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The effects of A23187 on the phospholipid phase transition of large unilamellar vesicles (LUVs) as detected by ultrasound spectroscopy

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The effect of the hydrophobic Ca^{2+} ionophore, A23187, on the phospholipid dynamics of large unilamellar vesicle (LUVs: 4:1 (w/w) mixture of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG)) membranes, as a function of A23187 content, was investigated using techniques sensitive to the phospholipid phase transition. The ultrasonic absorption per wavelength, $\alpha\lambda$, was determined with a double crystal acoustic interferometer, as a function of temperature and frequency for LUVs in the vicinity of their phospholipid phase transition. Differential scanning calorimetry (DSC) and electron spin resonance (ESR) were also employed to probe the thermodynamics and molecular environment of the hydrocarbon side chains. With increasing A23187 content, the phase transition temperature (T_m) of the LUV suspensions remained near 42.0 °C, while the amplitude of $\alpha\lambda$ at the phase transition increased dramatically. At T_m the relaxation frequency, where $\alpha\lambda_{max}$ occurs, decreased with A23187 content, suggesting that the relaxation rate of the event responsible for the absorption of ultrasound decreased. The ESR studies showed no change in the fluidity of the bilayer with the inclusion of 2 and 5 mol% A23187 in the C-12 region of the bilayer. Therefore, A23187 in LUV membranes slows the structural relaxation of the hydrocarbon side chains of the phospholipid bilayer at the phase transition.

Introduction

Ultrasound provides a dynamic probe of biological membranes that complements fluorescence, NMR, and temperature jump methods that are sensitive to membrane fluidity and dynamics [1]. The ultrasonic absorption may be studied, as a function of membrane composition and environment, to investigate the nature of molecular processes in biological membranes. Specifically, at a given temperature and pressure, ultrasonic absorption is dependent upon the molecular system to which it couples. Ultrasonic methods make it possible to determine the ultrasonic absorption per wavelength, $\alpha\lambda$ (the exponential reduction in the sound pressure amplitude in traveling one wavelength), in biological suspensions [1]. For a given system at equilibrium, $\alpha\lambda$ will have a maximum amplitude at the relaxation frequency of the chemical reaction, conformational change or structural change to which it couples, thus providing information on molecular states in the membrane. The frequency range of ultrasound used in these experiments was 0.58–5.2 MHz, which provides information on relaxation events on the timescale of 0.03–0.27 μs.

Ultrasonic absorption is also investigated in biological membranes in order to help determine mechanisms of ultrasonic energy loss in biological media. Such information is especially important in the investigation of the safety of diagnostic and therapeutic ultrasound. In this work, the ultrasound interaction with phospholipid bilayers is studied as a model of the interaction of ultrasound and biological membranes, as phospholipids make up a significant fraction of biological membranes. The further incorporation of different molecules, e.g., proteins, into phospholipid bilayers then allows the investigation of a system more closely approximating

Abbreviation: 12-NS PC, 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine.

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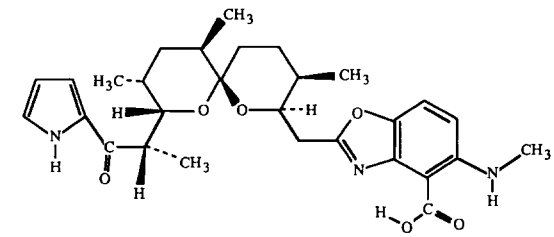
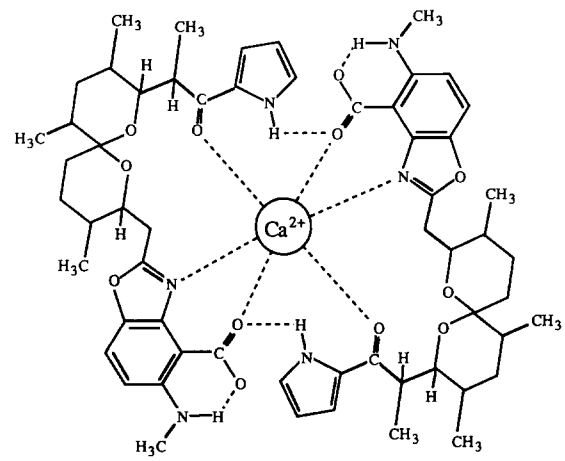
true biological membranes. In order to determine the site or sites of the interaction of ultrasound with biological membranes, agents with specific partitioning properties can be incorporated into the membrane bilayer structure. These agents should affect membrane dynamics at their site of interaction with the membrane, and these perturbations, if sufficient in amplitude, can be detected changes as in $\alpha\lambda$, as a function of temperature and/or frequency. Changes in the ultrasonic absorption should occur if the specific ultrasonic interaction is at the site of membrane perturbation and the event perturbed takes place on a timescale accessible to the specific acoustic interferometer. Observations of a change in $\alpha\lambda$ as a function of temperature and, especially, frequency then provide data from which is determined quantitatively the interaction of the ultrasound with biological membranes.

