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The effects of lipid composition on the binding of lasalocid A to small unilamellar vesicles

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The binding of the carboxylic ionophore lasalocid A (X537A) to small unilamellar phospholipid vesicles of varying composition was examined in an effort to determine what structural features of the phospholipid membrane influence the ionophore-membrane interaction. Apparent dissociation constants (K_{app}) were calculated for both the acidic and anionic forms of the ionophore using the change in fluorescence intensity observed for lasalocid A upon addition of phospholipid vesicles. The K_{app} for binding to fluid phase dimyristoylphosphatidylcholine (DMPC) vesicles is 46 μM for the anion and 14 μM for the acid. While the phase transition of DMPC had no effect on the K_{app} of the anion, an increase was observed in the K_{app} of the acid below the phase transition temperature. The K_{app} of the anion was not affected by the incorporation of 10% dimyristoylphosphatidylethanolamine (DMPE), but increased slightly upon incorporation of cholesterol. The pK_a values of the ionophore were the same in DMPC and DMPC/DMPE membranes. Incorporation of the negative lipids phosphatidylglycerol, phosphatidic acid, or phosphatidylethanolamine (at pH 9.4 where PE carries a negative charge) decreases binding of the anion in accord with the increase in surface potential estimated from Gouy-Chapman theory. The CD spectrum of membrane-bound lasalocid A anion indicated the ionophore to be in an extended acyclic conformation on the membrane surface with the C-1 carboxylate rotated out of the plane of the salicylate ring. The out-of-plane rotation of the carboxylate may be the result of facial binding by the amphiphilic ionophore on the membrane surface or of weak ion pairing to the polar lipid head groups. These results suggest that the primary determinants of binding of the anionic ionophore on the membrane surface are packing density of the polar head groups and membrane surface potential. There is no evidence of strong hydrogen bond formation between the lipid polar head groups and the ionophore as has previously been suggested.

Introduction

The carboxylic ionophores constitute a large class of membrane active natural products that transport cations across membranes by an electroneutral, exchange-diffusion mechanism [1]. Membrane-bound ionophores form uncharged lipid-soluble complexes with cations at the membrane surface by a sequential ligand interchange mechanism [2]. Transverse diffusion of the ionophore-metal ion complexes across the bilayer and subsequent decomplexation at the opposite interface result in net ion transport with high turnover and moderate cation selectivity [3]. The rate of ion translocation appears to be limited by transverse diffusion, as the complexation-decomplexation reactions at the membrane surface are extremely rapid [4].

Interactions between the phospholipid bilayer of a membrane and the carboxylic ionophore may modify both ion selectivity and transport rates [2,3,5]. The ion complexation affinities and transport activities of A23187 and lasalocid A, for example, are sensitive to the phospholipid composition of vesicles [4,6]. It has been suggested that this sensitivity of ion binding and

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Abbreviations: CD, circular dichroism; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DMG, dimethylglutaric acid; DMPA, dimyristoylphosphatidic acid; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DSC, differential scanning calorimetry; DPH, diphenylhexatriene; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EYPC, egg yolk phosphatidylcholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HLA, free acid of lasalocid A; LA^-, anion of lasalocid A; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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transport to membrane composition may reflect polarity-dependent changes of ionophore conformation [7–10]. In addition, specific interactions between ionophores and functional groups in the membrane may perturb cation binding, either by competitive inhibition or by the formation of ternary complexes that anchor the ionophore at the membrane surface [3]. Evidence for ion pairing interactions between lasalocid A and DMPC in solution has been reported from circular dichroism (CD) studies [3]. Infrared studies of grisorixin in phospholipid vesicles also suggest that there may be hydrogen bonding interactions involving the terminal carboxylate of the ionophore and the phosphate group of the phospholipid [11].

One of the major obstacles to the development of carboxylic ionophores as potential pharmacologic agents is the broad spectrum of activity they exhibit in biological systems. The activity of the ionophores must be limited to a specific site of action in order to eliminate undesirable side effects. One proposed strategy for achieving this goal is to identify the physiochemical basis of the effects of membrane composition on differential membrane binding, ion selectivity and transport efficiency, and to chemically modify an ionophore such that it binds to and functions most efficiently in a biomembrane of a specific composition [3].

