1992

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Received December 17, 1991; Revised Manuscript Received July 16, 1992

ABSTRACT: The magainins, peptide antibiotics secreted by the frog *Xenopus laevis*, have previously been shown to permeabilize phospholipid vesicles. To elucidate the mechanism of permeabilization, we have conducted detailed kinetic studies of magainin 2 amide (mgn2a)-induced release of 6-carboxyfluorescein from vesicles of phosphatidylserine. The results show that dye release occurs in (at least) two stages—an initial rapid phase, with $t_{1/2} \approx 3$ s, followed by a much slower phase that approaches zero leakage rate before all the dye is released. Light-scattering studies showed that mgn2a does not cause gross changes in vesicle structure. The peptide was found to rapidly equilibrate between vesicles; this was demonstrated by determining a binding isotherm for the peptide–lipid interaction, and by showing that addition of unloaded vesicles rapidly quenches peptide-induced leakage from loaded vesicles. Transient dye release in the presence of an equilibrating peptide can be explained in two ways: (1) the peptide exists only transiently in an active form; (2) the vesicles are only transiently leaky. Preincubation of mgn2a at assay concentrations in buffer alone or with unloaded vesicles did not inactivate the peptide. Therefore, rapid leakage is probably due to transient destabilization of the vesicle upon addition of mgn2a.

The magainins, a class of antimicrobial peptides secreted from the skin of the frog *Xenopus laevis* (Zasloff, 1987; Zasloff et al., 1988), are thought to kill cells by permeabilizing the plasma membrane. A total of 23 residues in length and bearing a positive charge of +3 to +5 at physiological pH, these peptides could potentially form amphipathic helical structures that are able to disrupt lipid bilayers or form oligomeric transmembrane channels [see Williams et al. (1990) and references cited therein].

Evidence that magainins can act directly on lipid bilayers comes from studies with planar bilayers and liposomes. Duclohier et al. (1989) found that magainin 1 forms anion-selective channels in planar lipid bilayers under conditions of high salt and high trans-negative potential. Several conductance states were identified, the lowest being 80 pS. The dose response curves suggested that the magainin channel consists of three to six monomers.

When magainin 2 amide (mgn2a)[1] or its analogs were added to respiring cytochrome oxidase-containing liposomes (which have an inside-negative membrane potential), Juretic et al. (1989) found that the peptides uncouple respiration and cause a stable (rather than transient) decrease in the steady-state membrane potential. The sigmoidal dose response curve was attributed to magainin monomers interacting cooperatively in the membrane to form an active aggregate of four to five subunits.

These two studies suggest that the mechanism of magainin action is similar to that proposed for other voltage-dependent pore-formers, such as alamecithin [reviewed by Stein (1986)]. According to the model, monomers or aggregates of the peptide initially bind to the bilayer interface (through a combination of electrostatic and hydrophobic interactions) as an inactive amphipathic α-helix. The inactive form is then driven into the bilayer by the membrane potential, whereupon the peptide forms an active transmembrane pore. Changes in aggregation state can occur before, during, or after voltage-induced transmembranal insertion.

There is, however, evidence to show that magainin effectively permeabilizes liposomes in the absence of a membrane potential: When magainin 1 was added to sonicated phosphatidylserine (PS) vesicles, Matsuzaki et al. (1989a) found that entrapped calcein was rapidly released, on a time scale of seconds to minutes. By measurement of the initial rate of leakage as a function of total peptide and total lipid concentration, an equilibrium binding isotherm for the peptide–lipid interaction was also determined. The resulting curve, which fit well to the Langmuir equation, suggested to Matsuzaki et al. that extensive aggregation of peptide on the vesicle does not occur. From this, they concluded that magainin 1 probably does not form oligomeric channels in the bilayer, and therefore functions instead as a bilayer destabilizer. An important caveat, which they noted, is that their binding data are not sensitive enough to rule out the possibility that a small fraction of the peptide forms oligomeric channels. In our opinion, binding isotherms are exceedingly difficult to interpret because steric effects and changes in membrane surface potential lead to large activity coefficient effects that are hard to predict quantitatively (Schwarz et al., 1986).

To help clarify the mechanism of magainin's lytic action, we have studied in detail the kinetics of mgn2a-induced release of 6-carboxyfluorescein (CF) from phosphatidylserine liposomes in the absence of a membrane potential. Our results show that the fast release of dye is a transient effect. After experimentally eliminating several explanations, we conclude that fast release results from transient destabilization of the bilayer upon initial interaction with the peptide. A unifying mechanism for magainin action is proposed.

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylserine (bovine brain) was purchased from Avanti Polar Lipids; Triton X-100, especially

† Supported by USUHS Protocol CO71AH.

