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Functional synergism of the magainins PGLa and magainin-2 in *Escherichia coli*, tumor cells and liposomes

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Xenopus laevis skin secretion contains a mixture of magainins, which are small positively charged oligopeptides with antimicrobial activity. In this study, we show that two of these peptides, i.e. magainin-2 and PGLa, are much more active in biological functions when added together than when added alone. This synergy applies for the antimicrobial activity of these peptides, and for the toxic effects on tumor cells. We show that this peptide combination is also synergistic when permeabilizing protein-free liposomes for glucose, when dissipating the membrane potential in cytochrome oxidase liposomes and *Escherichia coli*, and, reversibly, when stimulating respiration in the liposomes. The occurrence of synergy in these diverse systems (complex and simple) suggests that the biological synergy results from synergy in the primary activity of the magainin peptides, namely the permeabilization of free-energy transducing membranes, possibly by forming a multimeric transmembrane pore of mixed peptide composition. The antimicrobial activity of *X. laevis* skin secretions may be greatly enhanced by the application of this binary weapon.

Keywords. Antimicrobial peptides; binary toxins; cooperativity; *Xenopus laevis*; peptide-based immunity.

Glands in the skin of *Xenopus laevis* are responsible for the secretion of a rich mixture of peptides that are often carboxamidated (Richter et al., 1985; Giovannini et al., 1987; Langenberg and Middleton, 1969; Gibson et al., 1986; Zasloff et al., 1988). Of these, the magainins are potent antimicrobial agents (Zasloff, 1987; Soravia et al., 1988; Chen et al., 1988; Bevins and Zasloff, 1990; Boman, 1991). As such, the magainins are analogous to the cecropins (Boman and Hultmark, 1987) produced by the silk worm, sarcotoxin-1 produced by the fly (Okada and Natori, 1983) and defensins produced by human neutrophils (Ganz et al., 1985; Carroll and Martinez, 1981; Young et al., 1986). Magainins and, even more so, proteolysis-resistant variants are also active against various eukaryotic cells including spermatozoa (De Waal et al., 1991) and certain tumor cells (Cruciani et al., 1991; Lincke et al., 1990; Baker et al., 1993).

Through membrane-potential dissipation (Westerhoff et al., 1989b), magainins release respiratory control in rat-liver mitochondria (Westerhoff et al., 1989a) and cytochrome oxidase liposomes (Juretić et al., 1994). Moreover, magainins form channels in black lipid membranes (Duclohier et al., 1989; Cruciani

et al., 1992). Like sarcotoxin-1 (Okada and Natori, 1985), magainins interfere with membrane-mediated energy metabolism in *Escherichia coli* (Westerhoff et al., 1989b). Since this interference occurs at the same concentrations as the antimicrobial activity, we formulated the working hypothesis that membrane permeabilization followed by membrane-potential dissipation is the mechanism by which magainins kill their microbial targets (Westerhoff et al., 1989b).

In view of their tendency to form amphiphilic α -helices (Marion et al., 1988; Chen et al., 1988), the approximately 23-residue, basic, magainins conform to prototypes of channel-forming membrane peptides. Such channels would consist of complexes of peptide monomers (Guy and Raghunathan, 1988; Catterall, 1988; Kaiser and Kezdy, 1987; Lear et al., 1988; Vaz Gomes et al., 1993). Alternatively, the peptides populate the membrane-solution interface (Bechinger et al., 1991) and compromise membrane stability (Williams et al., 1990; Cruciani et al., 1992; Grant et al., 1992). Included in the magainin family are PGLa (Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-NH₂; Gibson et al., 1986; Giovannini et al., 1987) and magainin-2 (Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-NH₂; Zasloff, 1987). When studying the uncoupling activity of these peptides in rat liver mitochondria (Westerhoff et al., 1989a), protein-free liposomes (Vaz Gomes et al., 1993) and cytochrome oxidase liposomes (Juretić et al., 1994), we noted a higher than first-order dependence on peptide concentration. This reinforced the suggestion that a peptide oligomer was responsible for the uncoupling. The possibility

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Abbreviations. (cHxN)₂C, *N,N'*-dicyclohexyl carbodiimide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazide; magainin-2, Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-NH₂; PGLa, Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-NH₂; Ph₄P⁺, tetraphenylphosphonium ion.

that complexes of a number of magainin-2 molecules, or a number of PGLa molecules, are responsible for the action of these peptides, suggests that mixed complexes of magainin-2 and PGLa may also form (Williams et al., 1990) and disrupt membrane energization (Juretić et al., 1989b). In this interpretation, the results of this study show the heterocomplexes to be much more effective than their homocomplex counterparts in interfering with membrane-linked free-energy transduction and the viability of *E. coli* and certain human tumor cells.

