Electric Potentiation, Cooperativity, and Synergism of Magainin Peptides in Protein-Free Liposomes†

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ABSTRACT: Magainins, positively charged peptides present in the skin of *Xenopus laevis*, are known to permeabilize free-energy transducing membranes. Structural studies in otherwise protein-free model systems show α-helical magainins parallel to the membrane water interface. However, functional studies in biological membranes suggest that magainins operate as oligomeric complexes. Here we investigate whether magainins *function* as oligomers in protein-free liposomes also. We report that they do exhibit strong positive heterocooperativity. The magainins, magainin 2 and PGLa, act synergistically. Both activity and cooperativity are enhanced by *net* negative charge of the liposomal membranes. A transmembrane electric potential, negative inside, enhanced the activity of the peptides. We propose a model in which (i) binding to the surface of the membrane, mainly guided by electrostatic interactions, occurs and (ii) the bound form is in equilibrium with an *n*-meric complex of magainins spanning the membrane.

Magainins are 23-residue long, basic peptides found in the skin of *Xenopus laevis*. They belong to a general family of membrane-active peptides, including PGLa[1] and the precursor fragments of xenopsin, caerulein, and levitide (Zasloff, 1987; Andreu et al., 1985; Bevins & Zasloff, 1990). Recently, these peptides were also detected in the stomach of *X. laevis*, in addition to another peptide, PGQ (Moore et al., 1991). The frog skin as eukaryotic source of antibiotic peptides is not unique; also in phagocytes, and in insects' hemolymph, biologically active peptides (defensins, sarcotoxins, and cecropins) have been identified (Boman, 1991). Magainins were proposed to be cytotoxic because they permeabilize free-energy transduction membranes for ions (Westerhoff et al., 1989a,b; Juretic et al., 1989a). The hypothetical channel-forming properties of magainins were further studied in synthetic bilayers at the tip of patch-clamp pipettes (Duclohier et al., 1989; Cruciani et al., 1991, 1992). Duclohier described the "channel" as anion-specific. Later, Cruciani et al. (1991, 1992) proposed cation specificity for magainin 2 channels, on a ratio of cations over anions of 5:1.

Most studies aimed at the functional basis of the broad spectrum antibiotic activity of magainins have been performed in complex proteinaceous systems, such as whole cells or isolated mitochondria (Juretic et al., 1989a; Westerhoff et al., 1989a,b; de Waal et al., 1991). The effects of magainins in these very diverse model systems all suggested positive cooperativity in their mechanism of action. This cooperativity was revealed as supralinear concentration dependence in the stimulation of respiration of isolated rat liver mitochondria, cytochrome *c* oxidase reconstituted liposomes, and hamster spermatozoa (Westerhoff et al., 1989a,b; Juretic et al., 1989a; de Waal et al., 1991) or by the inhibition of hamster spermatozoa motility (de Waal et al., 1991). Such cooperativity suggests the formation of a complex (consisting of a certain number of monomers) constituting the active form of magainins (Westerhoff et al., 1989a,b). Due to their size and amphiphilic properties, it is conceivable that magainins form transmembrane α-helices.

A structural model for a magainin "channel" was proposed by Guy and Raghunatan (1988). In this initial model, the magainins would form a transmembrane complex. Later, a model in which the lining of the channel would be formed by lipid head groups, with the magainins forming a two-dimensional hexagonal array on the surface of the membrane, was proposed (Cruciani et al., 1991, 1992). The magainin peptides would stabilize the orientation of the lipid head groups lining the channel. The ion specificities observed in the studies of Cruciani et al. (1991, 1992) using black lipid membranes would constitute experimental evidence for such a channel.

Two-dimensional NMR (Marion et al., 1988), rotational-echo double-resonance (REDOR) NMR (Hing, et al., 1991), and circular dichroism (CD) (Chen et al., 1988; Matsuzaki et al., 1989) studies showed increased helical content for magainins in hydrophobic environments, such as trifluoroethanol, but also in lipid vesicles, a much more realistic model system for a membrane environment. Recently, solid-state NMR spectroscopy studies supported the idea that magainins lie in the plane of the bilayer (Bechinger et al., 1991). However, it remains uncertain if such a conformation corresponds to the active form of the peptides, observed in functional studies. At the moment we are left at the position that structural studies in protein-free systems have looked at

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[1] Abbreviations: DCP, dicetyl phosphate; $\Delta\Psi$, transmembrane electric potential; ΔpH, transmembrane pH gradient; DOPG, dioleoylphosphatidyl-DL-glycerol; DPPG, dipalmitoylphosphatidyl-DL-glycerol; magainin 2a, 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-$CONH_2$; magainin A, synthetic analogue of magainin 2, where Gly_1 is substituted by β-Ala and Ser_8, Gly_{13}, and Gly_{18} by Ala, carboxy amidated; magainin 1, the same sequence as magainin 2 except that Lys_{10} is substituted by Gly and Asn_{22} by Lys; PA, L-α-dipalmitoylphosphatidic acid; PC, fresh egg yolk L-α-phosphatidylcholine; PGLa, H_2N-Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-$CONH_2$; PGQ, H_2N-Gly-Val-Leu-Ser-Asn-Val-Ile-Gly-Tyr-Leu-Lys-Lys-Leu-Gly-Thr-Gly-Ala-Leu-Asn-Ala-Val-Leu-Lys-Gln-COOH; PS, L-α-phosphatidylserine; SA, stearylamine; SUVs, small unilamellar vesicles.

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predominantly monomeric magainin, whereas magainins seem to function as an oligomer.

The action of membrane-active peptides is generally described by an initial step of binding, thought to be driven by electrostatic forces (Beschiaschvili & Seelig, 1991; Kim et al., 1991; Madhavi Sekhraram et al., 1991; Tamm, 1991), followed by membrane insertion. The effect of cecropins, subtilin, and melittin is documented as following such a mechanism (Christensen et al., 1988; Kordel et al., 1989; Schwarz & Beschiaschvili, 1989). Electrophoretic effects due to a transmembrane electric potential (Kamp et al., 1988) may contribute to a more efficient insertion of the peptide into the membrane. Such potentiating effects of a transmembrane potential have been described for the incorporation of the presequence that directs import into the mitochondria of subunit IV of cytochrome *c* oxidase, a 25-residue long basic peptide (Roise et al., 1986), and also for an artificially designed basic hexa- or pentapeptide, bearing more than one positive charge (de Kroon et al., 1991; de Kroon, 1991).

