Fluorescence Indicates a Calcium-Dependent Interaction between the Lipopeptide Antibiotic LY146032 and Phospholipid Membranes

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ABSTRACT: LY146032 is one of the A21978C family of calcium-dependent antibiotics. This paper reports on its interactions with membranes as studied by its intrinsic fluorescence. The Trp residue was found to have a low fluorescence yield because of Förster-type energy transfer to the kynurenine residue (Kyn) (ϵ = 5000 at 364 nm). However, the Kyn fluorescence (λ_{max} = 465 nm in H_2O) was a sensitive probe of the membrane interactions, and it was used in steady-state fluorescence measurements including fluorescence polarization anisotropy. Initial binding of the peptide to phospholipid vesicles occurs in calcium-free solutions. When calcium is added, the resulting 10-fold fluorescent enhancement and 15-nm blue shift show that it causes the antibiotic to penetrate further into the lipid bilayer. Calcium is bound with an association constant of 151 M^{-1}, while a phospholipid titration in the presence of calcium gave an association constant of 5×10^3 M^{-1} for egg phosphatidylcholine. Magnesium and cadmium cause very slight fluorescence enhancements, but a more significant effect is caused by the trivalent lanthanide ions. Analysis of these data indicates that the calcium-selective site is on the peptide and that ion binding to the phospholipid headgroups has a secondary role. Comparison with the divalent cation dependent antibiotics bacitracin and amphomycin shows that LY146032 has a quite different activity and that a calcium-dependent membrane interaction could account for results obtained in vivo.

LY146032 (Figure 1), an acidic lipopeptide antibiotic produced by *Streptomyces roseosporus*, is effective against a range of Gram-positive pathogenic bacteria (Eliopolous et al., 1985). However, its in vitro antibiotic activity is unusual because it requires the presence of calcium ions. For example, it has been shown that in the case of *Streptococcus faecalis* the addition of 1 mM calcium chloride to the culture medium causes a 30-fold decrease in the minimum inhibitory concentration (Eliopolous et al., 1985). This effect was found to be unique to calcium, and media supplements of Ba^{2+}, Zn^{2+} and Mg^{2+} had no comparable ability to lower the minimum inhibitory concentration. LY146032 has been shown to inhibit test infections in mice, but clearly it was not possible to observe the effect of a varying Ca^{2+} concentration.

The possibility that its action was that of a calcium ionophore was excluded by experiments on black lipid films and phospholipid vesicles. These showed that although it did increase the bilayer conductivity, this increase was neither calcium dependent nor sufficiently large to account for the observed antibacterial activity (Lakey & Lea, 1986). These experiments did show, however, that in common with other lipopeptides, e.g., polymixins, octapeptins (Storm et al., 1977), and iturins (Maget-Dana et al., 1985), LY146032 interacts strongly with bilayer membranes (Lakey & Lea, 1986). This common property of lipopeptides is thought to be due to insertion of the fatty acyl moiety into the membrane, and as a group their modes of action are varied but always membrane based. If the action of LY146032 involves a membrane-based step, it serves a purpose other than directly increasing membrane permeability, and so another measure of this interaction is required. In this paper we use the intrinsic fluorescence of LY146032 to study its interactions with phospholipid vesicles and calcium.

The peptide sequence of LY146032 is identical with that of the A21978C family of peptides produced by *S. roseosporus* (Debono et al., 1987). They differ solely in the length of their fatty acyl tails, LY146032 having a straight-chain decanoyl side chain (Figure 1). As the chemical structure of the calcium-dependent antibiotic (CDA) from *Streptomyces coelicolor* (Lakey et al., 1983) is unknown, its relationship with LY146032 is unclear.

Experimental Procedures

LY146032 was a gift from Lawrence Day of Eli Lilly & Co. Egg yolk phosphatidylcholine was purified according to the method of Singleton et al. (1965). DMPC[1] and DPPC were purchased from Sigma, verified pure by thin-layer chromatography, and used without further purification. $GdCl_3$ was from UCB, Brussels, Belgium, and $EuCl_3$ was from Fluka. L-Kyn was purchased from Sigma, and all other chemicals were of the best available grade.

Small unilamellar vesicles (SUV) were prepared by sonication. Phospholipid in ethanol was dried as a thin film in a glass cup, a standard buffer solution (standard buffer, 200 mM KCl/10 mM cacodylate, pH 6.0) was added, and sonication by an MSE sonicator was carried out under nitrogen. Subsequently, the vesicles were centrifuged for 1 h at 40000 rpm (130000*g*) to remove multilamellar liposomes and titanium fragments. All stages of the preparation were performed above the phase transition temperature for the lipid concerned. PC

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[1] Abbreviations: SUV, small unilamellar vesicles; PC, phosphatidylcholine; DMPC, L-α-dimyristoyl-PC; DPPC, L-α-dipalmitoyl-PC; L-Kyn, free kynurenine; P-Kyn, kynurenine residue in LY146032; FPA, fluorescence polarization anisotropy; T_m, phase transition temperature.