Previous studies

Liposomes, which can be formed from a variety of lipids, provide a useful model of the cell membrane. The mechanical and thermodynamic properties of liposomes have been investigated using ultrasound [2–8]. Ultrasonic absorption of large unilamellar vesicles (LUVs) has been used to study the ultrasound interaction with biological membranes, and to determine the effect of perturbations to such membranes. LUVs, formed by the reverse phase evaporation method [9], assumed to be spherical with diameters in the range of 0.2 to 0.8 μm, can be produced from natural or synthetic phospholipids [2]. Previous ultrasound studies of liposomes showed that in MLV (multilamellar vesicle) and LUV suspensions, $\alpha\lambda$ exhibited a large increase near the phase transition temperature (T_m) of the phospholipids in the vesicle membrane [8,10]. In LUV suspensions this peak is correlated with structural changes in the membrane which lead to dramatic increases in the permeability of LUV membranes in the vicinity of T_m. Furthermore, at the phase transition temperature of the LUVs (DPPC/DPPG (4:1, w/w)), a maximum in $\alpha\lambda$ at 2.1 MHz occurs, probably identifying a relaxation frequency of the bilayer, where ultrasound presumably couples to conformational changes of individual phospholipids or groups of phospholipids in the vesicle membrane [2]. In studies of LUV suspensions in which specific regions of the LUV membrane are perturbed, alteration of the headgroup region of the phospholipid with Ca^{2+} or of the water structure with ^2H_2O did not change the relaxation frequency at which the maximum in $\alpha\lambda$ occurred although other characteristics of the phase transition were altered, suggesting that ultrasound interaction is not at the surface of the bilayer [11,12]. However, in experiments with the Na^+ ionophore, gramicidin, in LUV membranes, where the gramicidin dimer traverses the bilayer, the relaxation frequency was changed to 0.75 MHz, indicating an interaction of ultrasound with



Free Acid



A23187 Complex with Ca^{2+}

Fig. 1. Chemical structure of A23187 in its free acid form and its complexed form.

the hydrophobic region of the lipid bilayer [2]. It therefore was hypothesized that A23187, as a transport molecule partitioning in the hydrophobic portion of the lipid bilayer, should affect the kinetics of the event responsible for the relaxational absorption of ultrasound.

A23187

A23187 is a small molecular mass carboxylic acid ionophore of 523.6 daltons that is known to partition into the hydrophobic region of the phospholipid bilayer [13]. It is soluble in organic solvents such as chloroform, DMSO, and ethanol, and insoluble in water. The A23187 is a mobile carrier ionophore specific for divalent cations over monovalent cations with its order of affinity for $Mn^{2+} > Ca^{2+} > Mg^{2+}$. It forms a complex of two molecules in order to transport each divalent cation across the membrane. The exchange of ions is electrically neutral although the mechanism by which charge neutrality is maintained is not known. The structure of A23187 and its complex are shown in Fig. 1.

In the present study, A23187 was incorporated into the lipid membrane of LUV suspensions (in specified portions) to investigate the interaction of the Ca^{2+} ionophore with phospholipid membranes and to de-

termine the importance of the hydrophobic lipid region in the interaction(s) of ultrasound with LUV membranes. It is hypothesized that A23187 would perturb the relaxation time of the structural relaxation of the hydrophobic carbon side chains at the phospholipid phase transition. Since A23187 could perturb lipid structure in the hydrophobic side chain region without creating pores in the membrane (as with gramicidin), it would not necessarily change the thermodynamics of the transition itself. Differential scanning calorimetry (DSC) was employed to monitor the static thermodynamics of the lipid transition for any effects of A23187 and electron spin resonance (ESR) was used to provide information of the ionophore's effect on the mobility of individual acyl side chains in the phospholipid bilayer.