Magainins 1 and 2 showed stronger effects on negatively charged SUVs (PS,DOPG, DPPG, and mixtures of PC/PA, PC/PS, or PC/DCP), 9:1 each, in comparison to neutral membranes (Matsuzaki et al., 1989, 1991). For the action of magainin 2 on PS SUVs, Matsuzaki et al. (1991) focused on the interpretation that each monomer caused a small perturbation on the lipid packing and that the accumulation of such disturbed sites caused leaky patches rather than channels. Recently, Grant et al. (1992) studied the action of magainin 2 amide on PS liposomes. These authors observed magainin 2 amide-induced leakage of carboxyfluorescein-loaded liposomes and examined the binding between peptides and lipids. They concluded that magainin addition to small unilamellar liposomes leads to a transient destabilization of the bilayer, allowing for passage of carboxyfluorescein. None of these papers have considered the possibility of positive cooperativity between magainin monomers or possible potentiating effects of transmembrane electric potentials.

Electric fields around and in membranes depend on lipid composition, protein content (and composition), and transmembrane electric potential differences. In the functional studies of magainin action, the membranes contained protein, carried net negative charge, and had electric potentials across them. Hence, we considered the possibility that insertion of magainins as a transmembrane oligomer into the membranes depends on such electric fields and that only this transmembrane oligomer is functional. Other structures may exist and even be in excess, but these may not be responsible for the permeabilizing activity of magainins.

In this work, we therefore investigated whether in protein-free liposomes, devoid of a transmembrane electric potential, the action of magainins exhibits cooperativity. We have also studied how the net membrane charge and the transmembrane electric potential affect the cooperative action of the peptides.

We observed that the functional cooperativity and activity do not require the presence of other membrane proteins but are enhanced by electrostatic effects.

MATERIALS AND METHODS

Egg yolk L-α-phosphatidylcholine (PC), dicetylphosphate (DCP), stearylamine (SA), soybean asolectin, calcein, cobalt chloride, valinomycin, and nigericin were obtained from Sigma. Magainin 2 amide (referred to as magainin 2) and PGLa were synthetic peptides (Zasloff et al., 1988; Andreu et al., 1985) identical to the natural compounds.

Small unilamellar vesicles (SUVs) (New, 1990) were obtained after 1 h of sonication under nitrogen flow, in an ice bath, starting from a hydrated, vacuum-dried lipid film (10 mg of lipid *per* mL, at lipid composition indicated in molar ratios in the figure legends). Debris from the sonicator tip was removed by centrifugation. Homogeneity of the liposomal population was evaluated by negative staining, followed by electron microscopy. No significant number of multilayered liposomes were present. Media and lipid composition are specified in the figure legends. Untrapped medium was removed by minicolumn centrifugation through Sephadex G-50 (coarse grade) (Penefski, 1977). Phospholipid concentrations were determined by inorganic phosphorus analysis (Ames & Dubin, 1960).

Magainin-induced membrane permeability was monitored by quenching of calcein fluorescence by Co^{2+}, with calcein and Co^{2+} being initially in separate compartments (Oku et al., 1982). Measurements were performed at room temperature, in a Perkin-Elmer fluorescence spectrophotometer MPF-2A. The excitation and emission wavelengths were 495 and 515 nm, respectively, using excitation and emission slits of 4. An aliquot (50 μL) of the vesicle suspension was added to 2.5 mL of medium (180 μg/mL lipid concentration in the fluorescence assay). Throughout a series of assays, the fluorescence signal before addition of the peptides was the same. Upon addition of the peptides, the fluorescence signal decreased to a plateau value (see Figure 1). Complete quenching was only achieved after addition of 50 μL of 10% Triton X-100 or cholate. Initial rates of decrease in calcein fluorescence were measured.

A transmembrane K^+-diffusion potential, negative inside, was generated in soybean asolectin vesicles. In this case, the medium inside the vesicles contained 150 mM KCl. The external medium contained LiCl at the same concentration, instead of KCl, and the SUVs were added to this medium just before each assay. The diffusion potential was generated by addition of 1 μL valinomycin (final concentration 76 ng/mg of lipid) in dimethyl sulfoxide (DMSO) and confirmed by a Rhodamine 123 assay as described (Emaus et al., 1986). Unlike ethanol, DMSO did not affect membrane permeability.

RESULTS

We assayed magainin activity in (otherwise) protein-free liposomes by measuring the fluorescence quenching of entrapped calcein by external Co^{2+}. Quenching is dependent on permeabilization of the liposomal membrane. Figure 1 shows the effect on the fluorescence of the addition of magainin 2, PGLa, or the mixture (1:1) of the peptides to PC/DCP (9:1) liposomes. Upon addition of the peptide, fluorescence decreased to a lower level. Generally, adding more, or more active, peptides led both to an increase in the initial rate of fluorescence quenching and to a decrease in the ultimate level of fluorescence. In the following, we shall focus on the initial rate of fluorescence quenching. In terms of the scheme proposed by Grant and colleagues (1992), we only consider the first phase of magainin action. The effect of the same amount of magainin 2 plus PGLa (1:1) was much stronger than each of the other peptides alone. PGLa alone always caused stronger effects than magainin 2 alone.

If individual magainin molecules permeabilized the liposomal membranes, then the initial rate of fluorescence decrease should be proportional to the added magainin concentration at low magnitudes of the latter. Positive cooperativity between magainin monomers implies that, for the first magainin molecule to be active, a second molecule has to assist, a higher