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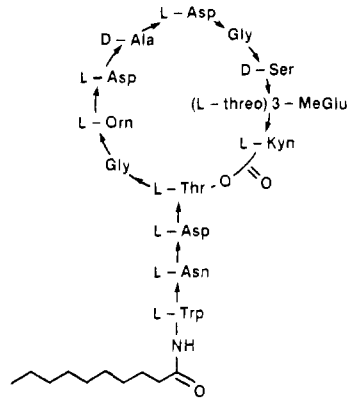

FIGURE 1: Structure of LY146032 (decanoyl-A21978C): Kyn, kynurenine; Orn, ornithine; Me-Glu, methyl glutamate.

concentrations were determined by measuring the lipid phosphorus according to the method of Chen et al. (1956).

Chemical Modification of LY146032. Acetylation of the aromatic amine on the kynurenine residue of LY146032 was carried out as follows: 0.5 mg of LY146032 was dissolved in a solution of 330 μL of distilled water, 100 μL of 1 M HCl, and 60 μL of acetic anhydride by vortexing. After the addition of 20 mg of solid sodium acetate, the mixture was vortexed again and then purified by the method of Bohlen et al. (1980) with 2-propanol/water (7/1) as the eluant.

Fluorescence Techniques. Fluorescence measurements were carried out on a Kontron SFM25 with slits set to 5 nm (excitation) and 10 nm (emission). The spectrofluorometer was interfaced with an Apple IIe microcomputer for data collection and manipulation. Temperature control was by circulating water through the four-cell holder in which the temperature was measured by a thermocouple placed in a buffer-filled cuvette.

The fluorescence results are expressed as either the relative fluorescence at a given wavelength (F) or, where possible, the relative quantum yield (Φ) calculated as the integrated fluorescence emission between 400 and 520 nm.

Polarization experiments used the SFM25 polarization accessory under remote control via the Apple IIe. The fluorescence intensity components (I_{vv}, I_{vh}, I_{hv}, I_{hh}), in which the subscripts refer to the horizontal (h) or vertical (v) positioning of the excitation and emission polarizers, respectively, were used to calculate the steady-state fluorescence polarization anisotropy (FPA):

$$\text{FPA} = (I_{vv} - I_{vh}G)/(I_{vv} + 2I_{vh}G) \quad (1)$$

where G is the grating factor that corrects for wavelength-dependent distortions of the polarizing system.

$$G = I_{hv}/I_{hh} \quad (2)$$

All experiments were carried out in 1 cm path length cuvettes containing at least 2.5 mL of sample with an absorption at 364 nm of <0.1. Absorption measurements were performed on a Beckman DU-8 or an LKB Biochrom spectrometer.

The association of LY146032 with phosphatidylcholine, Ca^{2+}, and Gd^{3+} (as ligands) was analyzed by the method of Lehrer and Fasman (1966). The association constant K_a is related to the sample fluorescence at a particular ligand concentration [S] by

$$K_a = [(F - F_0)/(F_\infty - F)](1/[S]) \quad (3)$$

where the fluorescence intensities of the peptide alone, the peptide at [S], and the peptide at an infinite concentration of S are denoted by F_0, F, and F_∞, respectively. F_∞ was calculated by extrapolation of the plot $1/(F - F_0)$ versus $1/[S]$ to $1/[S] = 0$. In all cases, the plot of $(F - F_0)/(F_\infty - F)$ versus [S] fitted to a straight line at nonsaturation concentrations of S, and K_a was calculated as the inverse of [S] at the intersection with $(F - F_0)/(F_\infty - F) = 1$ (Privat et al., 1974). The treatments according to Kolber and Haynes (1981) and Kauffman et al. (1983) were as described in the original references.

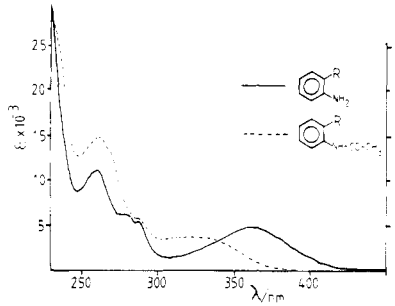

FIGURE 2: Absorption spectra of native and modified LY146032 in water: solid line, unmodified LY146032; broken line, LY146032 after acetylation of its kynurenine residue.

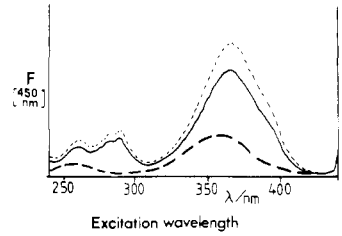

FIGURE 3: Excitation spectra of LY146032 kynurenine fluorescence in 1 mg/mL egg PC SUV: F(450 nm), relative fluorescence at 450-nm emission wavelength without subtraction of the SUV base line; (—) standard buffer plus 50 mM $CaCl_2$; (- - -) standard buffer plus 50 mM $CaCl_2$ and 15 mM $GdCl_3$. The excitation spectrum of free L-Kyn in standard buffer is also shown (– –) although not on the same scale.