As the first step in identifying structural and organizational properties that might influence differential association of carboxylic ionophores with biomembranes, we investigated the binding of lasalocid A (Fig. 1) to small unilamellar vesicles (SUVs) of defined lipid composition. The polarity-dependent fluorescence signal of the salicylate moiety was used to calculate apparent dissociation constants for the membrane-ionophore complex [12]. By varying the lipid composition of the SUVs, it was possible to determine the role of fluidity, surface charge, and specific head group composition in determining the extent of ionophore-membrane interactions. We also examined the effect of membrane composition on the conformation of the anionic ionophore using circular dichroism.

Methods

Lasalocid A was obtained from Sigma (St. Louis, MO) as a mixture of the sodium and potassium salts. To produce the free acid, 5 g of the mixed salts was dissolved in 200 ml of methylene chloride and washed three times with 200 ml aliquots of 10% aqueous HCl. The organic layer was then washed twice with 200 ml of thrice-distilled, deionized water and flash evaporated. The crude acid was recrystallized from ethanol and dried in vacuo, m.p. 109–110 °C (lit. 100–109 °C [13]). Flame emission photometry indicated that the sample contained less than 0.1% of the sodium or potassium salt. DMPC, DMPE, disodium DMPA and sodium DMPG were obtained from Avanti Polar Lipids (Birmingham, AL), DL-DMPC (*rac*-1,2-DMPC) was purchased from Fluka (Ronkonkoma, NY), cholesterol was obtained from Sigma. Lipids were used without further purification. The sodium salts of DMPA and DMPG were converted to their fully protonated forms by three extractions in chloroform/methanol (2 : 1, v/v) against 1 M citric acid followed by three washes in deionized water. Methanol was added as necessary to break any emulsions.

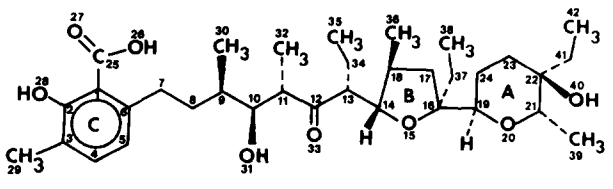


Fig. 1. Structure of lasalocid A.

The standard buffer consisted of 5 mM Ches (Aldrich, Milwaukee, WI), 5 mM Hepes (Aldrich), 0.1 mM EDTA, 0.1 mM EGTA, and 33 mM tetraethylammonium hydroxide (Alfa, Danvers, MA). For experiments at pH 4.5, the buffer also contained 5 mM citric acid or 5 mM dimethylglutaric acid (DMG). The pH was adjusted by the addition of $HClO_4$. Care was taken to exclude cations capable of forming inclusion complexes with lasalocid A from the buffer. All glassware was washed with deionized water, and 'Milli-Q' deionized water (Millipore, Bedford, MA) was used to prepare all solutions. Sodium concentration was determined in the buffer to be less than 1 μM by flame emission photometry. All solutions were cleared of particulates prior to use by filtration through 0.45 μM sterile fitlers (Millipore HA).

Small unilamellar vesicles were prepared essentially as described by Huang and Thompson [14]. Aliquots of the appropriate lipids in chloroform (20 mg/ml) were combined and flash evaporated followed by drying in vacuo for two hours. The dried lipids were suspended at 50 mg/ml in the standard buffer (vide supra) and mixed at 40–50 °C for 15 min. The suspension was sonicated with a Heat Systems W-375 sonicator using a microtip probe for 10–15 min at a 40% duty cycle until the solution appeared clear. The vesicles were then centrifuged at 80 000 × g at 30 °C for 60 min to remove titanium particles derived from the probe and residual large, multilamellar vesicles. The preparations were stored at 30 °C and used within two days of preparation. Vesicles containing cholesterol were prepared under argon. Examination of the preparations for light scattering between 300 and 500 nm showed the linear λ^{-4} dependence expected for small unilamellar vesicles [15]. Light scattering was measured periodically and found not to change significantly over two days, indicating that the vesicles did not coalesce in that time.

Phospholipid concentration in the vesicle preparations was determined by the method of Ames et al. [16]. Phase transition temperatures were determined by differential scanning calorimetry or DPH anisotropy [17]. The relative composition of mixed phospholipid vesicles was confirmed by ^{31}P-NMR [18]. Vesicles were diluted in a 10% deoxychoalte solution and ^{31}P-NMR spectra were acquired at 121.3 MHz with gated decoupling at 50 °C on a Varian XL-300 or VXR-300S spectrometer. The lipid composition of the mixed vesicles was found to be ±10% of the nominal value.