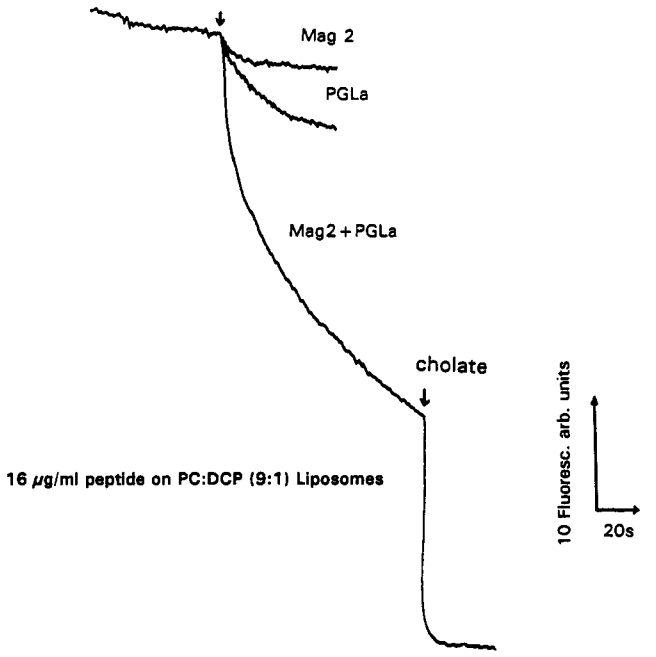

FIGURE 1: Magainin-induced permeability of PC/DCP (9:1) SUVs. Liposomes contained 10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 100 μM calcein whereas the external medium had calcein replaced by 200 μM $CoCl_2$. At the moment indicated by the arrow, 16 μg/mL (total peptide concentration) magainin 2, PGLa, or magainin 2 plus PGLa (1:1) were added. Initial rate of decrease in fluorescence (in arbitrary units/s) is measured. After addition of either Triton X-100 or cholate, total quenching of calcein fluorescence is observed.

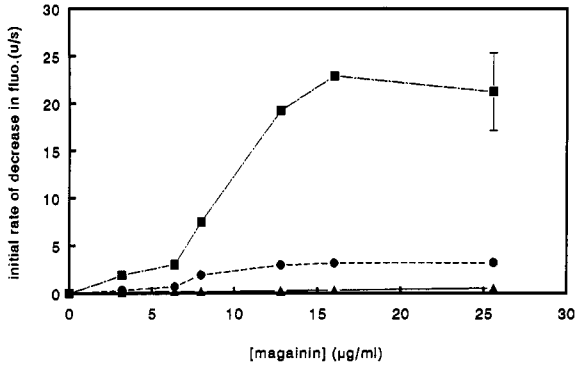

FIGURE 2: Magainin-induced membrane permeability of asolectin liposomes shows homo- and heterocooperativity. The experimental conditions are the same as for Figure 1, except for the different lipid composition. Symbols: triangles, magainin 2; circles, PGLa; squares, magainin 2 plus PGLa (1:1).

than first-order dependence on the magainin concentration should result. We determined how the initial rate of fluorescence decrease in experiments of the type depicted in Figure 1, varied with the concentration of added magainins.

The circles in Figure 2 show that the rate of fluorescence decrease varied sigmoidally with the concentration of added PGLa. Magainin 2 was much less active than PGLa, and this dependence was virtually linear (see below). When the magainins were added as an equimolar mixture of PGLa and magainin 2, their activity was much higher and again sigmoidally dependent on the total peptide concentration. As for the following figures, each point is a mean of three measurements of a typical experiment and has its standard deviation associated, although it can only be seen when it is larger than the size of the symbols. These results suggested that, at least in some cases, magainin monomers act cooperatively, as if a complex of monomers rather than the monomers themselves corresponds to the permeabilizing agent. At the higher concentrations, "saturation" seems to occur.

To obtain an indication of the number of monomers that would be involved in the active complex, we used a quantitative model in which a complex of n monomers is the sole permeabilizing agent. Since the Hill equation does not contain the concentration of the complex explicitly (Kurganov, 1982), we derived a quantitative model where such feature can be included. The permeability is supposed to be a saturating function of the concentration of the magainin n-mer. The concentration of total peptide added (T) is defined by the conservation law:

$$T = M + nC \tag{1}$$

where M is the concentration of monomers still present in the aqueous phase and n is the number of monomers forming the complex, which has a concentration of C. If K is the dissociation constant for the complex,

$$K = \frac{M^n}{C} \tag{2}$$

Equation 1 can thus be rewritten as

$$T = nC + \sqrt[n]{KC} \tag{1a}$$

Considering that the complex is the active form of magainins, we observed that the effect (X) caused by the added peptide (T) is a saturable function. If X_m is the maximal effect and B the concentration of complex corresponding to half-maximal effect, the observed effect (X) will be a function of complex concentration (B):

$$X = \frac{CX_m}{C + B} \tag{3}$$

Using eq 3 to express C into X and substituting this expression for C in eq 1a, we obtain

$$T = \frac{nBX}{X_m - X} + \sqrt[n]{\frac{KBX}{X_m - X}} \tag{4}$$

Equation 4 shows how, according to the model, the rate of initial fluorescence decrease (X) should vary with the amount of added peptide (T). More importantly, it shows how this variation depends on the number (n) of magainin monomers in the proposed complex. Consequently, by finding the value for n that makes the best fit of the experimental data to eq 4, we should find an indication for the number of monomers per complex. The first three lines of Table I show the results of this analysis for magainin 2, PGLa, and their mixture in asolectin liposomes: it confirms our tentative conclusions above, i.e., that magainin 2 itself is not cooperative in these liposomes (if at all significantly active), whereas PGLa and the combination of PGLa and magainin 2 are positively cooperative with an apparent stoichiometry close to 3 or 4.

In Figures 2–4, each point is the mean of three measurements, each belonging to a whole series of concentrations. Each of these series was fitted to eq 4 using MLAB™ (Knott, 1979), each giving rise to a value of n. For each peptide, the three estimates were averaged, and these are the values shown in Table I. When the three estimates corresponded to the same value, the standard error of the mean was consequently 0. Additional details about the fitting procedure are given in the Appendix.

In asolectin liposomes, the mean values of n for magainin 2, PGLa, or the mixture (1:1) were 1, 3.3, and 3.8, respectively. The absence of cooperativity ($n = 1$) observed for magainin 2 alone may have either of two meanings: (a) magainin 2 is active as a monomer, or (b) most of the added magainin is

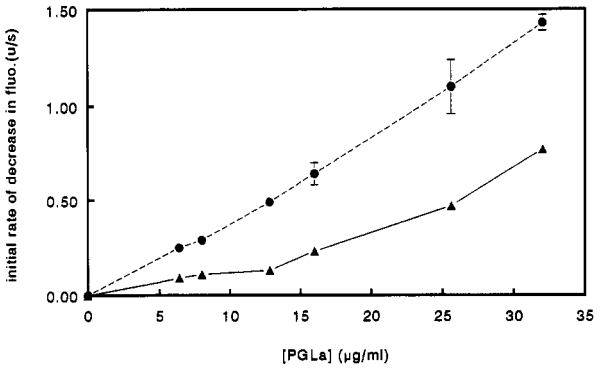

FIGURE 3: PGLa-induced membrane permeability is stronger in more negatively charged liposomal membranes. The experimental conditions are the same as for Figure 1, except for the lipid composition of the liposomal membranes, namely, PC/DCP (9:1), triangles, and (9:3), circles.