RESULTS

LY146032 Fluorescence Characteristics. As shown in Figure 1, the peptide contains four negatively charged groups [three aspartate and one methyl glutamate (Me-Glu)], one positively charged ornithine (Orn), and two fluorophores. These comprise a tryptophan residue situated between the lipid chain and the peptide headgroup and a kynurenine residue (Kyn) in the headgroup itself. The situation of the Trp should make it sensitive to insertion of the lipid moiety into a hydrophobic bilayer, but unfortunately, this residue has a very poor fluorescence yield. When a solution of LY146032 (10^{-5} M peptide in standard buffer) was excited at 285 nm, the Trp emission was only 3.5% that of free L-Trp under the same conditions. The absorption spectrum clearly indicates (Figure 2) a Trp (ϵ = 5000 at 286 nm) concentration equal to that of the peptide, while the ^{1}H NMR spectrum (D. Marion, A. Caille, J. Lakey, and M. Ptak, unpublished results) showed Trp and kynurenine to be present in equal proportions, thus excluding the possibility of Trp degradation having occurred (Creed, 1984). Furthermore, the excitation at 285 nm produced a second emission at 465 nm, which was more intense when excited at 365 nm, indicating that Kyn was the second emission source. The excitation spectrum (Figure 3) for this Kyn emission clearly shows a strong Trp component around 285 nm that is in between the Kyn absorbtion maxima at 256 and 364 nm. It was thus assumed that the poor Trp emission is at least partly due to energy transfer. This Forster type of transfer results from the combination of the proximity of the two fluorophores and the overlap of the Trp emission spectrum

Table I: Effect of Multivalent Ions on P-Kyn Fluorescence

	salt	concn (mM)	rel quantum yield	max emission (nm)
LY146032			1	465
(10^{-5} M in std buffer)	$CaCl_2$	50	0.80	465
	$CdCl_2$	50	0.92	465
	$MgCl_2$	50	0.98	465
	$GdCl_3$	15	2.62	456
LY146032			1.0	462
(10^{-5} M + 1 mg/mL	$CaCl_2$	50	10.7	450
egg PC in std	$CdCl_2$	50	1.7	462
buffer)	$MgCl_2$	50	1.2	462
	$GdCl_3$	5	5.4	452

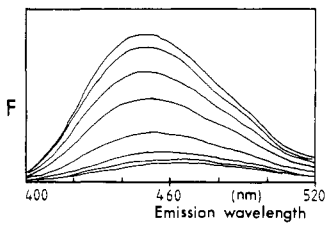

FIGURE 4: Emission spectra of LY146032 kynurenine fluorescence in 1 mg/mL egg PC SUV. F is the relative fluorescence after subtraction of the SUV base line. Spectra show the increase in fluorescence as the calcium ion concentration is increased stepwise from 0 to 10 mM $CaCl_2$ in standard buffer. Excitation wavelength, 365 nm.

and Kyn absorption spectrum (Weinryb & Steiner, 1971).

Chemical Modification of Kyn. In order to test this hypothesis and perhaps in so doing increase the available Trp signal, we changed the absorption characteristics of the Kyn residue by acetylation of the free NH_2 group. This resulted in a decrease in the Kyn absorbance (Figure 2), which together with the Kyn fluorescence was blue shifted by 20 nm. As the Kyn absorption band was initially at a longer wavelength ($\simeq$360 nm) than the Trp emission ($\simeq$350 nm), this shift alters only slightly the overlap of these two functions. However, the combined changes in the Kyn absorption result in a 25% increase in the Trp emission, which although confirming the energy-transfer hypothesis does little to enhance Trp's usefulness for studies in vesicles. Other modifications (carbamylation, benzoylation) were tried, but acetylation proved the most effective. All subsequent experiments were carried out on the unmodified peptide.

Kynurenine Fluorescence. Kyn shows only a very weak fluorescence as a free amino acid (L-Kyn), but when incorporated into the peptide (P-Kyn), its relative quantum yield is 7.6 times greater. As such it is slightly more luminescent than P-Trp at 10^{-5} M, although at higher peptide concentrations the ratio of fluorescence yield P-Kyn/P-Trp increases, possibly due to aggregation effects (^{1}H NMR in water at pH 6.0 reveals significant line broadening above 5 mM peptide). In 1 mg/mL egg PC vesicles the P-Kyn (10^{-5} M) yield increases by 2.5-fold and thus becomes useable in studies of lipopeptide/vesicle binding. This is further helped by the >80-nm difference in its absorption and emission wavelengths, which reduces problems due to vesicle light scattering.