MATERIALS AND METHODS

Materials. Cytochrome oxidase (10–11 nmol heme *a*/mg protein) was a gift from Dr W. Caughey. Asolectin was obtained from Fluka, purified as described by Darley-USmar et al., 1987, and stored under nitrogen at -70°C . The preparation of cytochrome oxidase liposomes followed the sodium cholate dialysis procedure (Hinkle et al., 1972; Wrigglesworth et al., 1987). The phospholipid/protein ratio was 50:1 and the respiratory control ratio was 10:1 or higher. Egg phosphatidylcholine, dicetyl phosphate and the glucose oxidase/peroxidase kit for glucose determination were obtained from Sigma.

Magainin-2 and PGLa were the synthetic compounds with carboxyamidated C termini (Soravia et al., 1988). Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and valinomycin were obtained from Fluka; tetraphenylphosphonium (Ph_4P^+) was from Aldrich.

Growth inhibition of *E. coli*. *E. coli* strain W3102 was grown overnight at 37°C with shaking in $0.5 \times$ medium 56 (Monod et al., 1951) supplemented with 0.4% glucose or 1 $\mu\text{g}/\text{ml}$ thiamine and 0.9% succinate. The culture was diluted and approximately 2×10^5 cells were plated in 2.5 ml molten 0.8% agar plates containing medium of the same composition. Subsequently, 10- μl samples of magainin were applied 2 cm apart and the plates were incubated for 2 days at 37°C .

Oxygen consumption by cytochrome oxidase liposomes. The respiratory rate was measured with a Clark oxygen electrode in 1.8 ml 50 mM potassium phosphate, pH 7.4, (Juretić et al., 1994). Background respiratory rates (liposomes absent) and coupled respiratory rates (in the absence of uncouplers or magainins) were also measured. Relative respiration rates were calculated as follows: the background oxygen consumption rate (J_B) was measured at 25°C in the presence of 1.8 ml 50 mM potassium phosphate, pH 7.4, EDTA (0.56 mM), sodium ascorbate (5 mM), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (83 μM), and cytochrome *c* (35 μM). The coupled respiratory rate (J_C) was recorded 3 min after cytochrome oxidase liposomes were added (0.14 mg/ml phospholipid in the oxygraph). The respiratory rate in the presence of peptide (J_P) was measured 1 min after the addition of each aliquot of peptide. The relative rate v_1 was calculated as $(J_P - J_B)/(J_C - J_B)$. This preparation of cytochrome oxidase vesicles had a respiratory control ratio of 12.8 [measured after valinomycin (0.5 μM) and FCCP (0.6 μM) addition]. The enhancement in the respiratory rate caused by the peptides was calculated as $v_0 = v_1 - 1$. v_0 is a normalized measure of the increase in respiratory rate caused by the peptide. The maximal v_0 (v_M) value was estimated to be approximately 10% higher than the actually recorded maximum due to the inhibition of electron flow that occurred with higher peptide concentrations. The parameter $Y = v_0/(v_M - v_0)$ was calculated for each titration point and plotted as $\log(Y)$ versus $\log(\text{peptide concentration})$. The peptide concentration corresponding to the Y value of 1 in the titration was divided by the lipid concentration in the oxygraph. The resulting activity parameter $D_{1/2}$ (μg peptide/mg phospholipid) was used to compare the capability of different

peptides to cause half-maximal uncoupling of respiration rate in the presence of a known amount of liposomes.

EDTA treatment of *E. coli*. *E. coli* D31 cells grown in Luria-Bertani broth (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 5 g/l NaCl, pH 7.4) were harvested in the stationary phase, washed twice, and suspended in ice-cold 120 mM Tris/HCl, pH 7.4. A 1-min incubation at 37°C in the same buffer was followed by 45 min incubation at 37°C in that buffer plus 1 mM EDTA. The incubation was ended by washing the cells in ice-cold 250 mM sucrose, 2 mM Hepes, 0.5 mM EDTA, pH 8 (medium D). After resuspending the cells in the same medium, glycerol was added [to make a 10% (by vol.) mixture] and cells were stored as aliquots at -70°C .