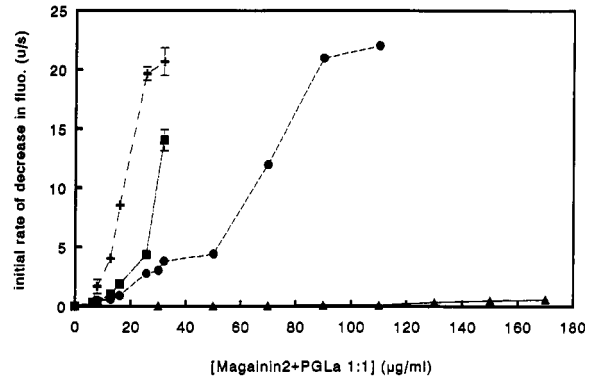

FIGURE 4: Heterocooperativity in magainin-induced [magainin 2 and PGLa (1:1)] membrane permeability increases with the amount of negative charge of the membrane (PC/DCP at different ratios). The conditions are the same as Figure 1. Symbols: circles, PC/DCP (9:1); squares, PC/DCP (9:2); crosses, PC/DCP (9:3); triangles, PC.

present in the form of a complex (of the same stoichiometry as the permeabilizing complex). We cannot distinguish between these two possibilities at this moment. Juretic et al. (1989a,b) determined the Hill coefficient for magainin 2 and PGLa, in cytochrome *c* oxidase reconstituted in asolectin liposomes. The Hill coefficients (being an estimate of *n*) were 5 and 3, respectively. In rat liver mitochondria, the values for magainin 2 amide and PGLa were 4 and 3, respectively (Westerhoff et al., 1989a). The presence of protein in liposomal and in mitochondrial membranes is expected to cause changes in the membrane surface potential. The presence of a transmembrane electric potential, negative inside, in such model systems may be an additional cause for such different values of *n*.

To investigate if changes in surface potential could affect the activity and cooperativity in our protein-free liposomes, we examined (cf. Figure 3) the effects of PGLa on liposomal membrane permeability, in PC/DCP (9:1) and (9:3) liposomes. Although the activity of PGLa increased significantly with increasing amounts of negative charge in the liposomal membrane (compare circles to triangles in Figure 3) the *n* values obtained were approximately 2 and 2.5 (see Table I), for PC/DCP (9:1) and (9:3) liposomes, respectively. Magainins thus show limited homocooperativity in protein-free liposomes.

Compared to asolectin liposomes, PC liposomes allowed for much lower magainin activities. Even the mixture of magainin 2 and PGLa (1:1) was only marginally active in this type of liposomes (triangles in Figure 4). For both the peptides

Table I: Estimates of the Possible Stoichiometry (*n*) of Magainin Monomers Constituting the Permeabilizing Magainin Complex, in Different Liposomal Populations[a]

	$n \pm$ SEM[b]
Asolectin Liposomes with Different Magainin Peptides	
magainin 2	1 ± 0
PGLa	3.3 ± 0.6*
magainin 2 + PGLa	3.8 ± 0.8*
PC/DCP Liposomes with PGLa	
9:1	2 ± 0*
9:3	2.5 ± 0.8*
PC/DCP Liposomes with Magainin 2 + PGLa (1:1)	
PC	2 ± 0*
9:1	2.3 ± 0.6*
9:2	3 ± 0
9:3	5 ± 0

[a] The estimates of *n* were obtained by fitting the data from Figures 2–4 to 4. In these figures, the mean of three measurements is plotted, but each measurement belongs to a series of measurements over the range of concentrations of peptide studied. For each series, the data were fitted to eq 4 using fixed integer values of *n*, letting the program search for the best values of *K*, *B*, and X_m. We then chose the *n* value corresponding to the lowest sum of residuals. As a result, the estimates of *n* presented here are the mean of three integer *n* values for that peptide. [b] Inside each group of *n* value estimates, the values were tested for being significantly different to the 5% level (Snedecor & Cochran, 1980). The values indicated by an asterisk showed overlapping 95% confidence intervals.

individually (not shown) and the mixture, however, negative charges among the lipids greatly enhanced the magainin-induced membrane permeability: Figure 4 also shows the activity of magainin 2 plus PGLa (1:1) in PC/DCP liposomes, at different ratios of the lipids (9:1, 9:2, and 9:3). Heterocooperativity in the action of magainin 2 and PGLa, as measured by the estimated *n* value, increased with increasing amount of negative charge in the liposomal membrane. The values for 9:1, 9:2, and 9:3 PC/DCP were 2.3, 3, and 5, respectively. In Figure 4 the results obtained with PC liposomes are also plotted. PC liposomes were susceptible to magainins only at much higher concentrations than PC/DCP liposomes, but some cooperativity is present ($n = 2$). We also prepared positively charged PC/SA (9:1) liposomes and investigated the magainin-induced permeability [magainin 2 plus PGLa (1:1)]. Even at high concentrations, these liposomes were not susceptible to the mixture of the peptides (results not shown).

In view of the small effects of magainin 2 plus PGLa (1:1) on PC liposomes, compared to PC/DCP liposomes, we decided to investigate whether the *amount* of negative charge or the *net* (i.e., negative minus positive) charge of the membrane determines the activity of magainins. SUVs consisting of PC/DCP/SA at the ratios 9:1:1 and 9:3:3 were prepared. We can observe (Figure 5) that liposomes of PC/DCP/SA (9:3:3) are somewhat more susceptible to the simultaneous action of magainin 2 and PGLa (1:1) than 9:1:1 liposomes. However, the pluses in Figure 5 and comparison to those in Figure 4 reveal that magainins are much more active toward liposomes with uncompensated negative charge than toward liposomes with nearly neutral charge. The membrane permeability of the neutral liposomes is affected only at high concentrations of the peptides (Figure 5). This observation also excludes the possibility that when PC/DCP/SA liposomes were prepared, extensive segregation of SA and DCP occurred, yielding a nonhomogeneous population of SUVs. Our results thus suggest that the action of magainins is determined, to a large extent, by the amount of net negative charge in the membrane.