Fluorescence measurements were made on a Perkin-Elmer 650-10 spectrofluorimeter or a Perkin-Elmer MPF-66 spectrofluorimeter operating in the ratio mode. Fluorescence titrations were performed with excitation at 312 nm and emission at 420 nm. Lasalocid A was added from a 0.1 mM stock solution in ethanol to 2.0 ml of buffer. The final concentration of ethanol was kept below 0.5% and the total volume change at the end of a titration with SUVs was less than 2%. Equilibrium fluorescence intensity values were measured 2–3 min after addition of an aliquot of vesicles when the fluorescence signal reached a constant value. We assume that this is a sufficient period, as indicated by the changes in fluorescence intensity, to account for redistribution of the protonated ionophore in all the intramembrane compartments. Control titrations were performed by adding the vesicles to a solution without lasalocid A.

The pK_a of bound lasalocid A was determined by fluorescence quenching due to protonation. Vesicles were prepared as above in Hepes/Ches/citrate buffer at pH 7.0. Aliquots of vesicles were diluted to 1 mM phospholipid and the pH adjusted with additions of tetraethylammonium hydroxide or $HClO_4$. The vesicles were then sonicated for 10 min to allow for equilibration of transmembrane pH gradients. The buffering capacity of the vesicle lumen was assumed to be adequate to compensate for deprotonation of the protonated ionophore in the vesicle interior. Small unilamellar vesicles are known to encapsulate small molecules such as the buffers used in this study with high efficiency [19]. The solution pH was recorded and fluorescence was measured in the presence and absence of 0.25 μM ionophore at 31 °C.

CD spectra were recorded with a JASCO J-600 spectropolarimeter (Japan Spectroscopic Co., Tokyo) in a 2-cm path length, thermostated cuvette (T = 31 °C). Vesicles were prepared from racemic DL-DMPC to reduce the CD background from the lipid. In order to minimize scattering effets, the cuvette was placed adjacent to the photomultiplier window [20].

Data analysis: determination of apparent dissociation constants. The extent to which lasalocid A binds to the phospholipid matrix of a membrane bilayer was determined by titration of SUVs against a fixed concentration of ionophore. The binding can be described by Eqn. 1.

$$K_{app} = [LA][PL]/[LA \cdot PL] \quad (1)$$

K_{app} is the apparent dissociation constant, [LA] is the concentration of free lasolocid A, [LA · PL] is the concentration of bound lasalocid A, and [PL] is the concentration of unbound phospholipid. K_{app} can only be considered an apparent dissociation constant in that it does not take into account the stoichiometry of the phospholipid/ionophore interaction.

The proportion of membrane-bound ionophore as described by Eqn. 1 is related to measured fluorescence intensity by Eqn. 2,

$$\Delta F/\Delta F_{max} = [LA \cdot PL]/[LA]_{tot} \quad (2)$$

where ΔF is the difference between the observed fluorescence and the fluorescence in the absence of phospholipid, ΔF_{max} is the fluorescence intensity at infinite phospholipid concentration, and $[LA]_{tot}$ is the total lasalocid A concentration. Measured ΔF values were corrected for the effects of light scattering [21].

If the total phospholipid concentration, $[PL]_{tot}$, is in large molar excess relative to $[LA]_{tot}$, it can be assumed that [PL] is approximately equal to $[PL]_{tot}$. Eqns. 1 and 2 can then be combined to give Eqn. 3,

$$\Delta F/\Delta F_{max} = [PL]_{tot}/\{K_{app} + [PL]_{tot}\} \quad (3)$$

K_{app} values were determined from a nonlinear, least-squares regression analysis of titration data using Eqn. 3. The error in the fit of each titration was generally less than ±5%. Eqn. 3 is only valid below lasalocid A concentrations of 0.5 μM where K_{app} was found to be independent of $[LA]_{tot}$.

Results and Discussion

The fluorescence spectra of the anionic and protonated forms of lasalocid A in aqueous solution are

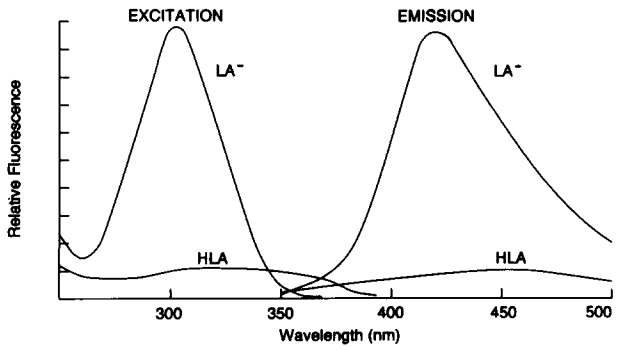


Fig. 2. The corrected excitation (em = 425 nm) and emission (ex = 303 nm) spectra of fully protonated (HLA) and fully deprotonated (LA^-) lasalocid A (5 μM) recorded in Hepes/Ches/citrate buffer at pH 2.0 and 9.4, respectively, in the absence of complexable cations at 23 °C.