Membrane-potential titrations. Ph_4P^+ was used as a probe for membrane potential developed upon energization of cytochrome oxidase vesicles or *E. coli* cells (Rottenberg, 1979). The uptake of Ph_4P^+ was monitored by a macroscopic Ph_4P^+ -ion selective electrode (modified from Hendler, 1991) in a stirred 25°C reaction chamber that also contained an oxygen electrode and a glass pH electrode (Hendler, 1991). All experiments were performed at 25°C with constant stirring. The aerobic state in the presence of excess substrate was maintained with mixtures of O_2 and Ar blown over the surface of the suspension. In each experiment, the Ph_4P^+ electrode was calibrated by adding Ph_4P^+ in several aliquots until the final concentration was reached. Peptides added in the absence of energy-transducing membranes did not show any effect on the Ph_4P^+ electrode. Cytochrome oxidase liposomes (0.7 mg/ml phospholipid) were added to 6.2 ml 50 mM KCl, 10 mM Hepes, pH 7.4, 0.6 mM sodium EDTA, 85 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 35 μM cytochrome *c*, 43 mM ascorbate, 21 mM potassium phosphate, and 20 μM Ph_4P^+ . To maintain the aerobic state during the titrations, O_2 was blown over the surface of the reaction mixture at a rate of 36 ml/min.

Binding of tritiated magainins to cytochrome oxidase liposomes. Tritiated magainin-2 (labelled at Gly1) was diluted 25 times in 2 mg/ml unlabelled magainin-2. 8 μl sample gave an average of 33000 dpm (ACS scintillation cocktail). 1-ml plastic centrifuge tubes were used. Each tube had 500 μl 50 mM potassium phosphate, pH 7.4, cytochrome oxidase vesicles at the indicated amount (mg dry mass phospholipid) and peptides. The respiratory control ratio was low in this preparation (7.7 after valinomycin and FCCP addition). This solution was mixed with 8 μl peptide stock (to give approximately 30000 dpm) on a vortex mixer, and centrifuged at 100000 rpm (Beckman TL-100), for 10 min at 15°C . The supernatant was decanted (the pellet was very tight) directly into the scintillation vial. The pellet was carefully mixed with a thin glass rod in the presence of 250 μl 1.7% SDS. The solution was stirred on the vortex mixer, decanted into the scintillation vial, and the entire operation was repeated with another 250 μl SDS.

Glucose efflux from liposomes. Liposomes of egg phosphatidylcholine and dicetyl phosphate in a molar ratio of 9:1, were prepared by the extrusion method as described in MacDonald et al. (1991). The lipid mixture was vacuum dried and hydrated with 10 mM Hepes, 50 mM glucose, pH 7.0, to a final lipid concentration in the suspension of 10 mg/ml. After freeze-thawing five times (Mayer et al., 1985), the lipid suspension was extruded five times through Nucleopore filters of 400-nm pore diameter, followed by another series of five extrusion steps through Nucleopore filters of 200-nm diameter. The Liposofast system was used as an extruding device. Before the glucose efflux assays were performed, the external medium was replaced by 10 mM Hepes, 25 mM NaCl, pH 7.0, by column centrifugation through Sephadex G-50 (Penefsky, 1977).

The efflux of glucose was monitored spectrophotometrically using the glucose oxidase/peroxidase assay. In this assay, the formation of reduced *o*-dianisidine is monitored at 450 nm. We have used the assay conditions suggested by Sigma, except the concentration of the glucose-oxidase/peroxidase enzymes was twofold higher, to allow for continuous monitoring at room temperature (approximately 25 °C). The reference cuvette contained 100 μ l 10 mM HEPES, 25 mM NaCl, pH 7.0, and 1 ml combined reagent (glucose-oxidase/peroxidase enzymes and oxidised *o*-dianisidine). The control for intrinsic glucose leakage of the liposome population contained 90 μ l buffer and 10 μ l lipid suspension (90 μ g/ml final lipid concentration), plus the combined reagent (1 ml). To study the membrane permeability induced by the different magainin peptides, the appropriate volume of peptide solution was added, subtracting accordingly the volume of buffer added. Monitoring started immediately after mixing the assay mixture by inversion five times. The initial rates were linear up to a concentration of 5 μ M glucose, and the response was immediate when an external standard of glucose (1.27 μ M) was added to an assay mixture containing liposomes and peptides.

Clonogenicity assay of mammalian cells. The human melanoma cell line BRO (Lockshin et al., 1985; for possible contamination with retrovirus-like activity see Schinkel et al., 1993) was cultured on HAM F10 medium (no. 074-1200, Gibco), supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere supplied with 5% CO₂ at 37 °C. The synergism between magainin-2 and PGLa was determined from dose-response curves using a slightly modified clonogenicity assay. Exponentially growing cells were treated with trypsin and transferred to wells of Terasaki microtest plates (3034F; Falcon) resulting in approximately 300 cells for each 30 μ l growth medium containing various concentrations of magainin-2, PGLa, or a mixture of these drugs. After incubation for 75 min (the microtest plate was incubated upside down to avoid anchorage), the cells were transferred again and plated in wells of a tissue-culture cluster (no. 3424; Costar, Cambridge, MA) to grow for 10 days in normal medium. After fixation and staining with 0.2% crystal violet (Merck 820603) in 3.7% glutaraldehyde, colonies of at least 100 cells were counted. The amount of surviving cells was determined as the number of such colonies grown from treated cells relative to those grown from untreated cells. From the dose-response curves, the *D*₁₀ values were obtained representing the concentration of magainin peptides that allow 10% of the cells to form a colony after a 75-min treatment.