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[1] Abbreviations: mgn2a, magainin 2 amide; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; CF, 6-carboxyfluorescein; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid).

purified for membrane research, was from Boehringer Mannheim. Carboxyfluorescein from Eastman (>99%) was used for most of these studies, without further purification; chromatographically pure CF (Molecular Probes) was also used in selected experiments, and gave results indistinguishable from those obtained with Eastman CF. Magainin 2 amide was a generous gift from Dr. Hao-Chia Chen.

Buffers. Buffers used in SUV studies were as follows: buffer A, 10 mM Pipes, 150 mM NaCl, 1 mM EDTA, and 100 mM sucrose, pH 7.0; buffer B, 10 mM Pipes, 150 mM NaCl, and 1 mM EDTA, pH 7.0; dye solution, 100 mM CF, 10 mM Pipes, 150 mM NaCl, and 1 mM EDTA, pH 7.0.

Preparation of Small Unilamellar Vesicles (SUVs). Because the phase transition of the bovine brain PS is 8 °C, SUVs and LUVs had to be prepared and stored above this temperature; for convenience, all procedures were carried out at or near room temperature.

Approximately 20 mg of PS in 2 mL of solvent was dried to a film in a 25-mL flask by rotary evaporation, redissolved in 5 mL of benzene, and lyophilized overnight under high vacuum. The lyophilized lipid was then suspended in 2 mL of dye solution by vortex mixing, transferred to a polycarbonate tube, and sonicated (under argon) at 25–30 °C to constant clarity in a cup horn using a Heat Systems ultrasonic sonicator (W-375). Unentrapped CF was removed from the solution by passing the sonicate through an Econo-Pac 10DG column (10 mL) using buffer A as the eluting buffer.

To obtain a homogeneous population of SUVs, larger particles were removed by sedimentation in an angle rotor (Ti50, Beckman ultracentrifuge), as described by Barenholz et al. (1977). Centrifugation at 45K rpm for 15 min was sufficient to produce a population of CF-loaded lipsomes that elutes as a homogeneous population of SUVs from a Sepharose 2B-CL column: A single, well-included peak was obtained, with a constant ratio of CF/phosphate across the elution profile. This result also shows that the vesicles are relatively stable, since vesicle breakdown or fusion usually produces a heterogeneous mixture of particles. Lipid phosphate was determined by the method of Bartlett (1959).

Preparation of Large Unilamellar Vesicles (LUVs). CF-loaded PS LUVs were prepared by the reverse-phase ether evaporation method (Düzgünes et al., 1983), using 100 mM CF, 10 mM Pipes, 10 mM NaCl, and 1 mM EDTA, pH 7.0 (296 mOsM), as the aqueous phase. The initially formed vesicles were extruded successively through Nucleopore filters of 0.4, 0.2, 0.1, and 0.08 μm. To remove unentrapped CF and contaminating SUVs, the vesicles were passed through an Econo-Pac 10DG column (10 mL) and then a Sepharose-2BCL column (1.6 × 60 cm), using 10 mM Pipes, 150 mM NaCl, and 1 mM EDTA, pH 7.0 (295 mOsM), as the eluting buffer.

CF Release Assay. Release of CF from the vesicles at 25 °C was determined fluorometrically by monitoring the decrease in self-quenching (excitation 490 nm, emission 520 nm; Aminco SPF-500C spectrofluorometer) as the dye is released from the vesicles (Weinstein et al., 1984).

Kinetic experiments were usually initiated by injecting 5–20 μL of peptide stock solution (0.37 mM mgn2a in buffer B) into a magnetically stirred, equilibrated cuvette containing 2 mL of 25–30 μM PS in buffer A. The mixing time was about 1 s. At the end of each run, the total releasable fluorescence was determined by the addition of 20 μL of 10% Triton X-100.

Progress of the reaction is reported as the percentage of CF quenched, $\%Q$, which is calculated from the formula $[1 - (F_t - F_0)/(F_\infty - F_0)]100$, where F_t is the fluorescence at time t, F_0 is the base-line fluorescence of the vesicles, and F_∞ is the fluorescence after Triton X-100 addition. As will be shown under Results, mgn2a induces the release of dye by an all-or-none mechanism. Consequently, $\%Q$ equals the percentage of total dye remaining in the vesicles.

The kinetics of dye release from SUVs were not significantly affected by the osmolarity of the bathing solution. This was determined by varying the osmolarity of buffer B from 331 to 804 mOsM with sucrose. The osmolarity of the dye solution was 600.

Isoosmotic conditions were used to measure the release of dye from LUVs, using the dye solution and external buffer described above in the section on LUV preparation.