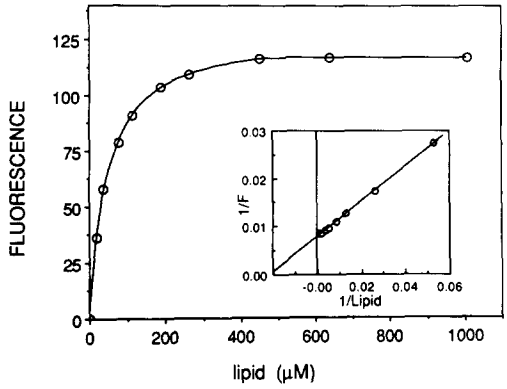


Fig. 3. Lasalocid A (0.25 μM) fluorescence intensity as a function of the concentration of DMPC SUVs. Excitation: 308 nm, emission: 420 nm. Fluorescence intensity, in arbitrary units, has been corrected for light scattering. Inset shows the double-reciprocal plot of data.

shown in Fig. 2. The fluorescence quantum yield of the salicylate chromophore increases with decreasing solvent polarity [12]. An analogous increase is observed in the presence of small unilamellar vesicles as the ionophore associates with the less polar membrane microenvironment. The quantum yield of the membrane-bound lasalocid A anion is similar to that measured for the anion in methanol solution, suggesting that the ionophore is binding in the polar head group region of the bilayer [12].

Binding of lasalocid A to DMPC membranes

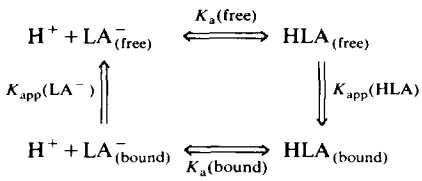
Fig. 3 shows the change in fluorescence intensity upon titration of lasalocid A with DMPC vesicles at pH 9.4. The solid lines show the fit to a binding isotherm with appropriate corrections for scattering effects. The calculated K_{app} at pH 7.0 and 31°C is 44.6 μM (Table I), significantly lower than the value of 100 μM reported for the binding of lasalocid A to DMPC monolayers at 30°C and pH 7.3 [15]. However, the monolayer system is significantly different from the SUVs used in this study, and the differences in K_{app} may arise from differences in membrane size and composition.

TABLE I

Lasalocid A-lipid vesicle apparent dissociation constants

The value of K_{app} with DMPC vesicles, pH 9.4, 31°C was calculated as the mean of all replicates ($n = 21$) weighted by the standard error of each replicate. The value was used as a standard for normalization. For each preparation of vesicles, a normalization factor was calculated as the ratio of the standard K_{app} to the K_{app} at pH 9.4, 31°C for titrations with a given preparation of vesicles. The mean values of K_{app} reported in Table I are the weighted means of the normalized values for each titration. The standard error shown for each mean was calculated to reflect both the error in the fit of Eqn. 4 for each titration and the spread in the estimates between replicate titrations.

Lipid composition	K_{app} (μM ± S.E.)		
	pH 4.5	pH 7.0	pH 9.4
DMPC	19.0 ± 0.3	42.4 ± 1.5	44.6 ± 1.4
10% Cholesterol	27.5 ± 1.6	–	55.0 ± 2.5
20% Cholesterol	176.7 ± 9.7	–	139.8
10% DMPE	19.1 ± 0.6	42.2 ± 4.0	79.2 ± 2.1
30% DMPE	–	91.4 ± 6.7	–
10% DMPG	101.8 ± 6.7	–	131.0 ± 12
10% DMPA	36.3 ± 1.8	–	148.1 ± 4.2



Scheme I. Binding and protonation equilibria for lasalocid A.

The quenching of lasalocid A fluorescence by ground state protonation [12] precludes direct measurement of binding of the fully protonated acid using fluorescence intensity. However, the binding of the protonated acid can be related to the binding of the anion as shown in Scheme I, and an apparent dissociation constant can be calculated using Eqn. 4,

$$K_{app}(HLA) = K_{app}(LA^-)(K_a(bound)/K_a(free)) \quad (4)$$

where K_a(bound) is the proton dissociation constant of the membrane-bound ionophore and K_a(free) is the dissociation constant of the ionophore in buffer.