RESULTS

Synergism between PGLa and magainin-2 in inhibiting bacterial growth. The magainins were discovered on the basis of their antimicrobial activity (Zasloff, 1987). To determine if the combination of magainin-2 and PGLa has additional activity when compared to either of the peptides alone, we measured the ability of *E. coli* strain W3102 to grow in the presence of these peptides. W3102 was grown overnight at 37 °C in minimal medium and was plated in top agar of the same medium. 10- μ l samples of magainin-2 or PGLa were spotted onto the surface of the plate and the plates were incubated at 37 °C. For each peptide, a circular zone (diameter approximately 2 cm) appeared where the peptides diffused through the top agar and prevented the growth of the cells (Fig. 1A). In the regions where magainin-2 and PGLa could interact with each other (Fig. 1A), additional inhibition was observed. No such zone of growth inhibition appeared at the junction of two spots of the same peptide (Fig. 1B). This demonstrates a synergistic antimicrobial effect of PGLa and magainin-2.

Fig. 1. Synergism between PGLa and magainin-2 in inhibiting the growth of *E. coli*. (A) PGLa (10 μ g/ml) (P) and magainin-2 (30 μ g/ml) (M). (B) Duplicate samples of PGLa or magainin-2 were placed as indicated. X, regions where synergism is observed; O, no synergism detected.

Fig. 2. Synergistic interference of magainin-2 and PGLa with a clonogenicity assay using human melanoma cells, BRO. Magainin-2 alone (●), PGLa alone (▲) and a 1:1 mixture (■) are based on two experiments each, performed in duplicate. Data for mixtures of M and P in the ratios 1:5 (○) and 5:1 (+) were obtained from single experiments in duplicate.

Synergistic action of PGLa and magainin-2 against tumor cells. Magainins have also been shown to compromise the viability of mammalian tumor cells (Cruciani et al., 1991; Baker et al., 1993). Combinations of magainin peptides have not been investigated by these authors for the presence of synergism. To examine whether PGLa and magainin-2 are also synergistic for tumor cells, we incubated cells of a human melanoma cell line for 75 min in the presence of either peptide, or of a mixture of the two and determined their clonogenicity. As judged after 10 min by microscopic inspection of the exclusion of Hoechst 33258, the synergistic combination of the peptides was more active than the individual compounds (data not shown).

In terms of the effect on proliferation capacity of the tumor cells, the synergism was even higher. Whereas 0.65 mg/ml magainin-2 and 1.0 mg/ml PGLa were required to reduce the number of colonies observed 10 days after plating of the cells to 10%, ten times less of the 1:1 mixture was sufficient to produce the same effect (Fig. 2). The synergism of the 1:1 mixture was much higher than that of the 1:5 mixtures.

Synergistic membrane-potential dissipation in *E. coli*. To examine at which level of complexity the two peptides act syn-

Fig. 3. Synergistic effect of magainin-2 and PGLa on membrane potential in *E. coli*. The titration of apparent membrane potential with magainin-2 (M) (40-nmol aliquots that increase magainin-2 concentration in 5.4 μM steps; upper trace) and with magainin-2 after PGLa (P) (10-nmol aliquot bringing the PGLa concentration to 1.4 μM ; lower trace). EDTA-treated *E. coli* D31 cells with an absorbance of 30 at 600 nm were added (0.4 ml) to 7 ml medium D (250 mM sucrose, 2 mM Hepes, 0.5 mM EGTA, pH 7.4) and 20 μM Ph_4P^+ . The solution was stirred and maintained at 25°C. A mixture of oxygen (12 ml/min) and argon (70 ml/min) was blown over the solution continuously except for the anaerobic period (▨) when oxygen was shut off. Other additions were succinate (S; 1.4 mM), (cHxN)₂C (D; 13.5 $\mu\text{g}/\text{ml}$), valinomycin (V; 0.13 μM) and nigericin (N; 0.07 μM).