Mode of Dye Release. Dye may be released from a population of vesicles by an "all-or-none" or a "graded" process; the all-or-none mode of release produces a mixture of fully loaded and completely unloaded vesicles whereas the graded mode produces a population of vesicles that are all partially unloaded to the same extent. These two modes of release can be distinguished by determining the percentage of fluorescence quenching remaining in the intravesicular medium, $\%Q_{tr}$, after a substantial fraction of dye has been released from the vesicle sample (Weinstein et al., 1984).

To determine the mode of release, mgn2a was added to SUVs (25–30 μM PS) containing either 30 mM or 100 mM entrapped CF. After 100 or 500 s, an aliquot of unloaded vesicles (6 μL of 85 mM PS) was added to quench the mgn2a-induced release of dye via intervesicular equlibration of peptide. To separate the vesicles from released dye, 1-mL aliquots of the mgn2a/SUV mixture were rapidly passed over 5-mL Sephadex G25 columns by centrifugation at a maximum speed for 15 s (at room temperature) in a clinical centrifuge. Vesicle fractions of 0.5 mL were then analyzed in the absence and presence of Triton X-100 to determine the percentage of fluorescence quenching. From these results, the intravesicular concentration of CF could be determined from a standard quench curve, which was obtained by measuring the fluorescence quenching of vesicle samples prepared in solutions ranging from 1 to 100 mM CF.

Light Scattering. To determine whether mgn2a induces gross structural changes of the vesicles by fusion, aggregation, or solubilization, changes in the 90° light scattering of vesicle solutions were monitored with the spectrofluorometer. An incident wavelength of 400 nm was used, with band-pass slit settings at 4 nm.

Even if no gross structural changes take place, a modest increase in light scattering is expected because bound peptide increases the mass of the vesicle and perhaps the refractive index increment as well. The predicted magnitude of these increases was calculated as described previously by Roseman et al. (1977) in a study of cytochrome b_5–vesicle interactions. Briefly, the Rayleigh equation shows that the relative increase in light scattering of an SUV–peptide complex compared to SUV alone depends on three ratios: the relative molecular weights, the relative concentrations in grams per milliliter, and the relative refractive index increments. From our experimentally determined mgn2a–lipid binding isotherm, the relative molecular weights and relative concentrations of particles could readily be calculated. The refractive index increments, which were not measured, had to be estimated from reported values for pure lipid (0.1478 mL/g) and protein (range: 0.15–0.2 mL/g); for these calculations, it was assumed that the refractive index increment of a complex is a simple weighted average of the refractive index increments of the pure components.

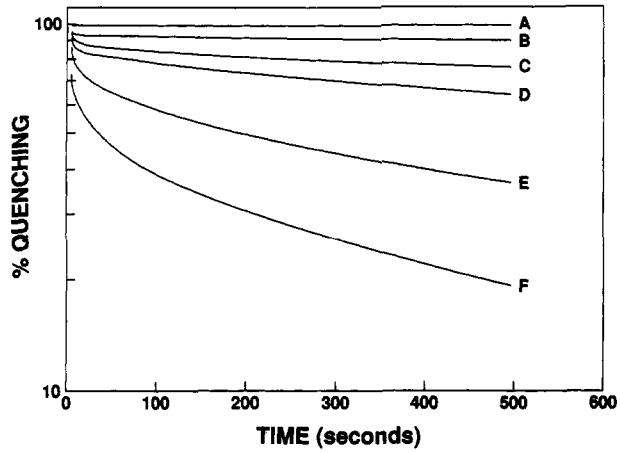


FIGURE 1: mgn2a-induced release of CF from PS SUVs. 5–20 μL of 0.37 mM mgn2a (in buffer B) was injected into a stirred cuvette containing a 2-mL solution of CF-loaded SUVs (30 μM in PS) in buffer A, and the increase in fluorescence intensity (excitation 490, emission 520) was monitored. See Experimental Procedures for further experimental details. The final concentrations of mgn2a in the six reaction mixtures A–F were, respectively, 0, 1.30, 1.85, 2.22, 2.78, and 3.70 μM.

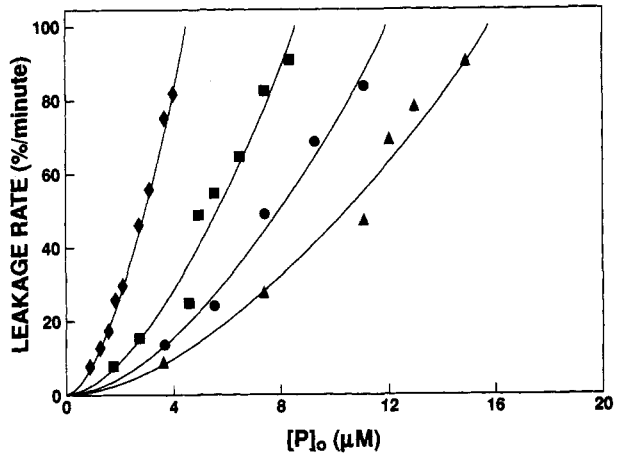


FIGURE 2: Leakage rate as a function of total peptide concentration at four concentrations of SUVs. The concentrations of PS were 25 (diamonds), 50 (squares), 75 (circles), and 100 μM (triangles). The leakage rate is defined as the percentage of dye released in the first minute.