Effect of Calcium Ions. When added to the free peptide in solution, divalent cations reduced the P-Kyn fluorescence, and the size of this quenching diminished in the order $Ca^{2+} \gg Cd^{2+} > Mg^{2+}$ (Table I). There was no observable effect on the wavelength of maximum emission, the absorption spectrum, or the extent of aggregation as viewed by light scattering. When mixed with egg PC vesicles, the result is the reverse with 50 mM $CaCl_2$, causing a 10-fold increase in

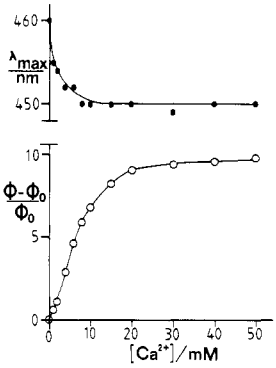

FIGURE 5: LY146032 kynurenine fluorescence enhancement in phosphatidylcholine SUVs by calcium ions. Samples contained 1 mg/mL egg PC SUV and 10^{-5} M LY146032 in standard buffer. Calcium was added from 1 or 3 M $CaCl_2$ stock solution and mixed, and the fluorescence yield was measured as the integral between 400 and 520 nm after base line subtraction. The fluorescence increase was instantaneous on this time scale. λ_{max}, wavelength of maximum emission; ϕ, fluorescence yield; ϕ_0, fluorescence yield in the absence of multivalent ions; λ_{ex}, 365 nm.

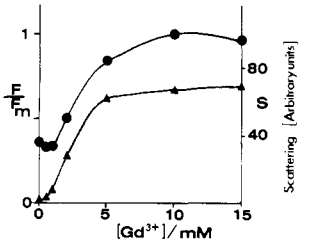

FIGURE 6: Fluorescence and light scattering increases in LY146032 solution on $GdCl_3$ addition. (●) Fluorescence expressed as the fraction of maximum fluorescence attained (F/F_m). The cuvette contained 2.5 mL of 10^{-5} M LY146032 in standard buffer. Gd^{3+} was added from 0.5 M $GdCl_3$ stock. λ_{ex}, 365 nm; λ_{em}, 455 nm. (▲) Same conditions as above except that $\lambda_{ex} = \lambda_{em} = 480$ nm so that the measured signal is a product of the solution's light scattering properties. The increases were not immediate, and those plotted (S and F) are the values 30 min after Gd^{3+} addition.

P-Kyn emission combined with a blue shift in its maximum (Table I; Figures 4 and 5). The effect was the same when calcium acetate was used instead. The order of size of effect was again $Ca^{2+} \gg Cd^{2+} > Mg^{2+}$ (Table I).

Effect of Lanthanide Ions. When their use as possible quenching agents was investigated, it was discovered that the lanthanide ions Eu^{3+} and Gd^{3+} also showed very specific interactions with LY146032. As shown in Table I, $GdCl_3$ has the opposite effect to $CaCl_2$ on the free peptide, causing an increase in P-Kyn's relative quantum yield. When followed simultaneously by light scattering measurements, the fluorescence increase was seen to be accompanied by the formation of aggregates (Figure 6), which may render P-Kyn into a more hydrophobic environment. At saturating concentrations, in egg PC SUV, $GdCl_3$ and $EuCl_3$ cause smaller increases and blue shifts in P-Kyn emission than does $CaCl_2$ (Table I; Figure 7). If $GdCl_3$ is added to 10^{-5} M LY146032, 1 mg/mL egg PC, and 50 mM $CaCl_2$, the fluorescence increases again with a second saturation at 5 mM $GdCl_3$ (Figure 3). A like result is obtained when calcium ions are added to a suspension containing 15 mM $GdCl_3$. Thus, it appears that the action of one species does not impede the activity of the other.

The data from Figures 5 and 7, when analyzed as described earlier, gave association constants (K_a) for the Ca^{2+} and Gd^{3+} effects as 151 M^{-1} and 6.6 × 10^3 M^{-1}, respectively. This assumes that the fluorescence enhancement is proportional to the quantity of bound ion.

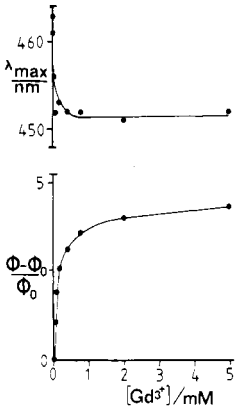

FIGURE 7: LY146032 kynurenine fluorescence enhancement in phosphatidylcholine SUVs by gadolinium ions. Samples contained 1 mg/mL egg PC SUV and 10^{-5} M LY146032 in standard buffer. Gadolinium was added from 0.5 M $GdCl_3$ stock solutions and mixed, and the resultant mixture was analyzed as in Figure 5. The fluorescence increase was instantaneous on this time scale.