DSC curves, measurement of excess specific heat, C_p, versus temperature in LUV suspensions, is qualitatively similar to that of $\alpha\lambda$ at ultrasonic frequencies near the relaxation frequency, indicating a relationship between the ultrasonic absorption of the lipid phase transition and the enthalpy of the phase transition. It is important to note, however, that DSC measures only the static thermodynamic variables of this system, while ultrasound measurements provide information on the relaxation times (in the microsecond time range) and therefore the kinetics of the system under study.

Electron spin resonance (ESR) was used to probe changes in the membrane caused by A23187. The ESR spin probe, 12-NS PC, a nitroxide attached to the twelfth carbon in the stearate chain of 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine, was chosen because it can provide information about the membrane structure and mobility at the hydrocarbon C-12 region of the LUV membrane [14]. The spectral features of 12-NS PC were used here to determine differences in individual phospholipid mobility in LUV membranes, with and without A23187. Such information complements changes in the ultrasound $\alpha\lambda$ caused by the presence of A23187 in the LUV membrane.

Materials and Methods

The control liposome preparations were made from mixtures of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG), in a 4 : 1 (w/w) DPPC/DPPG ratio. All lipids, including 12-NS PC, were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). The DPPG, whose headgroup has a net negative charge at pH 7.4, is thought to inhibit liposome aggregation and fusion due to surface repulsion of liposomes [4]. *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Hepes-buffered saline (buffer) was composed of 10 mM Hepes, 139 mM NaCl, 6 mM KCl, and distilled water, using 10 M NaOH to adjust the pH to 7.4 at room temperature. A23187 free acid was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Liposome preparation

LUV preparations without A23187 were made from DPPC/DPPG mixtures in a 4 : 1 (w/w) ratio, using the reverse phase evaporation method developed by Szoka and Papahadjopoulos [9]. This process results in liposomes with an average diameter of 0.21 ± 0.01 μm (S.E.) that range in size from 0.10 to 0.43 μm, as determined from electron microscopy. The average concentration of phospholipid in the preparation was 25.0 ± 0.1 mg/ml (S.E.), using the Bartlett phosphorus assay [15]. Samples of the original suspensions were diluted to 2 mg/ml phospholipid in buffer for acoustic measurements. This preparation is referred to as a standard LUV suspension. For A23187 LUVs, dried free acid A23187 was included in the organic phase (lipid + chloroform + ether) at the beginning of the reverse phase evaporation process, to result in 1, 2, or 5 mol% LUVs. A23187 incorporation did not produce an obvious change in the prepared liposomes, i.e., stable suspensions of liposomes, as determined by consistency and lack of aggregation or phase separation over three days of dialysis in the dark at 9 °C, were obtained.

For ESR measurements, LUV suspensions were prepared with the nitroxide-labelled phospholipid 12-NS PC (2 mol% in the LUV membrane), where the liposome suspensions had incorporated 2 or 5 mol% A23187 and the control LUV suspension was without A23187. The liposomes were not diluted before being used for ESR measurements. For All LUV suspensions, experiments were begun within 24 h of LUV preparation.

Apparatus

The acoustic interferometer used in this study is based on the design of Labhardt and Schwarz [16] and consists of two identical X-cut quartz transducers (diameter = 1 inch, fundamental resonance frequency, f_o = 4.0 MHz), positioned coaxially and parallel 5.5 mm apart forming the end walls of the measuring cell. One of the transducers, the transmitter, is excited electrically (cw) at a predetermined frequency to transmit ultrasonic waves through the fluid medium. The other transducer receives the sound wave and converts it into an electrical signal. The electrical input to the transmitting transducer is obtained from a synthesized signal generator (HP 8660B, Hewlett-Packard, Palo Alto, CA, USA), and the stable power drive is maintained by the HP 86601A RF section. The electrical output from the receiving transducer is monitored by a spectrum analyzer (HP 85521, HP 8553B). Ultrasonic intensities of less than 1 $\mu W/cm^2$ are used, which is many orders of magnitude below that needed to produce cavitation or a significant temperature increase [4]. The entire system resonates acoustically at certain input signal frequen-

cies. The mechanical quality factor Q of this resonance is related to the acoustic absorption per wavelength by [3]

$$\pi/Q = \pi\Delta f/f_o = \alpha\lambda$$

where α is the amplitude absorption coefficient per unit path length, $\alpha\lambda$ is the absorption per wavelength, λ, and Δf is the 3 dB bandwidth of the resonance, viz., the difference in the two frequencies for which the output power of the signal is one-half that at the resonance frequency f_o, and Q is the quality factor $f_o/\Delta f$.