The pK_a values of free and bound ionophore were determined by monitoring the fluorescence intensity as a function of pH in the absence and presence of a saturating concentration of SUVs, respectively (Fig. 4). The apparent pK_a of the DMPC-bound ionophore is 4.86, significantly higher than the pK_a of 3.36 measured in aqueous solution. The difference in pK_a presumably reflects the stabilization of the protonated ionophore in the less polar membrane environment.

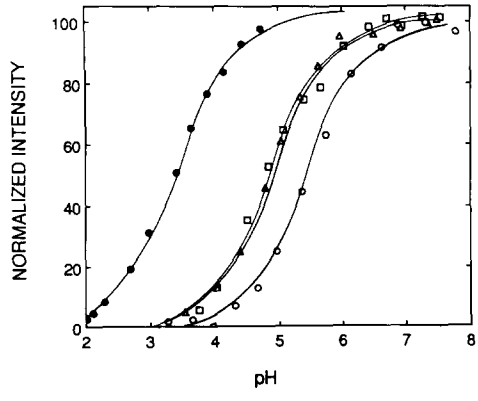


Fig. 4. pH titration of free and membrane bound lasalocid A. Solid lines shown are best fits to the data using a three parameter non-linear regression analysis. 5 μM lasalocid A in Hepes/Ches/citrate buffer (●). The pH was adjusted with HCl. 0.25 μM lasalocid A, 1 mM DMPC (□), 1 mM DMPG + DMPC (10% DMPG) (○), or 1 mM DMPE + DMPC (10% DMPE) (△) in Hepes/Ches/citrate buffer. Vesicles were sonicated at the indicated pH prior to addition of ionophore. Fluorescence was corrected for scattering by vesicles in the absence of ionophore. Excitation = 308 nm, emission = 420 nm.

Using proton dissociation constants of $1.38 \cdot 10^{-5}$ M for K_a(bound) and $4.37 \cdot 10^{-4}$ M for K_a(free), the calculated K_{app} for the free acid is 1.4 μM. This is a 30-fold increase in binding affinity over the anion. The measured K_{app} at pH 4.5 where the bound ionophore is approx. 70% protonated is an average of the protonated and unprotonated values weighted by the extent of protonation (Table I).

The increase in the apparent binding affinity of the protonated species may (i) reflect an increase in the intrinsic affinity of the free acid for the same binding sites as the anion due to the increased hydrophobicity of the neutral species, or (ii) be due to an increase in the number of binding sites available to the acid. The protonated ionophore can assume a cyclic conformation in the apolar membrane interior and consequently distribute across the membrane thereby making binding sites on the *endo*-face of the bilayer available as reported for A23187 [22].

It is not possible to determine the number of ionophore binding sites with confidence unless titration data is collected under conditions where the ionophore binding sites are near saturation. Ionophore concentrations were held well below that required to saturate binding sites as significant disruption of the membrane organization is known to occur at ionophore : lipid ratios as low as 1 : 50 [23,24]. Titrations at low ionophore : lipid ratios also minimize the effect of ionophore oligomerization [25], and the effect of the charged ionophore on surface potential [26]. Since the intrinsic binding affinity and the number of binding sites could not be accurately determined from the data in this study, the precise mechanism accounting for increased binding of the acid could not be determined.

The effect of temperature on the K_{app} for binding to DMPC vesicles was examined over a temperature range of 12–34° C. The van't Hoff plots for lasalocid A at pH 9.4 and 4.5 are shown in Fig. 5. While the plot for the anion is continuous through the phase transition with a small positive slope, the plot for the predominately protonated ionophore (pH 4.5) shows a sharp discontinuity at the phase transition. The binding enthalpies (ΔH) of the acid and the anion above and below the phase transition temperature calculated from the slopes of the van't Hoff plots are shown in Table II along with the values of ΔG and ΔS calculated at 23° C. Above the phase transition, the binding of both forms is moderately exothermic. However, the enthalpy of binding is small compared to $T\Delta S$. Thus, the binding of both species above the phase transition appears to be entropically driven, suggesting that binding is due primarily to hydrophobic interactions.