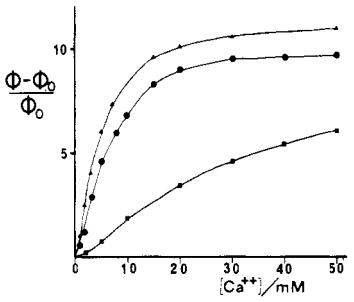

FIGURE 8: Influence of surface charge on LY146032 kynurenine fluorescence enhancement in phosphatidylcholine SUVs by calcium ions: (●) 2.5 mL of 1 mg/mL egg PC SUV and 10^{-5} M LY146032 in standard buffer; (▲) 2.5 mL of 1 mg/mL egg PC/stearylamine (9:1) SUV and 10^{-5} M LY146032 in standard buffer; (■) 2.5 mL of 1 mg/mL egg PC/PI (9:1) SUV and 10^{-5} M LY146032 in standard buffer. For other details see Figure 5.

The influence that surface charge has on the Ca^{2+} effect is indicated in Figure 8, which shows the results of adding a 10% mole fraction of phosphatidylinositol (PI) or stearylamine (ST) to the egg PC used to make the vesicles. The presence of negative charges (PI) reduces the response to Ca^{2+} addition, and the form of the curve is changed, with no observed saturation below 50 mM. The enhancement in positively charged bilayers is greater than in PC alone. The initial value of F has been normalized to 1 in all cases, and so differences due to charge in the absence of Ca have been ignored. On average (four experiments), the P-Kyn fluorescence at zero Ca^{2+} in negative bilayers was 88% that of the positive preparations. Gadolinium chloride was similarly affected by the charges, and its supplementary increase of P-Kyn emission in 50 mM $CaCl_2$ remained.

As the P-Kyn yield is dependent upon both Ca^{2+} and PC, it is important to consider whether the peptides' affinity for PC is calcium dependent. In this respect it is possible to measure an association constant between fluorophores and membranes by fluorometric titration of the molecule in solution with phospholipid vesicles (Kauffman et al., 1983; Kolber & Haynes, 1981). The data shown in Figure 9 were analyzed, like the ion binding data, according to the method of Lehrer and Fasman (1966), and this gave a K_a of 5.0×10^3 M^{-1} [application of the analogous method of Kauffman et al. (1983) and Kolber and Haynes (1981) gave results within 0.3 $\times 10^3$ M^{-1}]. Unfortunately, however, a comparison with the binding of PC in the absence of Ca^{2+} proved to be impossible due to the feeble fluorescence enhancement involved.

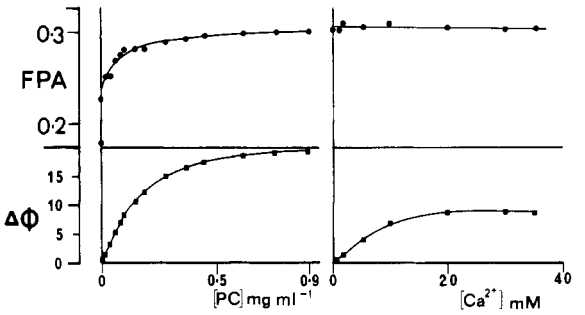

FIGURE 9: Effect of phosphatidylcholine and calcium ion titrations on LY146032 kynurenine fluorescence emission and fluorescence polarization anisotropy (FPA). DMPC titration (left-hand figure): (■) fluorescence increase (F) of 10^{-5} M LY146032 in standard buffer plus 50 mM $CaCl_2$ on addition of aliquots of 10.4 mg/mL DMPC SUV, 10^{-5} M LY146032, and 50 mM $CaCl_2$ in standard buffer; (●) FPA increase under the same conditions. Calcium titration (right-hand figure): (■) fluorescence increase of 10^{-5} M LY146032 in standard buffer plus 0.83 mg/mL DMPC on addition of aliquots of 3 M $CaCl_2$; (●) FPA under the same conditions. For calculation of FPA, see text; T = 30 °C.

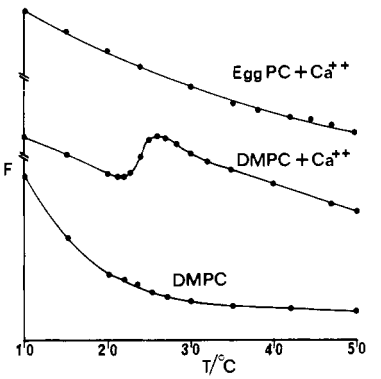

FIGURE 10: Dependence of LY146032 kynurenine fluorescence on lipid-phase transition. All samples contained 10^{-5} M LY146032. Top: 1 mg/mL egg PC SUV and 50 mM $CaCl_2$ in standard buffer. Middle: 1 mg/mL DMPC SUV and 50 mM $CaCl_2$ in standard buffer. Bottom: 1 mg/mL DMPC SUV in standard buffer (i.e., no calcium). Results are from temperature increases (10–50 °C) only.

In order to gain some insight into the role of Ca^{2+} in LY146032/PC binding, we carried out measurements of fluorescence polarization anisotropy (FPA). In 50 mM $CaCl_2$, P-Kyn shows a rise in FPA when PC is added that parallels the increase in P-Kyn emission (Figure 9). This indicates that the move of P-Kyn into a hydrophobic environment is associated with attachment to a larger structure, i.e., the phospholipid vesicle. At zero calcium, the FPA of LY146032 in 1 mg/mL PC is already at the maximum value of 0.30 reached in the PC titration. This confirms that the peptide is already fixed to the vesicles. An addition of Ca^{2+} causes an increase of the P-Kyn emission (Figure 9), which suggests a change in its environment but does not modify its FPA.