We also investigated whether the presence of a negative-inside transmembrane potential affected the magainin-induced

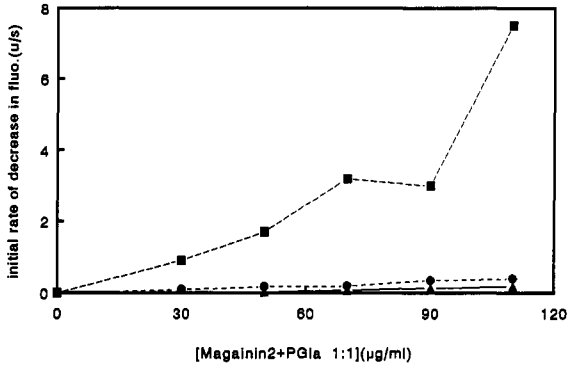

FIGURE 5: Total charge of the membrane lipids affects magainin-induced [magainin 2 and PGLa (1:1)] activity somewhat, but much less so than the net negative charge does. The same experimental system was used as described in the legend to Figure 1. Symbols: triangles, PC; circles, PC/SA/DCP (9:1:1); squares, PC/SA/DCP (9:3:3).

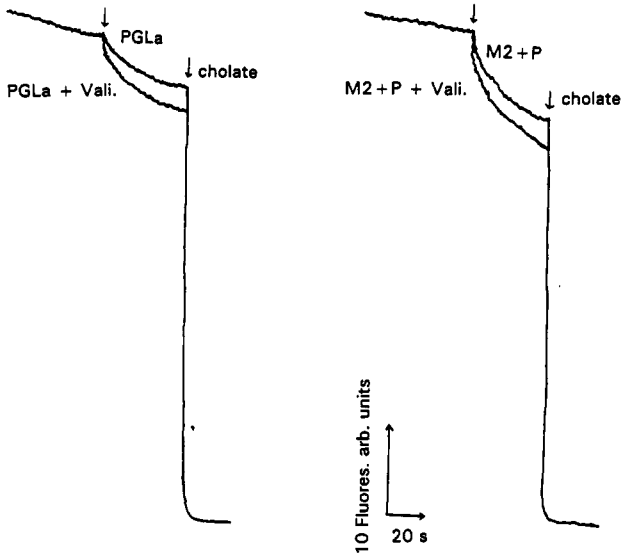

FIGURE 6: Transmembrane electric potential negative-inside potentiates the activity of PGLa and of magainin 2 and PGLA (1:1). Soybean asolectin SUVs were prepared in 10 mM Tris-HCl, 150 mM KCl, and 10 mM $CoCl_2$, at pH 7.0. The fluorescence assay was performed in 10 mM Tris-HCl, 150 mM LiCl, and 3.5 μM calcein, at pH 7.0. Additions of PGLa ("PGLa", left trace, 8 μg/mL final concentration) or magainin 2 and PGLa (1:1) ("M2+P", right trace, 3.2 μg/mL) were made alone or together with valinomycin (76 ng/mg of lipid). At the point indicated, 50 μL of 10% (w/v) cholate was added to achieve total disruption of the SUVs.

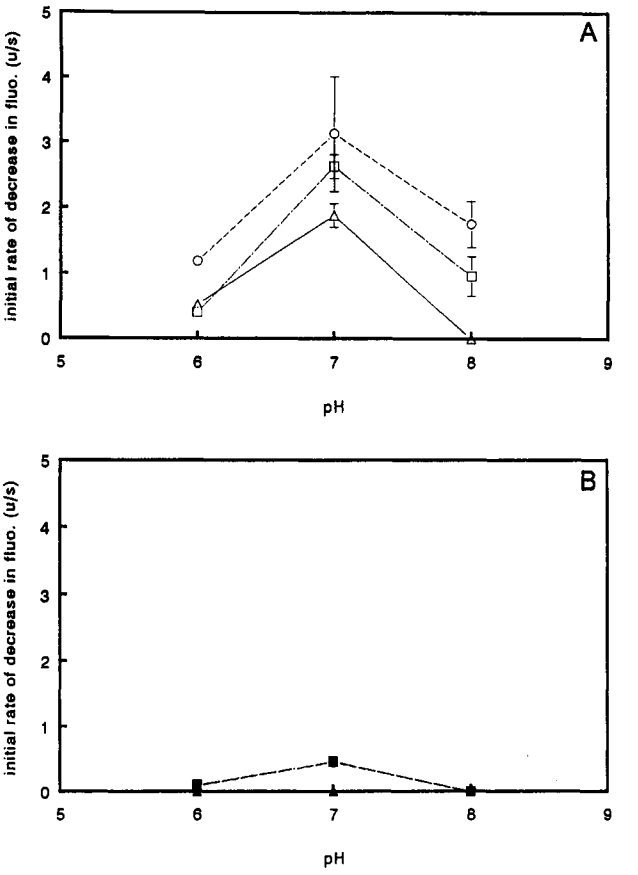

FIGURE 7: Activity of magainins is pH and ionic strength dependent. PC/DCP (9:1) liposomes were prepared in 10 mM HEPES at different pH values, with 1.5 mM NaCl (low ionic strength, open symbols; A) or 150 mM NaCl (high ionic strength, closed symbols; B). The internal medium (at the indicated pH values and ionic strength) contained 10 mM $CoCl_2$, while the external medium (at the same pH value and ionic strength as the internal medium) contained 3.5 μM calcein, 8 μg/mL magainin 2 (triangles), 8 μg/mL PGLa (circles), or 4 μg/mL magainin 2 plus PGLa (squares) was added.

membrane permeability. We produced a K^+-diffusion potential, using the quenching of rhodamine 123 fluorescence, as described by Emaus et al. (1986), as a control for the establishment of such potential. We added valinomycin (a K^+-ionophore) to KCl-containing, asolectin liposomes, suspended in LiCl medium. Figure 6 shows that when valinomycin and the magainins were added simultaneously, the former potentiated the latter. No such potentiation was observed when valinomycin was added after the magainins (not shown). We performed the following control experiments: in the calcein/Co^{2+} system, addition of valinomycin alone or the simultaneous addition of valinomycin and nigericin (in a concentration twice as high as that of valinomycin) did not cause any change in calcein fluorescence. When in the absence of valinomycin, or after preincubation with both valinomycin and nigericin, magainins were added to K^+-containing SUVs suspended in KCl or LiCl buffer, no rhodamine 123 response was observed and the calcein-quenching effect of the peptides was the same in the two buffers (results not shown).