The excess absorption due to the presence of LUVs in the suspension is obtained by subtracting the absorption coefficient of the reference buffer from that of the entire ensemble (reference buffer plus LUVs). For this situation, in which the acoustic velocity and impedance of the suspension are virtually the same as that of the reference buffer, correction for diffraction is unnecessary [3]. The excess absorption is

$$(\alpha\lambda)_{excess} = \pi(\Delta f - \Delta f_{ref})/f_o$$

Ultrasound measurement procedure

The excess absorption coefficient, $\alpha\lambda/c$, (c = concentration of phospholipid plus A23187 in g/ml; estimated $\alpha\lambda/c$ error = $\pm 1\%$ $\alpha\lambda/c$) was determined as a function of temperature and frequency. The temperature was varied from 38 to 48°C, while at each temperature the measurement frequency was varied from 0.58 to 5.2 MHz.

The temperature of the interferometer was maintained to within ± 0.05°C during data collection by immersion in a temperature controlled water bath (Exacal 500 with Endocal 350 refrigeration unit, and DCR-4 temperature Digital Controller, Neslab, Portsmouth, NH, U.S.A.). At least 30 min were allowed for the thermal stabilization of the interferometer after each temperature change. Remixing was performed after each temperature change to ensure that the LUV suspension was not experiencing liposome aggregation or fusion. Settling was not observed, as evidenced by the lack of change in $\alpha\lambda/c$ over time at any one temperature, and the lack of change in $\alpha\lambda/c$ after remixing of the suspension.

The ultrasound estimation of calorimetric transition enthalpy (ΔH_{cal}) for A23187 LUVs was determined by comparing the area of the $\alpha\lambda/c$ versus temperature curve with that for a standard LUV $\alpha\lambda/c$ curve, whose calorimetric enthalpy had been determined by differential scanning calorimetry (DSC). The Van't Hoff enthalpy ΔH_{VH} (cal/mol) of the transition may be calculated using the thermal width of the ultrasonic absorption coefficient from [5]

$$\Delta H_{VH} = 6.9 t_m^2/\Delta t_{1/2}$$

where t_m and $\Delta t_{1/2}$ are the phase transition temperature and half-width of the phase transition in Kelvin, respectively. These enthalpies may be used to determine the cooperative unit of the transition $\Delta H_{VH}/\Delta H_{cal}$ [2].

DSC procedure

The DSC-2 (Perkin-Elmer, St. Louis, MO) was used to obtain the specific heat at constant pressure, C_p, versus temperature on 50-μl samples of LUV suspension. The LUV suspensions in this study were 25.0 mg/ml phospholipid and the temperature range studied was 32 to to 52°C. The DSC experiments were performed (1) to compare the A23187 LUV DSC trace to standard LUV DSC traces and (2) to determine any changes, e.g., in T_m, ΔH, etc., caused by the addition of A23187 to the LUVs.

ESR procedure

All ESR spectra were taken at X-band (9.5 GHz) on a Varian E-4 spectrometer equipped with a Varian temperature controller. Sample temperatures were measured by inserting a copper-containing thermocouple into the sample before data collection. Sample temperatures were measured to within ± 0.03 C°. Liposomes were taken up in 1-mm (inside diameter) glass capillaries, sealed on both ends, and placed in an ESR tube which was subsequently placed in the ESR cavity dewar. The spectra were recorded in a standard first derivative mode with 100 kHz modulation and a microwave power of 5 mW. Data were collected and stored as arrays of 1024 points per spectrum with a PC computer [17]. Data collection and evaluation were performed using commercial EPR software (EPR Ware, Scientific Software Services, Bloomington, IL). The hyperfine splittings, i.e. the positions of the peaks, were determined by expanding the spectral feature of interest using the computer (estimated error $\pm$ 0.1G for 100 G scans). The ESR signal of LUVs without A23187 was compared to LUVs with 2 or 5 mol% A23187, to determine any changes in fluidity, rotation or mobility caused by the presence of A23187.

Results

The T_m, $\Delta T_{1/2}$, ΔH, ΔH_{cal} for 0, 1, 2, and 5 mol% A23187 LUVs are tabulated in Table I. Specific trends with increasing A23187 concentration are given below.