The interaction of LY146032 and PC was also followed as a function of temperature (Figure 10). In egg PC vesicles (50 mM $CaCl_2$) the fluorescence yield fell smoothly with increasing temperature due to greater nonradiative energy losses. This fall was, however, less than in aqueous solution due to the protective shielding of the lipid bilayer. In DMPC bilayers (50 mM $CaCl_2$) the fluorescence falls steadily until 21 °C when it rapidly increases to a value at 26 °C that is greater than its yield at 10 °C. It then drops almost linearly. In the absence of Ca^{2+} the P-Kyn yield drops as though in aqueous solution, with only a slight flattening of the curve around 21 °C. If DPPC bilayers are substituted, the fluorescence shows a rapid increase between 39 and 45 °C

(data not shown), while similar results were obtained from samples containing gadolinium ions rather than calcium ions. The increase in yield is thus associated directly with the "melting" of the lipid chains that occurs at the phase transition temperature (T_m) of the lipid in SUV [T_m(egg PC) < 0 °C; T_m(DMPC) = 20.8 °C (Gaber & Sheridan, 1982); T_m(DPPC) = 38 °C (Suurkuursk et al., 1976)]. Hence, in the absence of calcium the P-Kyn residues bound to SUVs are largely unaffected by the gel to liquid-crystalline transition of the fatty acyl chains. Whereas when calcium is present, it is clear that the P-Kyn fluorophore is very much more sensitive to the state of the hydrocarbon interior of the bilayer.

Discussion

The results presented in this paper clearly demonstrate that there is a calcium-dependent interaction between LY146032 and phospholipid vesicles that may aid our understanding of this antibiotic's in vivo calcium requirement.

In all the experiments described here we can assume that the lipopeptide is in monomer form. However, our ^{1}H NMR studies have shown that aggregation does occur at concentrations higher than 10^{-3} M. When mixed with SUV in the absence of $CaCl_2$, the P-Kyn yield is seen to increase by 2.5-fold, and the FPA study confirmed that the peptide does not require calcium in order to bind to these model membranes. The molecule is presumably anchored to phospholipid bilayers by insertion of its fatty acyl moiety into the nonpolar hydrocarbon layer. The existence of both negative and positive charges on the LY146032 headgroup should not be overlooked, and electrostatic interactions may also be involved at this stage. The high value of the FPA (0.30) indicates that the mobility of the P-Kyn residue is very restricted. However, the small change in the emission wavelength, the marked temperature dependence, and the insensitivity to the lipid phase transition all show that, while the peptide is certainly adhering to the membrane, this residue is situated at the lipid–water interface and is still accessible to the solvent. When calcium ions are added to the peptide/vesicle suspension, the blue shift of 10 nm and increase in relative quantum yield show that the polarity of the environment around P-Kyn falls considerably. This suggests that the neutralization of the peptide induces a motion toward the bilayer interior in which the P-Kyn comes in closer contact with the hydrocarbon chains. That such a change occurs is confirmed by the closely related yield enhancement that occurs on the melting of these chains at T_m.

The change in the position of the peptide caused by Ca^{2+} binding does not modify the FPA of its P-Kyn residue. Let us recall that, for a simple spherical molecule, the FPA is given by the classical Perrin equation

$$\mathrm{FPA} = 0.40[1/(1 + \tau/\theta)]$$

where τ is the fluorescence lifetime and θ the rotation correlation time. Usually, the major causes of FPA variations are changes in the correlation time θ.

In the present case, a FPA of 0.30 found in the absence of calcium indicates that the fluorescent probe linked to a slowly rotating vesicle already has a very restricted residual motion. A penetration of the peptide in the bilayer induced by Ca^{2+} could still slightly reduce this motion, but at the same time, there is an increase in the fluorescence lifetime (τ) that could compensate for an increase in θ. Qualitatively, one can only state that the change in the position of the peptide modifies the fluorescence parameters of P-Kyn in a manner that maintains the FPA nearly constant.

In fact, the P-Kyn residue probably has several lifetimes τ_1 and several correlation times θ_1, depending on its motions on the peptide cycle and in the bilayer environment. The binding of Ca^{2+}, which affects the conformation of the peptide and its position, modified the values and the distribution of such fluorescence parameters in a complex manner, which cannot be determined only by static measurements of anisotropy. Measurements of all lifetimes and anisotropies are then required and should be the next step of the present study.

