a positively charged peptide produced by flesh-fly larvae as a response to a challenge with bacterial antigens, also has been shown to interfere with bacterial free-energy transduction (7, 8). Small basic peptides isolated from rabbit and human serum and leukocytes have been shown to interfere with bacterial electron transport (32) or membrane permeability (32, 46). These and our experimental results suggest that a primordial antibiotic system that targets membrane-linked free-energy transduction of the intruding organisms extends from prokaryotes (4) through the insect kingdom to higher eukaryotes, and perhaps even (compare ref. 10) to man.

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