Differential scanning calorimetry

As is seen in Figs. 2A–2D, increasing concentrations of A23187 in LUVs did not greatly change the phase transition as detected by DSC. 1 mol% A23187 LUVs exhibited a DSC curve characteristic of standard LUV curves with a slightly increased T_m (42°C to 42.3°C) and a slightly decreased C_p (see Figs. 2A and 2B). 2 mol% A23187 LUVs in suspension showed only a

TABLE I

Characteristics of the lipid phase transition determined from ultrasound absorption coefficient measurements in LUV suspensions with varying percentages of A23187

ΔH_{cal} was obtained by comparison of the area under an $\alpha\lambda/c$ versus T (°C) curve of known enthalpy. The Van't Hoff enthalpy was calculated from the width of the ultrasound $\alpha\lambda/c$ peak at one-half its maximum height. The cooperative unit was estimated from the ratio of the Van't Hoff enthalpy to the calorimetric enthalpy. Estimates of error are given for each value.

LUV composition DPPC/DPPG (4:1, w/w), percentage of A23187	T_m (°C)	$\Delta T_{1/2}$ (C°)	ΔH_{VH} (kcal/mol)	ΔH_{cal} (kcal/mol)	Coop unit size	$\alpha\lambda_{max}$ freq(s) (MHz)
0 mol%	42.0±0.2	1.6±0.2	428±21	7.5±0.2	57±4	2.1
1 mol%	42.3±0.2	1.3±0.2	528±26	7.9±0.2	67±5	1.55
2 mol%	42.3±0.2	2.2±0.2	312±16	14.0±0.4	22±2	1.55 [a]
2 mol% (+0.5 mM Ca^{2+})	43.7±0.2	2.5±0.2	277±14	7.5±0.2	137±3	1.55
5 mol%	42.3±0.2	2.4±0.2	–	–	–	[b]

[a] Two additional peaks at 0.87 MHz and 1.13 MHz.
[b] Three peaks 0.57, 0.87, 1.13 MHz).

slightly increased T_m plus a slightly increased amplitude of C_p, as seen in Fig. 2C. 5 mol% A23187 LUVs in suspension resulted again in a phase transition similar to that of standard LUVs in suspension with a further increased amplitude of C_p (Fig. 2D). These results suggest that 1–5 mol% A23187 LUVs were thermodynamically not perturbed greatly in structure from standard LUV preparations.

DSC was also used to show the interaction of $CaCl_2$ with LUVs formed with and without A23187. Previously it was shown that the addition of $CaCl_2$ to standard LUV suspensions resulted in a phospholipid C_p versus temperature transition that was shifted to higher temperatures (see Fig. 3A) [11]. However, during the first through fifth heatings of the LUV suspension, two peaks were seen, presumably representing lipid not bound, and lipid bound, to $CaCl_2$, respectively [11]. As is shown from the experiments reported herein, with 1 mol% A23187 LUVs in suspension, (Fig. 3B), a somewhat different interaction with $CaCl_2$ addition occurred. Fig. 3B shows that the addition of $CaCl_2$ to 1 mol% A23187 LUVs resulted in one shifted peak, even upon the first heating, suggesting that A23187 in the LUV membranes allowed $CaCl_2$ equilibrium, across the bilayer, faster than the time elapsed in the first heating. This also demonstrated that the A23187 was incorpo-