RESULTS

Effect of mgn2a on the Permeability of PS SUVs. As shown in Figure 1, addition of mgn2a to PS SUVs causes the release of CF, but the kinetics are not simple first order; dye is released in a rapid initial phase, lasting about 100 s, followed by a much slower phase.

To narrow down the number of plausible explanations for these kinetics, it is necessary to know (1) whether the peptide readily equilibrates between vesicles, (2) the number of peptides bound per vesicle, and (3) whether the peptide induces gross structural changes in the vesicles.

Determination of a Binding Isotherm for the mgn2a–PS Interaction. A binding isotherm for the mgn2a–PS SUV interaction was obtained by the "indirect method", which has been used to determine the affinity of permeabilizing agents for cells or liposomes (Thron, 1964; Matsuzaki et al., 1988, 1989b). The first step, as shown in Figure 2, is to measure the fractional leakage rate ($\%Q/s$) as a function of peptide concentration at several fixed concentrations of lipid. Next, a set of lines parallel to the x axis is drawn through the four dose response curves, giving for each line four pairs of $[P_0]$,-

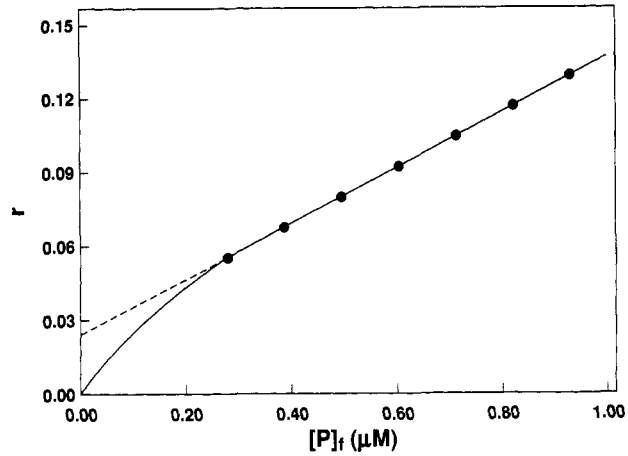


FIGURE 3: Binding isotherm for the interaction of mgn2a with CF-loaded PS SUVs.

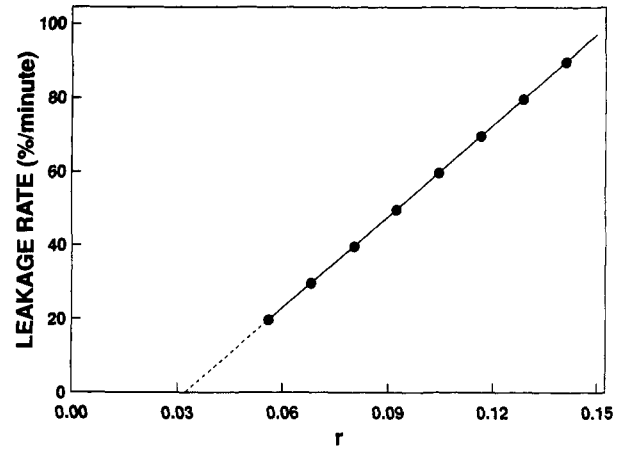


FIGURE 4: Dose response curve relating leakage rate to the ratio of moles of peptide bound per mole of lipid.

[L] values that produce an equivalent fractional leakage rate. $[P_0]$ and [L], the total molar concentrations of peptide and lipid, respectively, are functionally related to the concentrations of bound and free peptide by the conservation equation:

$$[P_0] = [P_f] + r[L]$$

where $[P_f]$ is the concentration of free peptide and r is the molar solution concentration ratio of bound peptide per lipid. Since all pairs of $[P_0]$,[L] values that produce a given fractional leakage rate must also produce the same value of r, plots of $[P_0]$ versus [L] at constant fractional leakage rates are linear, with slopes of r and intercepts of $[P_f]$. The set of r,$[P_f]$ obtained from these plots constitutes the binding isotherm, shown in Figure 3. From the slope of a line drawn through the experimental points, an apparent binding constant of 1.12×10^5 M^{-1} was obtained.

A dose response curve relating the fractional leakage rate to the amount of peptide actually bound is shown in Figure 4. The experimental points lie on a straight line that extrapolates to an intercept of $r = 0.03$, which is the "critical number" of peptide monomers that must be bound per mole of lipid to induce leakage. In our kinetic experiments, r varied from 0.06 to 0.14, which corresponds to 60–250 peptide molecules bound per SUV. Because this ratio is so high, no vesicle was devoid of peptide during a kinetic run.