The binding of the protonated free acid is significantly affected by the lipid phase transition, indicated by the sharp discontinuity in the van't Hoff plot and a nearly 2-fold decrease in K_{app} going from the fluid to the gel phase. This is consistent with a distribution of the neutral species in the bilayer interior. Increased packing density of the acyl chain region in the gel phase may effectively exclude the protonated ionophore from this region and limit its distribution to the exterior face of the vesicle. Kauffman et al. [22] observed a similar discontinuity at the phase transition in the van't Hoff plot for the protonated form of A23187. In contrast to binding to the fluid phase vesicles, the binding constant of the protonated species below the phase transition temperature is dominated by a large negative ΔH.

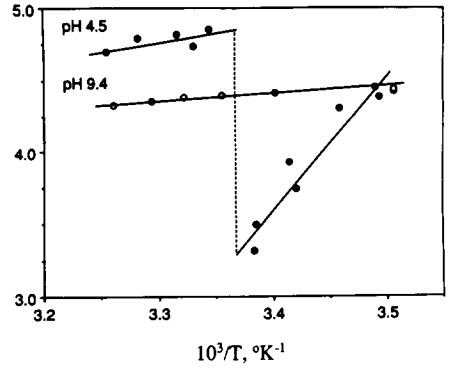


Fig. 5. Van 't Hoff plots of lasalocid A binding to DMPC vesicles. The logarithms of the binding constants K_b ($1/K_{app}$) are plotted vs. the reciprocal of the absolute temperature for pH 9.4 (○) and pH 4.5 (●). The curves drawn are calculated from the linear regression of the data. The break in the curve for pH 4.5 is drawn at 23° C, the main phase transition temperature for DMPC.

The relative insensitivity of the ΔG of anion binding to the lipid phase transition indicates a mechanism of binding which is different from the free acid. The insensitivity to the changes in lipid order at the phase transition is consistent with localization of the anion in the polar head group region of the bilayer, where we would expect comparatively small changes in the lipid packing order than in the acyl chain region of the membrane. Alternatively, the binding of the anion may be due to electrostatic adsorption to the surface dipole of the DMPC bilayer [27]. This interaction would be relatively insensitive to the phase transition, compared to the effect of acyl chain packing on hydrophobic

TABLE II

Thermodynamic parameters for lasalocid A binding to DMPC vesicles

The values of ΔG and ΔS are calculated from the lines fit to Fig. 5 extrapolated to 23° C. The values for HLA (fluid) are extrapolated from the data above 23° C and those for HLA (gel) from the data below 23° C.

	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol per deg)
LA^-	−5.96	−0.38	18.9
HLA (fluid)	−6.59	−0.98	19.0
HLA (gel)	−5.62	−7.51	−6.36

interactions as in the case of the free acid. Localization of the bound anion to the membrane surface is indicated in either case, consistent with similar results for A23187 [22].

The effect of membrane lipid composition on K_{app}

The effect of changes in lipid composition on lasalocid A binding to SUVs was investigated by incorporation of DMPE, DMPG, DMPA, or cholesterol into DMPC vesicles. K_{app} values are shown in Table I. The concentration of the included lipid in the mixed vesicles was kept low (10%) to favor homogeneous bilayer phases. In all cases, binding to the mixed phospholipid vesicles was measured above the phase transition temperature of the vesicles, as determined by differential scanning calorimetry.

The presence of 10% cholesterol in DMPC vesicles resulted in a 19% decrease in binding of the lasalocid A anion. At pH 4.5, the binding decreased 31% relative to binding to DMPC. Incorporation of cholesterol into PC vesicles tends to increase both the lipid packing density and the diameter of the vesicles [28]. The increased lipid packing density due to cholesterol may cause a decrease in solute partitioning into multilamellar vesicles, as in the case of benzene [29]. The observed decrease in binding with incorporation of cholesterol suggests that increased packing density also reduces ionophore binding. In contrast, specific binding interactions with cholesterol can increase solute binding to lipid membranes, as in the case of the polyene antibiotics [30]. The larger effect of cholesterol on binding of the free acid as compared to the anion may be due to the larger increase in packing density in the acyl chain region as compared to the polar head group region [31].

Incorporation of 10% PE into DMPC vesicles had little effect on binding below pH 9. In addition, the pK_a of the ionophore bound to 10% PE vesicles was not significantly different than that observed in pure PC vesicles (Fig. 4). Painter and Pressman [3] suggested that ion pairing with the primary amine of PE could result in a stronger interaction of lasalocid A at the surface of PE-containing membranes. Formation of a hydrogen-bonded ion pair would be expected to increase the binding and the apparent pK_a of the membrane bound ionophore. However, since there is no effect on either binding of pK_a upon incorporation of zwitterionic PE, it appears unlikely that lasalocid A forms an ion-paired complex with the PE primary amine. Hydrogen-bonded ion pair interactions of PE head groups with the phosphates of adjacent phospholipids may make the PE amine unavailable for complex formation with the ionophore [32].