Addition of nigericin, instead of valinomycin, together with magainins did not potentiate the peptides' action: the decrease in fluorescence was the same as if the peptides were added alone (not shown). This result indicated that the valinomycin-induced magainin potentiation was not a consequence of establishment of a ΔpH, as was the case with the designed monopositive hexapeptide of de Kroon et al. (1989, 1991). This possibility was also excluded by preparing liposomes with entrapped medium at pH 6.0–8.0 and the permeability assay performed with all combinations of these same pH values in the external medium: when the external pH value differed from the internal pH, the activity of the peptides corresponded to the activity at the external pH value (not shown).

We did observe pH-dependent activity of each of the magainin peptides; in Figure 7, the results obtained with PC/DCP (9:1) liposomes with the same value of pH inside and outside are shown. At pH 7, magainins were much more active than at pH 6 or 8.

The observed potentiation of magainin activity by negative membrane charge observed at physiological ionic strength suggested that lowering the ionic strength should enhance the magainins' activity toward negatively charged liposomes. Studies at high and low ionic strength (10 mM HEPES plus 150 mM NaCl or 1.5 mM NaCl, respectively), at various pH values, were performed. As expected, magainins were more

active at low ionic strength, at all the pH values studied (cf. Figure 7A,B).

DISCUSSION

The role of magainins as membrane-active peptides is widely established. Nevertheless, the molecular mechanism of action of the peptides has not yet been elucidated. In particular, there is an apparent contradiction between structural studies in protein-free systems that suggest that magainins are present as, possibly, monomeric α-helices, adsorbed parallel to the membrane, and functional studies with proteinaceous membranes, suggesting an oligomeric magainin complex as the functional unit.

One possible resolution of this paradox would be that in proteinaceous membranes magainins form a complex, whereas in protein-free membranes they do not. Since homo- and heterocooperativity is the functional evidence for the oligomeric nature of the active magainin species, we decided to investigate whether such properties would also be present in protein-free liposomes. The liposome work of Matsuzaki et al. (1989, 1991) established membrane charge effects on magainin activity, but they did not consider the presence of homocooperativity in their systems, and neither did they address the question of heterocooperativity or synergism. In the recent work of Grant et al. (1992), once again these important features of the action of the magainin peptides were not explored.

In liposomes constituted by zwitterionic and acidic phospholipids, we observed that magainin-induced membrane permeability did exhibit positive heterocooperativity. This was observed for asolectin liposomes and also for PC/DCP liposomes, as measured by the n value, obtained by fitting the experimental data to eq 4 (see Table I). Homocooperativity was somewhat lower. Heterocooperativity increased with the negative charge of the liposomal membrane (see Figure 4), while homocooperativity seemed to be only marginally potentiated. In the "virtually neutral" PC liposomes, heterocooperativity was also present, but a much higher concentration of the peptides must be present for functional effects to be observed.

The rather strong dependence of magainin action on net negative charge of the liposomal membrane may reflect the apparent cooperativity between negative lipid molecules and the peptides, due to reduced dimensionality in the electrostatic interactions (Mosior & McLaughlin, 1992a,b). We wish to stress that these effects can only cause positive cooperativity in the dependence on the lipid concentration but not in the dependence on peptide concentration. If anything, these effects would cause negative cooperativity in the latter.

Synergistic effects of magainin 2 and PGLa (1:1) had been observed in cytochrome c oxidase liposomes (Juretic et al., 1989; Juretic, 1991). Magainin A and PGLa (1:1) also showed synergism in their action on hamster spermatozoa (de Waal et al., 1991). In the present work, we observed that synergism between magainin 2 and PGLa (1:1) occurs also in a system devoid of a transmembrane electric potential (as opposed to the previously described model systems). Synergism seemed also to be potentiated by increasing amounts of negative membrane charge, i.e., at higher ratios of DCP (not shown). This suggests that, for cooperativity and synergism and hence for the functional unit to be oligomeric, neither the presence of membrane proteins nor the presence of catalytic activities such as those causing a transmembrane electric potential difference are essential. This conclusion may be valid only for high magainin activities. In liposomal preparations where magainins are less active, magainin action seemed less cooperative and the functional unit seemed closer to being monomeric. Factors potentiating magainins might also enhance their cooperativity. Indeed, heterocooperativity increased with activity, as the membrane charge was increased.

The studies with "neutral" vesicles, where the negative charge was compensated by the same amount of positive charge (see Figure 5), showed that although the number of charges of a membrane does influence the effectiveness of the peptides, the net amount of negative charge has a stronger effect. The observation that SUVs of 9:3:3 PC/DCP/SA were more susceptible than those of the 9:1:1 mixture may be due to electrostatic interactions at localized negative charges.

We investigated how a transmembrane electric potential, negative inside, affected the activity of the peptides. We observed that such a transmembrane electric potential did potentiate the magainins and that the synergism was retained in the potential-stimulated case. The studies in the presence of a K^+-diffusion potential (KCl inside, LiCl outside), showing potentiation of the action of magainins by a negative inside transmembrane potential, were in agreement with the studies on mitochondrial presequences (Roise et al., 1986), artificially designed basic peptides (de Kroon et al., 1991, de Kroon 1991), and some natural basic peptides (de Kroon, 1991). In the latter studies it was observed that peptides that have only one positive charge were activated by ΔpH rather than $\Delta\Psi$ (the neutral form penetrates) (de Kroon, 1991; Chakrabarti et al., 1992), whereas peptides having more than one positive charge were activated by $\Delta\Psi$ (de Kroon, 1991). In line with this conclusion, the present study showed that ΔpH did not potentiate the activity of the magainins. We think that the results at different pH and ionic strength substantiate the importance of the electrostatic component in the mechanism of action of magainins.

We conclude that electric potentials near and across the target membrane affect the activity of magainins and that the more active magainin forms are oligomers. We do not exclude the possibility that monomers also have some membrane-permeabilizing activity. In fact, most of the concentration dependences in this paper could be explained by superposition of a monomeric and a stronger oligomeric membrane-permeabilizing magainin form.