Although binding isotherms are frequently used in attempts to deduce the state of peptide aggregation on the vesicle surface, definitive interpretations are not always possible because it is difficult to take into account steric effects and changes in

Table I: mgn2a-Induced Increases in Light Scattering of SUVs[a]

[mgn2a] (μM)[b]	predicted changes in i_c/i_v: with no fusion or aggregation[c]	with fusion or aggregation[d]	observed
0.93	1.17–1.23	>2.34	1.31
1.30	1.25–1.34	>2.50	1.23
1.85	1.34–1.47	>2.68	1.54
2.22	1.44–1.61	>2.88	1.91
2.78	1.56–1.78	>3.12	1.92
3.70	1.77–2.09	>3.54	2.38

[a] The relative increase in 90° light scattering (i_c/i_v) was determined with a spectrofluorometer as described under Experimental Procedures. [b] 5–20 μL of 0.37 mM mgn2a was added to SUVs ([PS] = 30 μM) in buffer A; vesicles contained 100 mM entrapped CF. An immediate increase in light scattering was observed, which showed no further change over 500 s. [c] Range of values reflects uncertainty in the refractive index increment (see Experimental Procedures). [d] Lower limit estimate, assuming all peptide–vesicle complexes double in mass. The values were obtained by multiplying the smaller number in the previous column by a factor of 2.

Table II: Mode of mgn2a-Induced Dye Release from SUVs[a]

[mgn2a] (μM)	duration of run (s)	%CF release	predicted %Q: all-or-none release	graded release	observed
0	100	0			80
1.85		13	80	78	82
2.22		17	80	77	80
2.78		28	80	74	80
3.70		64	80	56	79
0	500	0			80
1.85		18	80	77	80
2.22		30	80	73	80
2.78		50	80	67	79
3.70		82	80	36	76

[a] SUVs (25–30 μM in PS) started with 30 mM entrapped CF. Details of how both the predicted and observed values were obtained are given under Experimental Procedures. The error in %Q is ±1.5%.

vesicle surface potential upon peptide binding (Schwarz et al., 1986). We shall avoid any attempt to do so here.

For our purposes, the most important result is that the mgn2a–vesicle interaction appears to be reversible. This was substantiated by the following experiment. To a mixture of 3.7 μM mgn2a and loaded SUVs (30 μM PS) was added 5 μL of unloaded vesicles (11.5 mM PS) at 10, 30, and 60 s after the peptide was injected. In each case, leakage stopped almost instantly after injection of unloaded vesicles. The most reasonable conclusion is that the level of mgn2a bound to the loaded vesicles was reduced by peptide equilibrating between loaded and unloaded vesicles.

Light-Scattering Studies. To determine whether mgn2a causes gross structural changes in the SUVs, the 90° light scattering of a vesicle solution was measured before and after addition of peptide. As shown in Table I, peptide causes a dose-dependent increase in the relative light scattering, up to a value of 2.38. This increase can be attributed mainly to the increase in mass and perhaps refractive index increment of the particles contributed by the peptide, as shown by the theoretical calculations in column 2.

Column 3 shows the theoretical lower limit for a light-scattering increase that would occur if all the initial SUVs (with bound peptide) uniformly increased in size by undergoing a single fusion event. Clearly, these values are substantially higher than the ones observed. Moreover, this lower limit prediction is highly conservative, if not unrealistic, since it has never been observed (to our knowledge) that bilayer-destabilizing peptides or proteins cause such a controlled, limited aggregation or fusion of vesicles; in all reported studies of protein- or peptide-induced fusion [reviewed in Maezawa et al. (1989)], the protein or peptide causes massive aggregation of vesicles, or the formation of a heterogeneous dispersion of vesicles that results from multiple fusions. It should also be noted that the kinetics of aggregation or fusion are usually sufficiently slow (with half-times of a few seconds to minutes) that time-dependent changes in light scattering are observed upon addition of peptide or protein. In our experiments, however, the changes in light scattering appear to be instantaneous and do not increase with time.

From our results, calculations, and the reported effect of known fusogens on lipid vesicles, we conclude that mgn2a does not induce a significant fusion or aggregation of PS SUVs.

Kinetic Studies of the Biphasic Release of CF. Since mgn2a (including any lytic form) rapidly equilibrates between vesicles, a simple model whereby the peptide reversibly forms channels cannot explain why only a fraction of the dye is released in the fast phase. One possibility is that magainin is inactivated during the initial 100 s by interaction with the vesicles. This was tested by preincubating mgn2a (1.85–3.7 μM) with unloaded vesicles (5 μM in PS) for 100 s before addition of loaded vesicles (final [PS] = 25 μM). Fast release was still observed (data not shown), of a magnitude predicted for this ratio of loaded to unloaded vesicles. Evidently, the peptide remains active.