Increasing the PE content of DMPC SUVs to 30% results in a 50% reduction in binding affinity at pH 7.4 (Table I). Haynes et al. [6] have previously observed that under similar conditions lasalocid A fluorescence decreases by 50% relative to 100% PC. This was attributed to reduced binding to the PE incorporated vesicles. The large effect of 30% PE as compared to 10% PE may be explained by the observation that PE incorporation into PC bilayers has an effect on membrane structure that is nonlinear with respect to %PE. The phase transition temperature of mixed PE/PC vesicles, for example, is nearly constant at 23°C from 0 to 10% PE but increases to 29–30°C at 30% PE, reflecting an increase in lipid packing density. Thus, the decreased binding of lasalocid A to 30% PE vesicles may be due to an increase in surface packing density.

The effect of negative lipids on the binding of lasalocid A

The development of a negative surface charge on the vesicle would be expected to result in a decrease in the effective concentration of the lasalocid A anion near the membrane surface. Indeed, binding of lasalocid A anion was significantly decreased with incorporation of DMPE, DMPG, or DMPA (Table I), lipids which confer a negative surface potential on the membrane at pH 9.4. The pK_a for the primary amino group of PE in PE/PC vesicles is approximately 10 [32].

The effect of the negative surface potential (ψ_0) on binding of the lasalocid A anion can be estimated by assuming that the change in K_{app} is proportional to the decrease in the effective concentration of lasalocid A at the membrane surface ($[LA]_0$) due to the repulsive interaction of the charged surface and the lasalocid anion. The relative concentrations may be determined by the Boltzmann distribution,

$$[LA]_0/[LA] = \exp(e\psi_0/kT) \quad (6)$$

The surface potential can be estimated from Gouy-Chapman theory as

$$\psi_0 = (2kT/ze)\sinh^{-1}(A\sigma/\sqrt{C}) \quad (7)$$

where k is the Boltzmann constant, T is the temperature, z is the charge on the solution electrolyte, e is the electronic charge, C is the concentration of electrolyte in the bulk phase, and σ is the surface charge density (unit charges per Å^2) [33]. The constant A, which is related to the dielectric constant of the medium, has a value of 137.0 $M^{1/2} \cdot Å^2$ [33].

At pH 9.4 the charge per molecule is approx. −0.25 for PE, −1.0 for PG, and −1.5 for PA [32]. The average molecular area of the fluid phase vesicles is assumed to be 70 Å^2 [34]. PE, PG, and PA concentrations are 10 mole percent in the vesicle, but an unsymmetrical distribution of PA is assumed with approx. 7–8% incorporation in the *exo*-face [32]. Using Eqns. 6 and 7, the calculated decrease in the concentration of lasalocid A anion at the membrane surface (relative to the neutral PC membrane) is 43% for PE, 33% for PG,

and 20% for PA. The observed decreases in binding affinity relative to PC were 56% for PE ($K_{app} = 79.2 \pm 2$ μM), 34% for PG ($K_{app} = 131 \pm 12$ μM), and 30% for PA ($K_{app} = 148 \pm 4$ μM).

Incorporation of 10% PG also resulted in an increase in the pK_a of lasalocid A from the value of 4.86 observed in DMPC vesicles to 5.49 (Fig. 4). The effect of surface charge on the pK_a of the bound ionophore can be estimated by calculating the decrease in the effective pH at the membrane surface. The calculated change in the pK_a of lasalocid bound to 10% PG vesicles is approximately 0.5 pH units, very close to the observed change of 0.63 pH units.

Estimates of the effects of surface potential on the distribution of mobile ions based on Gouy-Chapman theory are subject to a number of errors, particularly in the calculation of the surface charge density due to uncertainty in surface area per lipid, the symmetry of distribution of the negative lipid, and the charge per head group. Additionally, adsorption of tetraethylammonium, ClO_4^-, or other ions may alter the effective surface charge of the negative vesicles [35,36]. Thus, the general agreement between the measured effects of membrane surface charge and predictions of Gouy-Chapman theory suggests that electrostatic interactions alone can account for the effects of negative lipids at the low levels of incorporation examined in this study.