Fig. 4. Synergistic effect on membrane potential in cytochrome oxidase liposomes. Peptide additions were magainin-2 (M represents addition of 8 nmol or 1.1 μM), or PGLa (P represents addition of 5 nmol or 0.7 μM). 2.5 M and 2P represent addition of 2.5 times and 2 times these amounts of M and P, respectively.

gistically, we also determined the combined activity in the processes presumed to be responsible for magainin action. We showed previously that PGLa or magainin-2 alone can dissipate the membrane potential in *E. coli* (Westerhoff et al., 1989b). Again we used Ph_4P^+ uptake, now to probe for synergistic effects on this membrane potential. The initial upward shift in Fig. 3 corresponds to a decrease in the Ph_4P^+ concentration due to binding to the cells and to the equilibration of the medium

Fig. 5. Synergistic effect of PGLa and magainin-2 on respiration in cytochrome oxidase liposomes. (A) Uncoupling of respiration with magainin-2 (Δ), PGLa ($\circ$), and with PGLa after magainin-2 addition (4.4 $\mu\text{g}/\text{ml}$) ($\bullet$). (B) Evaluation of cooperativity parameters from these titrations. The slope n in this log/log plot reflects the degree of cooperativity between peptide monomers (Juretić et al., 1994).

with the cytoplasm, which has a negative electric potential with respect to the outside of the cells. Addition of succinate produced a further decrease in the external Ph_4P^+ concentration, because of the increase in the apparent membrane potential. Addition of dicyclohexyl carbodiimide [cHxN)₂C] appeared to decrease the membrane potential slightly. The onset of anaerobiosis decreased the membrane potential further. At the end of the titration with peptides, valinomycin and nigericin were added to ensure the complete dissipation of the membrane potential.

The effect of the first addition of magainin-2 alone on the membrane potential of *E. coli* (Fig. 3) was quickly reversed, most probably due to proteolytic cleavage of magainin-2 (Juretić et al., 1989a). The same amount of magainin-2 added after a much smaller amount of PGLa had been added (Fig. 3) caused a profound membrane-potential decrease, also suggesting a significant synergism between PGLa and magainin-2 in this action. Changes in the reading of the Ph_4P^+ ion-selective electrode can be converted into apparent membrane potential changes (Hendler et al., 1983). We estimated (data not shown) that the second addition of magainin-2, in the magainin-2 titration (Fig. 3), caused a 10% decrease in the apparent membrane potential. The first addition of magainin-2 in the mixed titration, i.e. after PGLa, caused a 25% decrease in the apparent membrane potential.

Table 1. Half-maximal uncoupling of respiration in cytochrome oxidase liposomes by different mixtures of PGLa and magainin-2. The calculation of $D_{1/2}$ (μg peptide/mg phospholipid) for the titration curves obtained by adding increasing amounts of different PGLa/magainin-2 mixtures was performed as described in the Materials and Methods section.

Experiment	Magainin-2	PGLa	$D_{1/2}$
	%		
			$\mu\text{g}/\text{mg}$
1	100	0	69
2	70	30	14
3	50	50	11
4	30	70	13
5	0	100	17

Magainin-2 enhances the uncoupling and depolarizing activities of PGLa in cytochrome oxidase liposomes.

The membrane potential of intact *E. coli* cells is a complex function of the activity of a multitude of processes. To probe for the basis of the synergistic effect, we directed our attention to a model system for studying the interference of magainins with membrane-mediated free-energy transduction, namely cytochrome oxidase liposomes (Juretić et al., 1989a, 1994). Cytochrome oxidase vesicles develop a membrane potential in the presence of ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and cytochrome *c*. In Fig. 4, we show the effects of magainin-2 and PGLa, added separately, and in combination, on the apparent membrane potential. In the presence of 20 nmol magainin-2, 5 nmol PGLa (0.7 μM in the reaction chamber) caused a quick

and pronounced decrease in the membrane potential. In the absence of magainin-2, the kinetics of the membrane-potential change provoked by PGLa were slower and the extent was less pronounced.

Since the rate of oxygen consumption in well-coupled cytochrome oxidase liposomes is strongly inhibited by membrane potential, the stimulation of that respiration rate by magainin is expected. Indeed, magainin-2 and PGLa increased the rate of oxygen consumption (Fig. 5). That synergism exists between PGLa and magainin-2 is also evident from Fig. 5A. In the presence of a small amount (4.4 $\mu\text{g}/\text{ml}$) of magainin-2, PGLa was approximately ten times more effective than in the absence of magainin-2.

The sigmoidal character of the curves, which is indicative of cooperativity between peptide monomers, can be quantified as a cooperativity parameter (Juretić et al., 1994; Vaz Gomes et al., 1993), which is in this case (Fig. 5B) 4.3 for magainin-2, 2.5 for PGLa, and 1.7 for PGLa titration after magainin-2 addition. $D_{1/2}$ was defined as the peptide to phospholipid ratio (by mass) causing half-maximal uncoupling of respiration. From the results presented in Fig. 5, $D_{1/2}$ decreased from 99 $\mu\text{g}/\text{mg}$ for the titration of respiratory rate with magainin-2 alone to 39 $\mu\text{g}/\text{mg}$ for the titration with PGLa alone, and to 4 $\mu\text{g}/\text{mg}$ when a small amount of magainin-2 was present during titration with PGLa. Additional evidence for the synergism between these two peptides is presented in Table 1. The peptides were most active in uncoupling the respiration when their ratio was 1:1 (by mass).