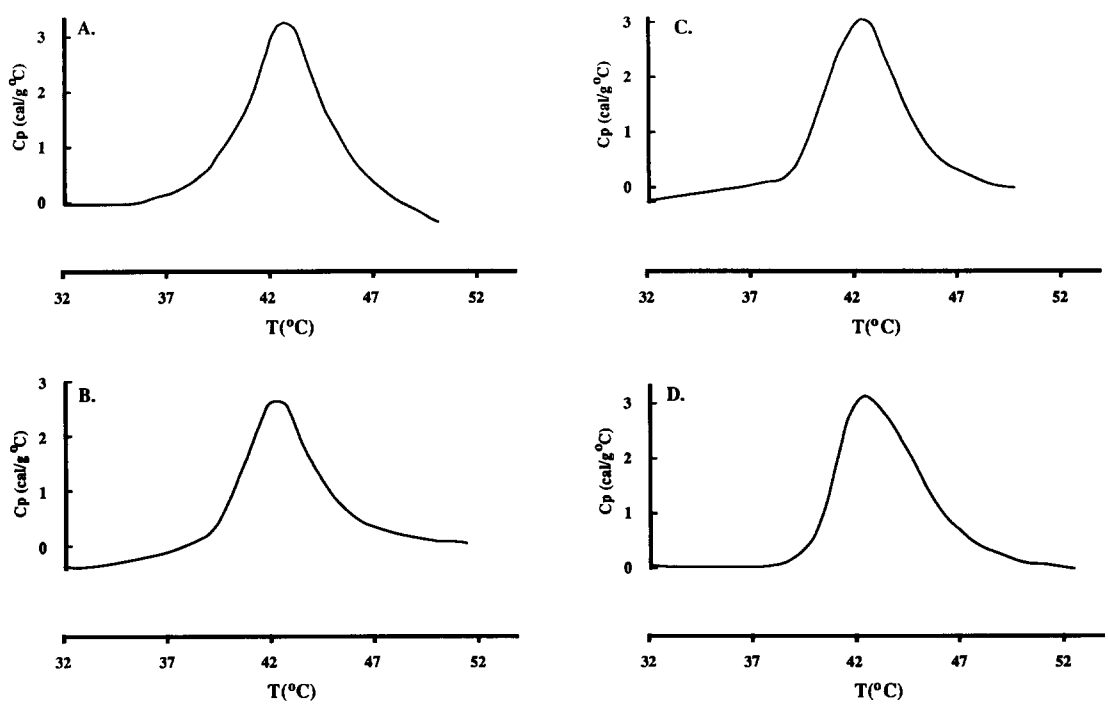


Fig. 2. C_p versus temperature in: (A) Standard LUVs in suspension, (B) 1 mol% A23187 LUVs in suspension, (C) 2 mol% A23187 LUVs in suspension, (D) 5 mol% A23187 LUVs in suspension.

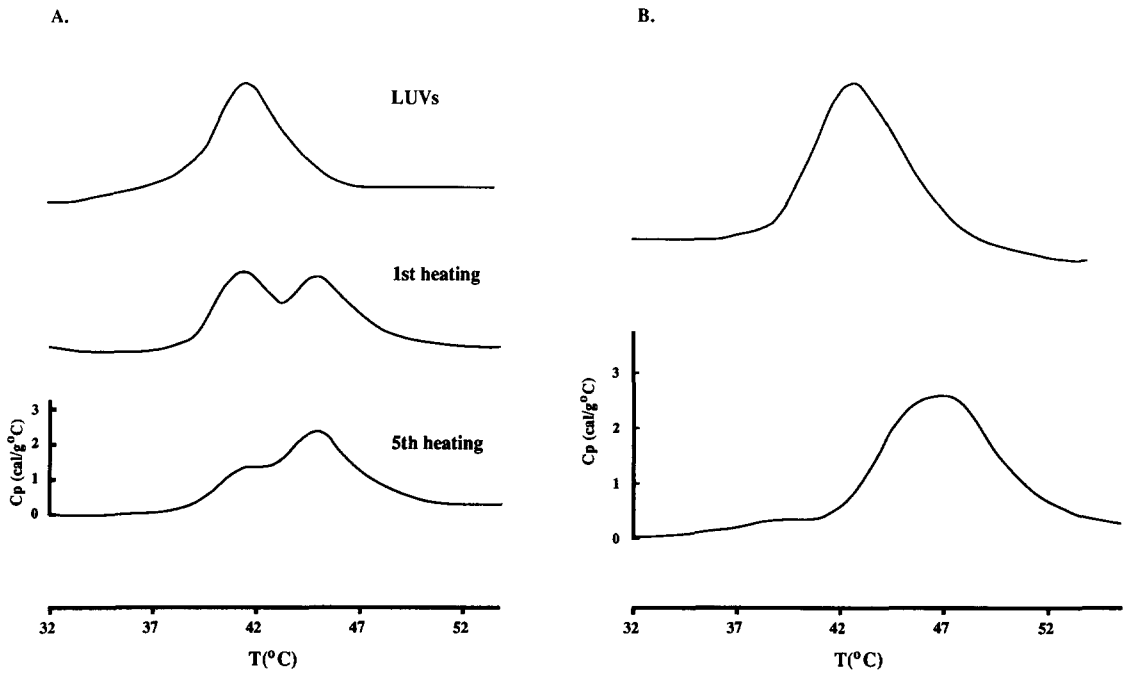


Fig. 3. C_p versus temperature in: (A) LUVs (60 mg/ml). The LUVs are shown (from top to bottom) without $CaCl_2$, with 2.5 mM $CaCl_2$ (first heating), 2.5 mM $CaCl_2$ (fifth heating). (B) 1 mol% A23187 LUVs (25 mg/ml) without $CaCl_2$ and with 2.5 mM $CaCl_2$ (first heating).