Biphasic kinetics could also arise from putative highly lytic peptide aggregates that exist in the concentrated stock solution, but which disaggregate in the assay mixture. This was tested by preincubating mgn2a for up to 24 h in the cuvette at assay concentrations before adding vesicles. The release kinetics were essentially the same, making this explanation unlikely.

[It should be noted that in these experiments the cuvette was rinsed with mgn2a solution before adding the preincubation solution. Without the prerinse, the activity of mgn2a decreases slowly if the solution is allowed to stand and decreases rapidly if the solution is continuously stirred. The simplest explanation is that in the absence of lipid, the cationic peptide adsorbs to the glass walls of the cuvette, and that stirring accelerates this process by eliminating diffusion as a rate-limiting step. Tosteson et al. (1985) have previously reported that melittin, a positively charged peptide, also binds to glass.]

If the intrinsic activity of the peptide remains constant, then it must be the vesicles that undergo transient changes. Some insight into the nature of these events can be obtained from determining the mode of dye release.

As discussed earlier, dye may be released in an all-or-none or a graded fashion. These mechanisms are distinguished by measuring the percent quenching remaining in the vesicles, %Q_{tr}, after a significant fraction of total dye has been released; if release is all-or-none, %Q_{tr} stays constant, whereas if release is graded, %Q_{tr} decreases by an amount that can be determined from a standard quench curve.

After addition of mgn2a to the vesicles, the residual quenching of entrapped dye was determined near the end of the fast phase (100 s) and at a later time point (500 s) in an attempt to discern the mode of release for each phase of the reaction. The results are shown in Table II. By comparing the experimental results with those predicted for the two modes of release, it is clear that release is all-or-none during the first 100 s.

A similar comparison of %Q_{tr} at 500 s appears to indicate that all-or-none release continues through the slow phase as well. However, before drawing such a conclusion, it must be shown that in theory, a purely all-or-none release would

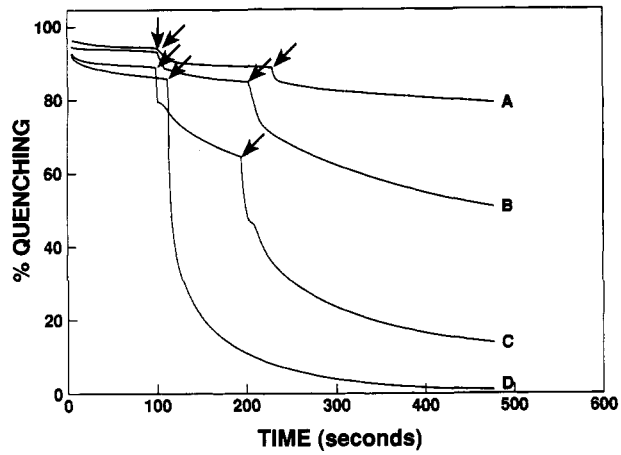


FIGURE 5: Effect of adding successive additions of mgn2a to loaded SUVs. mgn2a was added to SUVs of 29 μM PS. Curve A: 1.11-nmol additions were made at t = 0, 109, and 234 s. Curve B: 1.85-nmol additions were made at t = 0, 109, and 204 s. Curve C: 2.59-nmol additions were made at t = 0, 99, and 194 s. Curve D: 3.70-nmol additions were made at t = 0 and 115 s. Arrows indicate the times of addition.

produce a significantly different $\%Q_{tr}$ than a process involving a change from all-or-none to graded. Using a standard quench curve, a calculation showed that it is possible to discern the mode of release during the slow phase, but only in the runs where the two highest levels of mgn2a were used, i.e., 15 and 20 μg. In those cases, a sequential bimodal release (all-or-none followed by graded) should have produced final $\%Q_{tr}$ values of 74 and 64%, respectively; these differ sufficiently from the starting $\%Q_{tr}$, so that a bimodal release could have been detected if it had occurred. From the experimentally determined values of 79 and 76%, we conclude that release over the 100–500-s interval is primarily all-or-none.

There are two ways that lysis of the vesicles could arise. Interaction with peptide could *induce* instability into the vesicles, which then relax to stable proteoliposomes accompanied by lysis of a fraction of the vesicles. (Stability is defined here as the lowest free energy for vesicles of a given size.) Alternatively, the vesicles might start out in a metastable form before the peptide is added. If so, the peptide would function solely as a catalyst that facilitates relaxation of the unstable vesicles to a more stable structure, accompanied by lysis of a fraction of the vesicles. Preexisting metastability could result from the method of preparation, such as sonication.