Conformation of the membrane bound ionophore

Circular dichroism studies of lasalocid A in solution have suggested that specific interactions could occur with the polar head groups of lipids in biological membranes [3]. In nonpolar solvents the ionophore forms hydrogen-bonded ion-pairs with tri-*n*-alkyl ammonium ions and electrostatic ion pairs with tetra-*n*-alkyl ammonium ions [9]. Electrostatic ion pairing with the choline moiety of DMPC in 80% aqueous dioxane has been inferred from the large increase in intensity of π-π^* transitions of the salicylate chromophore at 240 and 310 nm [3]. The increase in intensity of the π-π^* bands is due to rotation of the carboxylate out of the plane of the aromatic ring in order to accommodate the bulky tetraalkylammonium group of the lipid. Ion pairing has no effect on the n-π^* transition of the C-12 ketonic carbonyl.

The effect of binding to PC and 30% PE/PC membranes on the CD of lasalocid A is shown in Fig. 6. In aqueous buffer the spectrum is dominated by the 288 nm n-π^* transition of the C-12 ketone [9]. The absence of π-π^* bands indicates there is no ion pairing with the tetraethylammonium cations present in the solution. Addition of DMPC or mixed PE/PC vesicles results in the development of a weak negative peak at 239 nm and a small red shift of the major peak to 290.5 nm. The decrease in intensity of the 290.5 nm band suggests a minor reorganization around the C-12 carbonyl consistent with a slight shift in the conformational equilibrium toward the acyclic form favored on the membrane surface [9]. A similar change has been observed in the CD spectrum of narasin A upon binding to DMPC membranes [10]. In the PC spectrum there is an additional small positive peak at 254 nm. Positive going peaks are indicative of lasalocid A dimers [37], suggesting that there may be some dimer formed at the PC membrane surface. The dimer may represent the second minor population of membrane-bound ionophore observed by Haynes et al. in fluorescence lifetime studies [6]. The CD spectra are essentially unchanged above and below the lipid phase transition, consistent with the absence of an effect of phase transition of K_{app}.

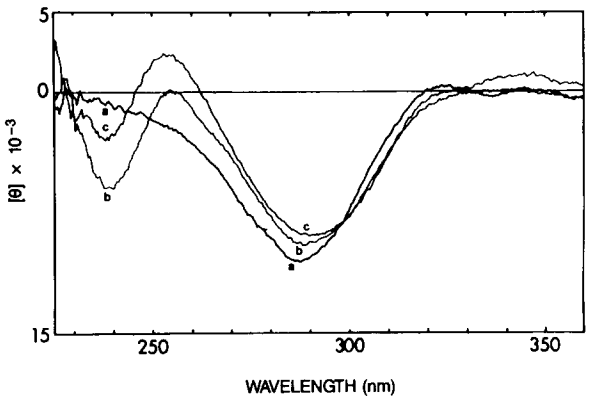


Fig. 6. CD spectra of lasalocid A (10 μM) at pH 7.0 and 31° C in: (a) Hepes/Ches/DMG buffer; (b) plus 50 μM DMPC vesicles; (c) plus 50 μM DMPC vesicles containing 30% DMPE.

The intensity of the π-π^* band at 239 nm in the presence of DMPC or DMPC + DMPE vesciles is significantly less than that seen in 80% dioxane in the presence of DMPC [3]. Thus, while the changes in CD spectra indicate some conformational changes associated with binding to membranes, we see no evidence for a strong ion pair interaction with either choline or ethanolamine head groups. This conclusion is in accord with the observations that changes in pK_a can be accounted for by polarity and also that the enthalpy of binding of the lasalocid A anion is small.

Conclusion

The binding of the carboxylic ionophore lasalocid A is affected by general features of membrane composition and organization. The anionic ionophore binds at the membrane surface where it is sensitive to lipid packing density and the charge on the membrane surface; it is relatively insensitive to the organization in the acyl chain region. In contrast, the protonated free acid distributes across the bilayer and is consequently more sensitive to changes in the organization of the bilayer interior. Binding of the anion and the proto-

nated ionophore to fluid phase membranes is dominated by hydrophobic interactions.

There is a small change in the conformation of the ionophore upon binding to the membrane surface. The conformation of the bound anion is unaffected by the lipid phase transition and little affected by substitution of PE for PC. The C-1 carboxylate of the salicylate moiety is rotated out-of-plane, possibly due to a facial preference for binding or to weak ion-pair interactions with the choline head groups.

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