In these experiments calcium ions can interact with two classes of site, (a) the negatively charged groups of the lipopeptide and (b) the zwitterionic groups of phosphatidylcholine. Calcium does bind to pure phosphatidylcholine bilayers and induce changes in organization that can be monitored by a variety of techniques [for a review, see Altenbach and Seelig (1984)]. Such studies indicate that the binding is weak (1–100 M^{-1}) and that it provokes a rigidification of the bilayer (Chapman et al., 1977) and establishes a positive surface potential on these zwitterionic bilayers (Altenbach & Seelig, 1984; Shah & Schulman, 1965). Much greater Ca^{2+} interactions are seen on negatively charged films. This is most apparent at low calcium concentrations, where the originally negative surface potential is rapidly neutralized. However, the Ca^{2+} ions do not bind directly to individual lipids but lie in the membrane potential trough with their effects shared among all the surface components (Macdonald & Seelig, 1987). The degree of selectivity between Ca^{2+}, Cd^{2+}, and Mg^{2+} manifested by LY146032 is not shown by pure PC systems alone (Chapman, 1977), while the $Ca^{2+} \gg Cd^{2+} > Mg^{2+}$ order of both solution quenching and membrane enhancement points to the peptide's involvement in the binding. The association constant for calcium is outside the range quoted for Ca–PC binding, but it should be made clear here that all the K_a values are first approximations only and use bulk solution concentrations rather than those existing in the double layer. Nevertheless, the binding constant is independent of the peptide concentration (results not shown) and is therefore a genuine feature of this lipid–peptide interaction rather than a function of the number of peptides per unit area of membrane.

The role of Ca^{2+} in the membrane interaction is clarified by the experiments with Gd^{3+}. It is known from previous studies (Akutsu & Seelig, 1981; Hauser et al., 1975) that Gd^{3+} interacts more strongly with phospholipids than does calcium, and this has been shown to be due to close association of the trivalent lanthanides with the phosphate group of the lipid headgroup (Hauser et al., 1975).

Hence, the fluorescence enhancement induced by Gd^{3+} in SUVs already saturated with Ca^{2+} is to be expected as the trivalent ions will replace the divalent calcium at the interface. However, this means that the fluorescence enhancement that occurs when Ca^{2+} is added to a Gd^{3+}-saturated sample must be due to the existence of a site that is selective for Ca^{2+} over Gd^{3+}. Our knowledge of the phospholipid headgroup preference for gadolinium thus confirms that a calcium-selective site must exist on the peptide.

The aggregation of LY146032 in free solution by gadolinium ions does indicate a degree of direct association that is not entirely unexpected between two such species. The relevance of this result to the membrane effect is however not yet clear.

At this stage it is reasonable to propose a model for the observed fluorescence changes in which the peptide, initially bound to the membrane by its lipid tail, is drawn further into the membrane by calcium bound to its negative residues and calcium associated with the neighboring phospholipid interface. Such a peptide will provoke a localized negative potential that

loosely associated Ca^{2+} will neutralize. When in competition with Gd^{3+}, these will be replaced, and only the tightly bound calcium ion(s) will remain. This may explain the slight sigmoidal nature of the Ca^{2+} titration curve with the peptides high-affinity binding site effective in the 0–2 mM range and the phospholipid binding saturating at 30 mM. Unfortunately, the induced fluorescence enhancement does not allow a serious analysis of this region, and the calculated binding constants assume one type of site. The proposed role for the lipid phosphate groups may also explain why LY146032 is less effective in increasing the conductivity of bilayer lipid membrane (BLM) formed from nonphospholipids (Lakey & Lea, 1986).

In the presence of negatively charged lipids, localized negative potentials will not be effective in obtaining the calcium ions necessary for charge neutralization and the membrane penetration will be lessened as seen under Results. The effect of cationic lipids may therefore be explained as an enhancement of the local negative potential well around the peptide.

Further study is required on the role of the positively charged ornithine in these experiments. It may be neutralized by a close union with one of the negative residues, but if free, it could possibly play an important role in the more electrostatically neutral calcium/peptide complex.

Polymixin, a positively charged lipopeptide antibiotic, might be expected to show binding to the phosphates of zwitterionic phospholipids but instead is entirely dependent on negatively charged lipids for its membrane binding (Hartmann et al., 1977). Its lipid chain does enter the membrane (Hartmann et al., 1977), but the charge interaction is less specific than LY146032 in which a positive membrane surface charge cannot replace calcium.

Two other calcium-sensitive acidic peptide antibiotics are known, bacitracin (Stone & Strominger, 1971) and amphomycin (Matsui et al., 1963). Bacitracin, a cyclic peptide with one Asp residue, is in fact sensitive to a range of divalent cations (Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}; Stone & Strominger, 1971). It has been shown to inhibit the dephosphorylation of C55 isoprenyl pyrophosphate by complexing with it, thus interrupting cell wall synthesis. This complex, stabilized by the divalent cation, is also dependent on the pyrophosphate's two negative charges and a hydrophobic interaction with the rest of the lipid carrier (Storm & Strominger, 1973). Amphomycin is closer to LY146032 in structure, being an Asp-rich lipopeptide (Bodansky et al., 1974). Like bacitracin it complexes a lipid carrier, dolichol monophosphate, but only with Ca^{2+} ions (Banerjee et al., 1981; Lakey et al., 1988). In spite of the marked similarities, LY146032 does not appear to act upon these carrier lipids because it inhibits a much earlier stage of peptidoglycan synthesis, possibly even in the cytoplasm (Allen et al., 1987). If it is required to cross the cytoplasmic membrane, then a calcium-specific interaction with phospholipids may be involved. No firm evidence exists for such transport, but the involvement of an electrically silent complex could account for the inconclusive results from planar bilayers (Lakey & Lea, 1986). Further studies are under way to clarify this problem.