Synergistic increase in glucose permeability. Magainins have been shown to increase the permeability of membranes in a non-selective manner (Duclohier et al., 1989; Westerhoff et al., 1989a; Matsuzaki et al., 1990; Grant et al., 1992; Vaz Gomes

Fig. 6. Magainin-2-induced and PGLa-induced increases in glucose permeability of protein-free liposomes. The external appearance of glucose is monitored spectrophotometrically at 450 nm, by reduction of *o*-dianisidine (glucose oxidase/peroxidase). The upper trace shows the intrinsic glucose efflux of the liposomal population (egg phosphatidylcholine/dicetyl phosphate molar ratio 9:1). At the times indicated, 20 μl Triton X-100 was added or an external standard of glucose to a final concentration of 1.27 μM . The lower traces show the efflux of glucose induced by addition of 8 $\mu\text{g}/\text{ml}$ of magainin-2, of PGLa or of a 1:1 mixture of the two peptides, respectively. Also indicated, on each trace, is the extent of reduced *o*-dianisidine formed after a 30-min incubation. 1 unit corresponds to an A_{450} of 0.01.

Fig. 7. In the presence of liposomes, PGLa protects magainin-2 from pronase action. In the lower trace, pronase can reverse the uncoupling of respiration caused by magainin-2 (11 μ M). After sodium EDTA, sodium ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, cytochrome *c*, and pronase (Pr; 0.056 mg/ml), magainin-2 (M; 11 μ M) was introduced 2 min before the addition of the oxidase liposomes (L; 0.17 mg/ml), and again, as indicated. Valinomycin (V; 0.56 μ M) and FCCP (F; 0.56 μ M) were added when indicated. In the upper trace, PGLa prevents the reversal by pronase. The second magainin-2 addition (11 μ M), added 2 min after PGLa (P; 1.4 μ M), produced a constant uncoupled respiration rate, that did not change significantly even after two additional aliquots (0.056 mg/ml) of pronase, and the usual amounts of valinomycin and FCCP, were added (data not shown).

Table 2. Binding of tritiated magainin-2 to cytochrome oxidase liposomes in the presence and absence of PGLa. The amount of added label in the supernatant and pellet fractions and total recovery after centrifugation are reported, with the standard error of the mean in parentheses. The volume was 0.5 ml.

Lipo- somes	Maga- inin-2	PGLa	Super- natant	Pellet	Reco- very	<i>n</i>
mg	μ g		%			
0	16	0	89	5	94	1
0.25	16	0	81	6	87	1
0.25	16	50	72	9	81	1
2.5	16	0	75(6)	19(4)	94(3)	3
2.5	16	50	55(6)	34(3)	89(5)	3

et al., 1993). To examine if the synergism extended to include this non-selective action we determined the effects of magainin-2, PGLa and their combination on the efflux of glucose from protein-free liposomes.

The magainin-induced (8 μ g/ml) efflux of glucose out of egg phosphatidylcholine/dicetyl phosphate (9:1 molar ratio) large unilamellar liposomes is shown in Fig. 6. When no magainin peptides were added, this liposomal preparation showed an intrinsic glucose efflux of 0.15 arbitrary absorbance units/min (one absorbance unit corresponds to A_{450} 0.01). The initial rates of glucose efflux, measured for magainin-2, PGLa and the mixture (1:1) of the two peptides at the same total concentration, were 0.21, 0.35 and 0.90 absorbance units/min, respectively. The synergism between magainin-2 and PGLa is revealed by these values. After 45 min, the extent of glucose leakage for magainin-2, PGLa or the mixture was steady and amounted to 19, 25 and 28

absorbance units, respectively. Apparently, the synergism between the two peptides is more pronounced in terms of the rate of permeabilization than in terms of the extent of permeabilization of the liposomes.

PGLa protects magainin-2 from pronase inactivation in the presence of cytochrome oxidase liposomes. The increased effectiveness of magainin-2 in dissipating the membrane potential and inhibiting bacterial growth in the presence of PGLa, could be due to a decreased rate of magainin-2 degradation in the presence of PGLa, and/or to an enhanced permeabilizing activity of a presumed PGLa/magainin-2 complex. Our experiments with the reconstituted system suggest that the second explanation is valid, but we wanted to investigate whether PGLa can also protect magainin-2 from hydrolytic degradation in the presence of the lipid phase.