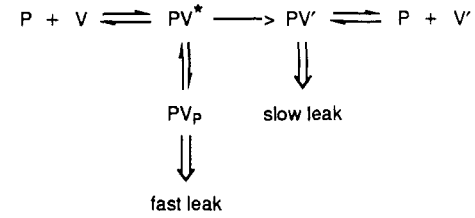
These alternatives can be distinguished by adding more magainin at the end of the fast phase. If the peptide induces instability, another fast phase should be observed, but if it acts catalytically, the vesicles should all be relaxed by the time of the second peptide addition; consequently, no additional fast-release phases should occur.

As shown in Figure 5, a new fast phase is induced with each addition of peptide, indicating that lysis arises from peptide-induced vesicle instability rather than preexisting vesicle instability.

In further support of this conclusion, we find that mgn2a induces biphasic release of dye from LUVs (data not shown), the only difference being that approximately half as much mgn2a is required to achieve the same $\%Q$ as with SUVs. Since LUVs are not produced by sonication, and are too large to be affected by curvature-induced strain, whatever might cause the putative metastability of SUVs is unlikely to be present in LUVs.

Kinetics of Detergent-Induced Dye Release. As shown earlier, mgn2a does not begin to induce measurable CF release

Scheme I



until the level of bound peptide becomes quite high, at r = 0.03. To compare the intrinsic permeabilizing activity of mgn2a with that of detergents, the effect of octyl glucoside and Triton X-100 on CF release from PS SUVs was determined. From the leakage kinetics and the published values of the bilayer/water partition coefficients for these detergents (Paternostre et al., 1988), dose response curves relating r to the fractional leakage rate can be obtained. Our results (not shown) are essentially the same as those reported by Lasch et al. (1990) in a study of the effect of these detergents on the release of CF from LUVs of egg PC: measurable leakage was not observed until the r values for Triton X-100 and octyl glucoside reached about 0.07 and 0.175, respectively. [The leakage rate sharply increases at r = 0.42 (Triton X-100) and r = 0.23 (octyl glucoside); these are the upward turning points of the sigmoidal dose response curves.]

When compared on a weight basis (grams per mole of lipid), the r values at leakage onset for mgn2a, Triton X-100, and octyl glucoside are remarkably similar—81, 44, and 51, respectively.

DISCUSSION

The simplest model that is at least qualitatively consistent with all of our results is the bilayer destabilization mechanism, shown in Scheme I.

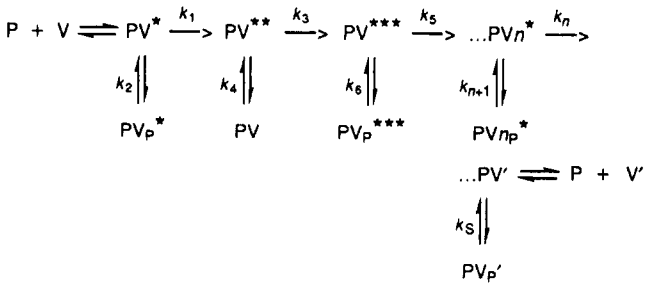
In the first step, peptide reversibly binds to the vesicles to form a destabilized vesicle, PV*. Destabilization is suggested to arise from a packing imbalance created by the accumulation of peptide on or in the outer monolayer of the vesicle. PV* then undergoes a rapid, irreversible relaxation, perhaps by lipid translocation, to a more stable proteoliposome, PV′, which exists in equilibrium with free peptide. Although the scheme suggests that the stable vesicle V′ is devoid of protein, V′ represents a proteoliposome with an equilibrium level of peptide bound to the outer monolayer; the packing and distribution of lipid in this proteoliposome is a variable that is dictated by the amount of protein bound to the surface. At the end of the fast release phase, the only species present are PV′, P, and V′.

During the lifetime of PV*, fluctuating pores (V_p) form within the lipid bilayer. In the scheme, the conversion of PV* to PV_p represents the rate of forming pores large enough to release CF. It is assumed that once a pore this large is formed, all the dye is released from that vesicle before the pore dissipates. Therefore, the rate-limiting step in the release of dye from PV* is the conversion of PV* to PV_p; the arrow leading back from PV_p to PV* represents the dissipation of a pore in an unloaded vesicle.

This model accounts for the limited fast release of dye by an all-or-none mechanism in a system with a persistently active peptide that exchanges between vesicles. To account for the observation that further addition of peptide at the end of the fast phase induces another fast phase (Figure 5), we suggest that V′ must undergo further destabilization and relaxation to accommodate a higher level of peptide.