The n values estimated are worth commenting at this point. For asolectin liposomes, the 95% confidence intervals of the estimated values of n for PGLa and magainin 2 plus PGLa (1:1) overlapped (Snedecor & Cochran, 1980). The same occurred for PGLa on 9:1 and 9:3 PC/DCP liposomes, and for magainin 2 and PGLa (1:1), on 9:1 PC and PC/DCP liposomes; in all other cases, inside the same group, the n values estimated were significantly different to the 5% level. Because of the uncertainties in the model, we do not wish to emphasize implications of these differences in cooperativity or the differences in the precise number of monomers in the permeabilizing complexes.

In view of our results, and considering the physicochemical properties of magainins, the tentative mechanism of action, depicted schematically in Figure 8, is proposed. Magainins are known to acquire an α-helical conformation in a hydrophobic/membrane environment. When bound to the surface of a membrane (electrostatic interactions playing an important role), the peptides may change their conformation from random-coil (in solution) to α-helix. Transmembrane reorientation (not yet clearly demonstrated) and formation of a complex (as the basis of the cooperative effects observed here) can in principle occur in the absence of a transmembrane electric potential, although our observations substantiate a

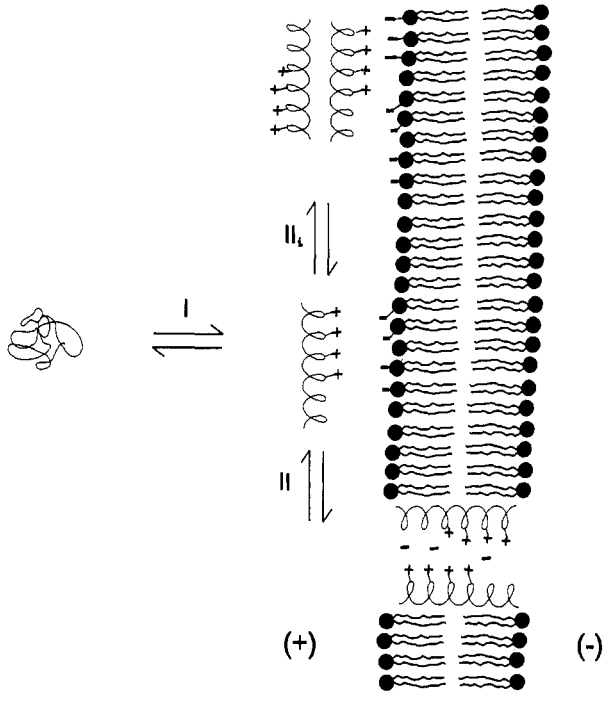

FIGURE 8: Model for aspects of the mechanism of action of the magainin peptides. The peptides exist as a random coil in aqueous solution, attaining helical conformation when bound to the surface of the membrane (step I). The negative charge of membrane phospholipids may facilitate this binding process and induce a change in peptide conformation. In step II, the peptides may transverse the membrane and acquire the *n*-mer, active form. The presence of a transmembrane potential, negative inside, may favor this insertion. In the scheme, the peptide and the membrane show immobile charges, while in the transmembrane complex some free negative charges are shown in a possible movement across the pore. An alternative to step II is the formation of an inactive aggregate (step II_i) on the surface of the membrane or also the insertion of the peptide parallel to the membrane surface, with its hydrophobic side into the lipid bilayer and the positive side chains contacting the negative phospholipid head groups (not depicted in the figure).

more effective or faster formation of the membrane-disturbing structure when an electric potential is present. The possibility of formation of intermediary structures of the oligomer, each with different intrinsic activity, before attaining the most active structure, or even formation of inactive complexes (step II_i), cannot be excluded. Also, the possibility that the magainin monomers insert into the lipid tails zone, parallel relative to the membrane surface, cannot be excluded. In this conformation, the hydrophobic side of the helix would be facing the fatty acid chains, while the positively charged side chains of the peptide would contact the negatively charged lipid heads. Such a structure might constitute an inactive form of the peptides, not contributing to functional effects. Our tentative mechanism of action does not confirm or contradict the mechanism of action proposed by Grant et al. (1992), where no "pore" would be formed by the magainin peptides, but only membrane disturbance would occur. We share the opinion expressed by Grant et al. that no experimental evidence exists yet to rule out either hypothesis. Indeed, a pore formed by magainins could be metastable and relax to a more stable and less permeable state at a time scale of tens of seconds. Alternatively, the nonexponential change in time of carboxyfluorescein (Grant et al., 1992) or calcein (this paper) fluorescence may be due to heterogeneity of the liposome preparation (e.g., in terms of surface tension and differences between lipid distribution between inner and outer monolayer as a result of size differences) as amplified by the nonlinear dependence on concentration of added magainins.

The equilibrium of step II would be shifted most strongly with a transmembrane electric potential difference. However, such a transmembrane electric potential does extend somewhat away from the core of the bilayer (Kamp et al., 1988). Hence we cannot be quite certain which step is potentiated by the transmembrane electric potential. Neither can we explain the synergistic action of magainin 2 or magainin A with PGLa, at the molecular level. We observed that the synergism of magainin 2 and PGLa was not so strong at low ionic strength. To clarify whether a faster binding process occurs, or a more efficient (maybe larger pore) is formed, further studies are required.

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APPENDIX

In this section, we will elaborate more extensively on two important details of the fitting procedure:

(1) Equation 4 was derived, assuming that the magainin form causing functional effects is a complex of monomers. As described in the text, the experimental data was fitted to fixed values of n from 1 to 6. But $n = 1$ corresponds to a total absence of complex! In this situation, the best fit of eq 4 does not give rise to unique estimates of K and B, but of the product (cf. the root argument of eq 4). Multiple combinations of values of K and B will correspond to such a solution. Nevertheless, since we did not want to state the implications of a specific value of K or B, in the situation of $n = 1$ (but also for the other values of n), we could still obtain the best estimate of n, using the criterion that is described below.

Another possibility of overcoming the ambiguity of evaluating K and B for $n = 1$ is to fit the data to eq 3, substituting C for T.

(2) In the legend of Table I, it is mentioned that when a fixed integer of n, from 1 to 6, is assigned, the best estimate for n was chosen according to the lowest value of the sum of residuals. This procedure is worth describing in more detail: at the end of a run of fitting with a particular value of n, we obtained the sum of residuals not only for the y values but also for the x values. For a set of experimental data, we thus obtained six sets of n values with the associated sum of residuals for x and y values for that particulate estimate of n. The best estimate of n was chosen as the minimum of the sum of residuals in both x and y values.