The uncoupling of respiration, which can be measured as an increase in the relative respiration rate (Fig. 5 A) or as a decrease in the respiratory control ratio, is a convenient assay for measuring the activity of small concentrations of magainins (Juretić et al., 1989a, 1994). In Fig. 7, magainin-2 alone and magainin-2 added after a small amount of PGLa are compared with respect to their uncoupling activity in the presence of the proteolytic enzyme pronase. In Fig. 7, magainin-2 was added in an amount (11 μ M, i.e. 27 μ g/ml) sufficient to produce the fully uncoupled respiratory rate, provided liposomes were already present (compare the second addition to first addition in the lower trace; see also Juretić et al., 1994 and Fig. 5A). However, in the absence of PGLa the inactivation of magainin-2 by the previously added pronase, quickly returned this rate to the level of coupled respiration. Subsequent valinomycin and FCCP addition were still capable of increasing the respiratory rate to the maximal level for this preparation in the absence of pronase. This maximal level was the same as that reached if magainin-2 was added alone (data not shown).

When a small amount of PGLa had been added prior to the addition of magainin-2 (the upper trace in Fig. 7), the increase in rate persisted, indicating that in this case, magainin-2 was protected from pronase activity. The addition of valinomycin and FCCP (data not shown) did not change the respiratory rate reached after PGLa and magainin-2 addition. It should be noted in Fig. 7 that the synergistic protection of magainin against pronase depended on the presence of liposomes at the moment of addition of the peptides.

Binding of tritiated magainin-2 is enhanced by the presence of PGLa. Protection of magainin-2 from pronase action could be due to the formation of an aqueous complex between the two peptides that is more resistant against pronase digestion. Since the protection depends on the presence of liposomes however (Fig. 7), this possibility is unlikely. Rather, PGLa addition may enhance the binding of magainin-2 to the lipid phase where it is inaccessible to pronase action (Juretić et al., 1989a).

Table 2 shows that magainin-2 bound to cytochrome-oxidase-containing liposomes, when the latter were presented at 5 mg phospholipid/ml. Added unlabelled PGLa enhanced the binding of magainin-2. This suggests that at least part of the functional synergism results from an increased affinity of the presumed PGLa/magainin-2 complex, to membranes.

It may be noted that at a concentration of phospholipids of almost three times that used in Fig. 7 (0.17 mg/ml), only a fraction of magainin-2 was pelleted when centrifuged, even in the presence of excess PGLa. When the liposome concentration was increased tenfold, only one third of magainin-2 was pelleted by centrifugation. Our liposomal preparation is probably hetero-

geneous and magainin-2 also binds to liposomes (e.g. those that lack cytochrome oxidase) that are lighter than the medium.

DISCUSSION

The antimicrobial activity of the secretion of the granular gland of *X. laevis* is not only due to magainin-1 and magainin-2 (Zasloff, 1987), but is also due to other small cationic peptides such as PGLa and xenopsin precursor fragment, XPF (Soravia et al., 1988). These peptides uncouple free-energy transduction in bacteria, mitochondria and cytochrome-*c* oxidase vesicles most probably by permeabilizing the membranes to small ions (Westerhoff et al., 1989a,b; Juretić et al., 1989a, 1994; Duclozier et al., 1989; Cruciani et al., 1991; Vaz Gomes et al., 1993). In this study, we report that in the case of magainin-2 and PGLa the combination of these two peptides has a much stronger activity than either peptide alone. The synergism in membrane depolarization was also observed between magainin-1 (non-amidated) and PGLa (data not shown).

The key aspect of the present study is that the peptides act synergistically in their biological activities. First, strong synergism appears where the peptides are supposed to function; i.e. in antimicrobial action. Secondly, this functional synergism extends to cytotoxicity for other cell types, including tumor cells where magainins may have a pharmaceutical future (Cruciani et al., 1991; Baker et al., 1993; compare De Waal et al., 1991). In addition, we showed that the synergism occurs at the mechanistic level, i.e. with respect to their biochemical and biophysical activities *in vitro*. The combination of magainin-2 and PGLa stimulated respiration in cytochrome oxidase liposomes more than either peptide alone (compare Juretić et al., 1989b). Moreover, glucose permeability of protein-free liposomes was induced synergistically. The latter result is in agreement with studies with the artificial permeability probes carboxyfluorescein and calcein (Williams et al., 1990; Vaz Gomes et al., 1993).

The present study focuses on the occurrence of synergism between PGLa and magainin-2. This should not obscure the fact that synergism is a special rather than a common phenomenon with membrane-active peptides. Various magainin peptide combinations do not exhibit synergism. Indeed PGLa in combination with any of a set of magainin-2 analogues is particularly active in terms of synergism (compare De Waal et al., 1991; Vaz Gomes et al., 1993). This phenomenon may identify not only a biological function of these two naturally occurring peptides, but also may provide information with respect to the structural requirements for the proposed membrane-active peptide complex.