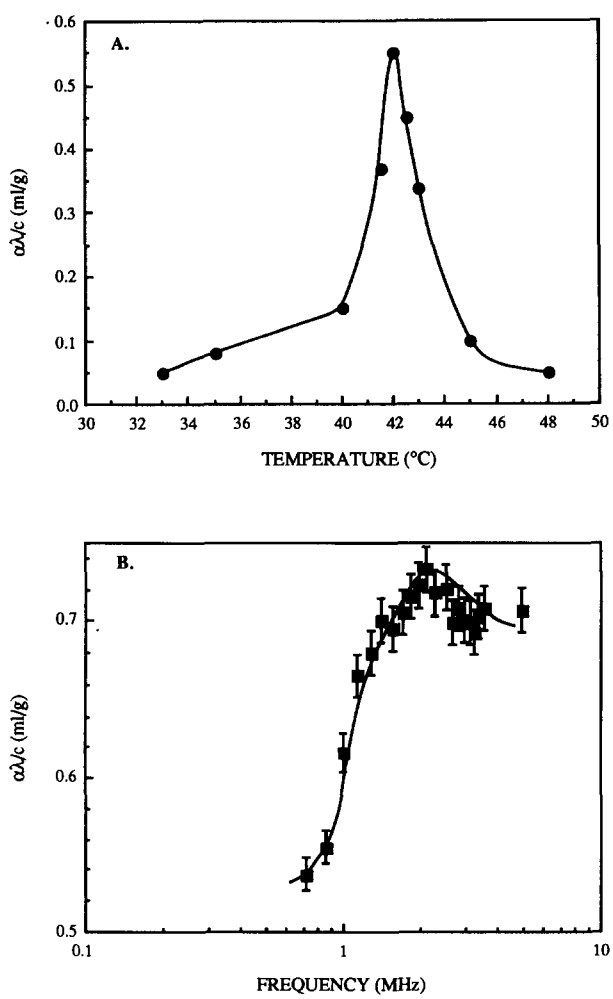


Fig. 4. (A) $\alpha\lambda/c$ versus temperature in standard LUVs in suspension. (B) $\alpha\lambda/c$ versus frequency in standard LUVs in suspension.

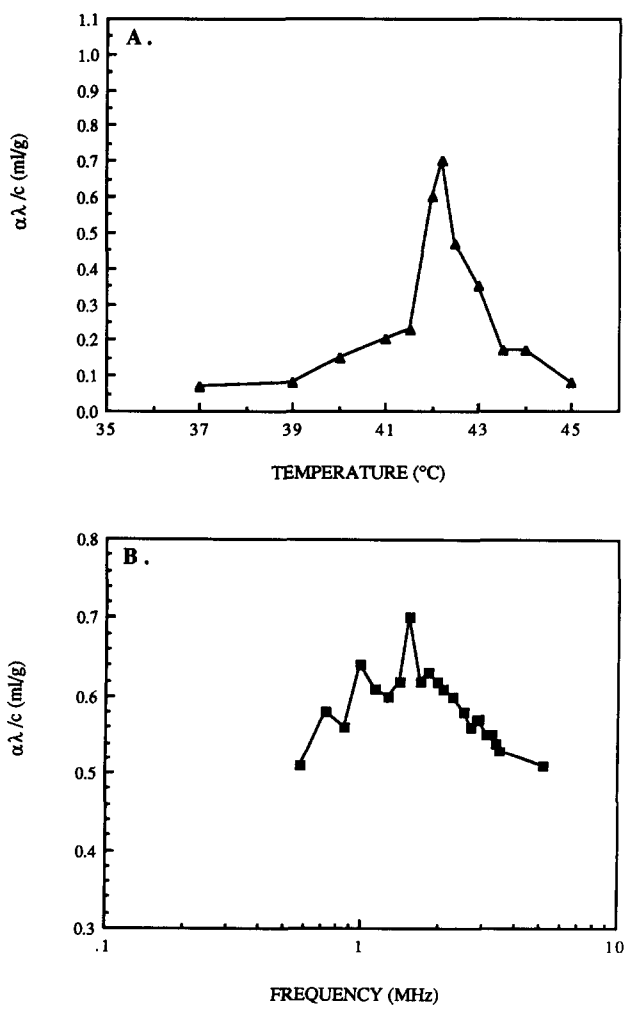


Fig. 5. (A) $\alpha\lambda/c$ versus temperature in 1 mol% A23187 LUVs in suspension. (B) $\alpha\lambda/c$ versus frequency in 1 mol% A23187 LUVs in suspension.

rated into, and was functional as an ionophore in, the LUV membrane.