REFERENCES

Andreu, D., Aschauer, H., Kreil, G., & Merrifield, R. B. (1985) *Eur. J. Biochem. 149*, 531–535.

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Bechinger, B., Kim, Y., Chirlian, L. E., Gesell, J., Neumann, J.-M., Montal, M., Tomich, J., Zasloff, M., & Opella, S. J. (1991) *J. Biomol. NMR 1*, 167–173.

Beschiaschvili, G., & Seelig, J. (1991) *Biochim. Biophys. Acta 1061*, 78–84.

Boman, H. G. (1991) *Cell 65*, 205–207.

Bevins, C. L., & Zasloff, M. (1990) *Annu. Rev. Biochem. 59*, 395–414.

Chakrabarti, A. C., Clark-Lewis, I., Richard Harrigan, P., & Cullis, P. R. (1992) *Biophys. J. 61*, 228–234.
Chen, H.-C., Brown, J. H., Morell, J. L., & Huang, C. M. (1988) *FEBS Lett. 236*, 462–466.
Christensen, B., Fink, J., Merrifield, R. B., & Mauzeral, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5072–5076.
Cruciani, R. A., Barker, J. L., Raghunathan, G., Durell, S., Guy, R., Chen, H.-C., & Stanley, E. (1991) *Biophys. J. 59*, 372a.
Cruciani, R. A., Barker, J. L., Durell, S. R., Raghunathan, G., Guy, H. R., Zasloff, M., & Stanley, E. F. (1992) *Eur. J. Pharmacol. 226*, 287–296.
de Kroon, A. (1991) Membrane Insertion and Translocation of Peptides, Ph.D. Thesis, Rijksuniversiteit, Utrecht, The Netherlands.
de Kroon, A., de Gier, J., & de Kruijff, B. (1989) *Biochim. Biophys. Acta 981*, 371–373.
de Kroon, A., Vogt, B., van't Hof, R., de Kruijff, B., & de Gier, J. (1991) *Biophys. J. 60*, 525—537.
de Waal, A., Vaz Gomes, A., Mensink, A., Grootegoed, J. A., & Westerhoff, H. V. (1991) *FEBS Lett. 293*, 219–223.
Duclohier, H., Molle, G., & Spach, G. (1989) *Biophys. J. 56*, 1017–1021.
Emaus, R. K., Grunwald, R., & Lemasters, J. J. (1986) *Biochim. Biophys. Acta 850*, 436–448.
Grant, E., Jr., Beeler, T. J., Taylor, K. M. P., Gable, K., & Roseman, M. A. (1992) *Biochemistry 31*, 9912–9918.
Guy, H. R., & Raghunathan, G. (1988) in *Transport Through Membranes: Carriers, Channels and Pumps* (Pullman, A., Jortner, J., & Pullman, B., Eds.) pp 369–379, Kluwer Academic Press, Dordrecht, The Netherlands.
Hing, A. W., Schaefer, J., Ferguson, M., & Blazyk, J. (1991) *Biophys. J. 59*, 300a.
Juretic, D. (1991) *Stud. Biophys. 138*, 79–86.
Juretic, D., Chen, H.-C., Brown, J. H., Morell, J. L., Hendler, R. W., & Westerhoff, H. V. (1989a) *FEBS Lett. 249*, 219–223.
Juretic, D., Hendler, R. W., Zasloff, M., & Westerhoff, H. V. (1989b) *Biophys. J. 55*, 572a.
Kamp, F., Chen, Y.-d., & Westerhoff, H. V. (1988) *Biochim. Biophys. Acta 30*, 113–132.
Kim, J., Mosior, M., Chung, L. A., Wu, H., & McLaughlin, S. (1991) *Biophys. J. 60*, 135–148.
Kordel, M., Schüller, F., & Sahl, H.-G. (1989) *FEBS Lett. 244*, 99–102.
Kurganov, B. I. (1982) in *Allosteric Enzymes Kinetic Behaviour*, pp 32–37, John Wiley & Sons, New York.
Madhavi Sekharam, K., Bradrick, T. D., & Georgiou, S. (1991) *Biochim. Biophys. Acta 1063*, 171–174.
Marion, D., Zasloff, M., & Bax, A. (1988) *FEBS Lett. 227*, 21–26.
Matsuzaki, K., Harada, M., Handa, T., Funakoshi, S., Fujii, N., Yajima, H., & Miyajima, K. (1989) *Biochim. Biophys. Acta 1063*, 130–134.
Matsuzaki, K., Harada, M., Funakoshi, S., Fujii, N., & Miyajima, K. (1990) *FEBS Lett. 267*, 135–138.
Moore, K. S., Bevins, C. L., Brasseur, M. M., Tomassini, N., Turner, K., Eck, H., & Zasloff, M. (1991) *J. Biol. Chem. 266*, 19851–19857.
Mosior, M., & McLaughlin, S. (1992a) *Biochim. Biophys. Acta 1105*, 185–187.
Mosior, M., & McLaughlin, S. (1992b) *Biochemistry 31*, 1767–1773.
New, R. R. C. (1990) in *Liposomes: A Practical Approach* (New, R. R. C., Ed.) pp 44–47, IRL Press, Oxford.
Oku, N., Kendall, D. A., & MacDonald, R. C. (1982) *Biochim. Biophys. Acta 691*, 332–340.
Penefski, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5*, 1327–1334.
Schwarz, G., & Beschiaschvili, G. (1989) *Biochim. Biophys. Acta 979*, 82–90.
Snedecor, G. W., & Cochran, W. G. (1980) in *Statistical Methods*, 7th ed., pp 64–65, Iowa State University Press, Ames, IA.
Tamm, L. (1991) *Biochim. Biophys. Acta 1071*, 123–148.
Westerhoff, H. V., Hendler, R. W., Zasloff, M., & Juretic, D. (1989a) *Biochim. Biophys. Acta 975*, 361–369.
Westerhoff, H. V., Juretic, D., Hendler, R. W., & Zasloff, M. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6597–6601.
Zasloff, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5449–5453.
Zasloff, M., Martin, B., & Chen, H.-C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 910–913.