For this minimal model, only one transiently destabilized form of the vesicle (PV*) is postulated. If relaxation occurs

Scheme II



through a series of intermediates, the reaction scheme can be expanded according to the general form shown in Scheme II where the rate constants with even-numbered subscripts represent the leak rate from each intermediate and k_s is the final leak rate in the slow phase. If the free and bound peptides are in rapid equilibrium, the rate equation for this mechanism simplifies to a sum of first-order exponentials:

$$F = A\exp[-(k_1 + k_2)t] + B\exp[-(k_3 + k_4)t]...+ Z\exp(-k_2t)$$

where F is the percentage of the vesicle population that contains dye and is equal to $\%Q$ because the mode of release is all-or-none. The preexponential terms are complex expressions of rate constants.

A nonlinear least-squares analysis (Bevington, 1969) of our $\%Q$ vs t curves indicates that at the highest levels of added mgn2a (where the signal/noise ratio is most favorable) the kinetics are closely fit by a triexponential, $\%Q = A\exp(-k_at) + B\exp(-k_bt) + C\exp(-k_ct) + D$, with the following values for the constants, in s^{-1}: $A = 41.6$, $k_a = 0.192$; $B = 26.9$, $k_b = 0.00756$; $C = 23.2$, $k_c = 0.0011$; $D = 5.71$. These results suggest that vesicle relaxation is a multistep process.

The alternative to bilayer destabilization is that the peptide forms transient oligomeric channels in the bilayer, but the only reasonable explanation for the transient nature of such a putative channel is that it exists only in a transiently destabilized bilayer. If so, the function of the channel is superfluous since a destabilized bilayer itself is a breach of the permeability barrier. Therefore, explanations based upon peptide-lined pores are hard to rationalize.

Since we favor a bilayer destabilization mechanism, it would be informative to compare the characteristics of mgn2a-induced leakage with the characteristics of detergent-induced leakage. In particular, we would like to know whether detergents ever induce biphasic release kinetics, cause leakage by an all-or-none mode of release, or have a specific activity comparable to that of mgn2a.

Some detergents do show biphasic release kinetics: Schubert et al. (1986) and Lasch et al. (1990) have found that cholate and deoxycholate release only a fraction of entrapped solute (the extent of which depends on the amount of detergent added) even after prolonged times. Schubert et al. suggest that leakage is due to the formation of transient holes in the bilayer, which is essentially what we are proposing to account for mgn2a action.

To our knowledge, all-or-none release has not been looked for in studies with liposomes. However, it has been shown that detergents can induce an all-or-none lysis of cells (Scharff, 1960; Maas, 1969; Silhankova, 1959) and some detergents cause discrete conductance fluctuations in planar bilayers (Blumenthal & Klausner, 1982).

The specific activities of mgn2a and detergents are comparable. Leakage onset does not occur until the levels of bound magainin (on a weight basis of grams per mole of phospholipid) equal or exceed the high critical values observed with detergents: for mgn2a, 81; Triton X-100, 44; octyl glucoside, 51. Although it seems fortuitous that a cationic amphipathic peptide should have a comparable activity to two neutral detergents, it is interesting to note that cholate, with an amphipathic architecture and charge that is different from Triton X-100 and octyl glucoside, also shows a similar critical r value: the release of raffinose from egg PC LUVs does not occur until r equals about 0.15 (Schubert et al., 1986), which is 65 g of cholate/mol of phospholipid.

As noted earlier, magainin forms channels in highly polarized (>100 mV) planar lipid bilayers composed mainly or exclusively of neutral phospholipids (Duclohier et al., 1989). In contrast, we have observed (unpublished observation) that addition of mgn2a to planar bilayers composed of PC/PE/PS under a trans-negative potential of <80 mV increases the macroscopic conductance without evidence of discrete conductance states. Also, although liposomes of the same lipid composition are readily permeabilized by mgn2a, when mgn2a was added to the planar bilayers in the presence of liposomes (to maintain the same protein:lipid ratio that induces rapid dye release from the liposomes), no significant change in membrane conductance was seen.

From our results and those obtained by others, we can explain the action of magainin in a variety of model membrane systems by making three assumptions. First, an increase in the negative charge of the membrane increases the affinity of the bilayer for the cationic peptide, which promotes transient bilayer destabilization and leakage. Second, an increase in the negative surface charge of the membrane from anionic phospholipids inhibits voltage-induced channel formation by causing the peptide to remain bound in an electrically neutral (and therefore potential-insensitive) complex at the vesicle surface or interface. Third, planar bilayers are more resistant than liposomes to peptide-induced transient destabilization because planar bilayers can relieve the strain by transferring lipid into or out of the torous or by bending in either the cis or the trans direction.

To summarize, mgn2a can permeabilize membranes via bilayer destabilization or by channels. Which mechanism predominates depends on the membrane system, composition, and degree of polarization.

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Registry No. mgn2a, 114317-44-1.