Although additional effects of magainins should not be excluded, their membrane permeabilization and depolarization and consequent interference with membrane-mediated free-energy transduction is a well-documented chain of events that can account for their cytotoxicity. Additional or even alternative mechanisms for the cytotoxic action cannot be excluded however. It is now pertinent to ask what extent the observation of synergism between magainin-2 and PGLa will provide information on the mechanism of cytotoxic action of the magainin peptides. First, the fact that the synergism between the two peptides was observed in all experimental systems, from the liposomal permeation of neutral molecules to anti-tumor cell activity, is in agreement with the concept that the permeabilization is responsible for the cytotoxicity. Indeed, it is quite feasible that the combination of the two peptides has increased membrane-permeabilizing activity due to the enhanced formation of a membrane-active complex, or to a higher intrinsic permeability of a membrane-active complex consisting of both peptides.

Secondly, that the active magainin form is a complex of magainin peptides (possibly including lipids) rather than a monomer, was suggested on the basis of the higher than first-order concentration dependence of the uncoupling of respiration in mitochondria and cytochrome oxidase liposomes (Westerhoff et al., 1989a; Juretić et al., 1994) and the permeabilization of protein-free liposomes (Vaz Gomes et al., 1993). Due to the lack of assays that are a linear function of peptide concentration, it has been more difficult to demonstrate (homo)cooperativity in terms of biological function. The synergism shown in Figs 1 and 2 demonstrates that the effect on biological function of the formation of a magainin multimer may also be relevant.

Thirdly, our experiments with a simple reconstituted system, cytochrome-*c* oxidase vesicles, demonstrated that (a) the presence of a membrane phase retards the inactivation of these peptides by added proteolytic enzyme, and (b) a mixture of PGLa and magainin-2 is less sensitive to protease than magainin-2 alone. This observation is most easily understood in terms of enhanced binding of a PGLa/magainin-2 heterocomplex as compared to a magainin-2/magainin-2 homocomplex to the phospholipid membrane, where both peptides are better protected from pronase hydrolytic cleavage. Accordingly, the binding of tritiated magainin-2 to liposomes was enhanced in the presence of PGLa (Table 2).

A fourth and quite crucial conclusion stems from our observation that the uncoupling action of a PGLa and magainin-2 mixture was reversed by pronase (with suitably chosen peptide concentrations), i.e. magainin action is not a lytic event of irreversible nature.

In view of the functional activity of possible heterocomplexes, the finding of mRNA (actually cDNA) molecules that each contain the information for a large prepro-polypeptide comprising a number of magainin molecules (Zasloff, 1987; Terry et al., 1988; compare Hunt and Barker, 1988) may be relevant; the different magainin species encoded by a single mRNA might function synergistically. However, at least in one case, the mRNA encoded one complete magainin-1 and five complete magainin-2 sequences (Terry et al., 1988), whereas these two magainins do not synergize much (De Waal et al., 1991).

With respect to these possibilities, the results of the present study suggest that complexation of different transmembrane peptides greatly enhances their membrane permeabilization effect as compared to the complexation of identical peptides. As for *X. laevis* itself, the putative PGLa/magainin-2 complex may be the antimicrobial binary weapon of choice in the frog; either of the peptides alone may be relatively harmless to both target and host, but in response to infection, *Xenopus* may solicit the combination of the two peptides so as to produce the more active peptide mixture only when and where it is needed. PGLa and magainin-2 immune reactivity have been detected in single granules (Moore et al., 1992). The toxic synergistic combinations may arise only after processing of the prepro-polypeptides.

The *in vivo* synergistic action may therefore be the biological reason for the diversity of antimicrobial peptides found in the granular gland secretions. There is no compelling reason to suspect that synergism between magainins is confined to the combination of PGLa and magainin-2. Indeed, there is the possibility that the putative most active antimicrobial complex consists of six different magainins. Such a heterocomplex could be superior to the presumed homocomplexes by an increased tendency to adsorb onto or into the target membrane, providing the advantages of being less accessible to aqueous proteases and closer to the site of action.

A different type of synergistic combination was shown by Darveau et al. (1991), when the simultaneous action of magainin-2 and β -lactamic antibiotics was studied in *E. coli*. This type

of synergism suggests initial weakening of the bacterial outer membrane by the β -lactam, facilitating the action of the magainin peptide, and not the actual formation of a mixed complex.

It remains to be established if other natural-peptide-based defense systems also exhibit synergism and if this phenomenon may be exploited when using peptides to eliminate undesired cell types (De Waal et al., 1991; Baker et al., 1993).

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