It remains to be seen whether the $K_a(Ca^{2+})$ value of 151 M^{-1} provides for a sufficiently large activity at physiological calcium concentrations (2.5–5 mM) to account for the in vivo effectiveness of its antibacterial action. If a cytoplasmic high-affinity site is the target, the kinetics could be more advantageous than they appear here, but on the other hand, the large amount of negative charge present on bacterial membranes will probably act as in the SUVs to reduce the penetration of LY146032 into the bilayer.

In conclusion, it appears possible that the observed calcium effect is related to the unique antibacterial action of LY146032. This result is important not only for its implications in antibacterial chemotherapy but also because a useful new model is now available for studies of lipid–protein interactions.

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Registry No. LY146032, 103060-53-3; Ca, 7440-70-2; Cd, 7440-43-9; Mg, 7439-95-4; Gd, 7440-54-2.

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Lymphoma–Vesicle Interactions: Vesicle Adsorption, Membrane Fragmentation, and Intermembrane Protein Transfer†

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Appendix: Plasma Membrane Composition of the Cultured Murine Lymphoma Line BL/VL3

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ABSTRACT: Sonicated dimyristoylphosphatidylcholine vesicles interact with cultured murine lymphoma (BL/VL3) to generate complexes of vesicle and cell membrane components. Cell-free supernatants harvested after cell–vesicle incubations contain three distinct lipid species that can be separated by density gradient centrifugation. Analysis of protein and lipid composition and assays for cell and vesicle lumen contents reveal that the densest of the three lipid species comprises sealed plasma membrane fragments complexed with vesicles, while the least dense species is indistinguishable from pure phospholipid vesicles. The third, intermediate density species consists of topologically intact vesicles with associated plasma membrane proteins but without detectable cell lipids or cytoplasmic components. The membrane fragmentation and cell-to-vesicle protein transfer observed during lymphoma–vesicle incubations are examined as functions of cell and vesicle concentrations and incubation time.

Liposomes interact with cells in a variety of ways. Lipid may transfer between the two membranes, liposomes may adsorb to the cell surface or be incorporated by endocytosis, or the two species may fuse, pooling cytosolic and encapsulated vesicle contents [for reviews, see Pagano and Weinstein (1978), Huang (1983), and Margolis (1984)]. In a fifth mode of interaction, protein may transfer between cell and liposome membranes (Newton et al., 1983; Huestis & Newton, 1986). The nature and extent of such interaction(s) are dependent on the cell type, the liposome composition, and experimental parameters such as cell and liposome concentrations and length of incubation. For example, the major mode of interaction between murine P388 cells and dioleoylphosphatidylcholine (DOPC)[1] vesicles is stable adsorption of intact vesicles to the cell surface (Blumenthal et al., 1982). In contrast, when human erythrocytes are incubated with vesicles composed of the more hydrophilic lipid dimyristoylphosphatidylcholine (DMPC), vesicle lipid transfer, vesiculation of the cell membrane (Ott et al., 1981; Ferrell et al., 1985), and spontaneous cell-to-vesicle protein transfer (Newton et al., 1983; Huestis & Newton, 1986) are observed.

Erythrocyte-to-vesicle protein transfer has been characterized by analysis of the resulting protein–vesicle complexes. The composition of these complexes and the function and proteolytic sensitivity of their bound proteins (in particular, the anion transporter band 3) showed that transferred protein inserts into the vesicle bilayer in native orientation; exofacial inhibitor sites are exposed to the suspending medium and the cytofacial protein segment contacts the vesicle lumen (Newton et al., 1983; Huestis & Newton, 1986; Newton & Huestis, 1986). Protein transfer in the reverse direction, from vesicles to cells, has been demonstrated at a low but detectable level. Erythrocyte band 3 transfers in native orientation from protein–vesicle complexes into the membranes of erythrocytes and human and murine lymphoma, conferring increased anion transport capacity on the cells (Newton et al., 1983; Newton & Huestis, 1988).

This paper examines the generality of intermembrane protein transfer in a study of interactions between DMPC vesicles and cultured murine lymphoma (Lieberman et al., 1979). Incubation of BL/VL3 lymphoma with sonicated DMPC vesicles results in the generation of two types of protein–lipid complexes that differ in density and lipid and protein composition. Separation and compositional analysis of these species

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[1] Abbreviations: BTEE, *N*-benzoyl-L-tyrosine ethyl ester; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; FDA, fluorescein diacetate; FITC, fluorescein isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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