Ultrasound experiments

The incorporation of 1 and 2 mol% A23187 did not qualitatively affect the $\alpha\lambda/c$ versus temperature curve of LUVs in suspension. Increasing percentages of A23187 in LUV membranes caused only a small perturbation in the T_m, from 42.0 °C to 42.3 °C, which was independent of the concentration of A23187 from 1 to 5 mol% (see Figs. 4A–7A). However, the amplitude of $\alpha\lambda/c$ versus T(°C) increased with increasing concentration of A23187 as did the ΔH of the transition, suggesting an enhancement of the ultrasound absorption of the phospholipid bilayer phase transition. The presence of A23187 alone was probably not causing simply an offset of $\alpha\lambda/c$, as the baseline (measured ± 5 C° from T_m) had the same value (0.06 ± 0.01 ml/g) as seen in standard LUV suspensions for 1 and 2 mol% A23187 LUVs (see Figs. 4A–6A). The width of the phase transition. $\Delta T_{1/2}$, while slightly decreased at 1 mol% A23187, increased with higher A23187 concentrations. This suggests that, in 2 and 5 mol% A23187 LUVs, the A23187 decreased the cooperativity of the lipid bilayer phase transition, as evidenced by broadening of the $\alpha\lambda/c$ versus temperature curve (see Figs. 6A and 7A). For 5 mol% A23187 LUVs in suspension (1 mg/ml), $\alpha\lambda/c$ was greatly increased in amplitude, especially at low frequencies (see Figs. 4A and 7A). At frequencies from 0.58 to 1.27 MHz, $\alpha\lambda/c$ was above the normal baseline (0.06 ± 0.01 ml/g) throughout the temperature range of 38 °C to 46 °C. At higher frequencies (2–5 MHz), $\alpha\lambda/c$ temperature dependence was qualitatively like that of an LUV curve, but with a significantly higher amplitude of $\alpha\lambda/c$ compared to standard LUV suspensions.

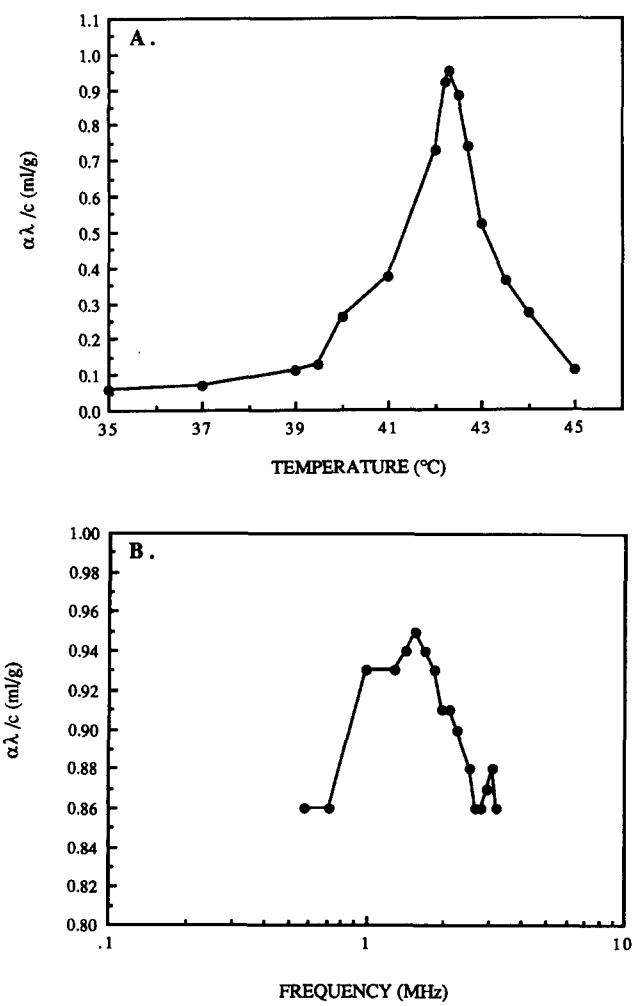


Fig. 6. (A) $\alpha\lambda/c$ versus temperature in 2 mol% A23187 LUVs in suspension. (B) $\alpha\lambda/c$ versus frequency in 2 mol% A23187 LUVs in suspension.

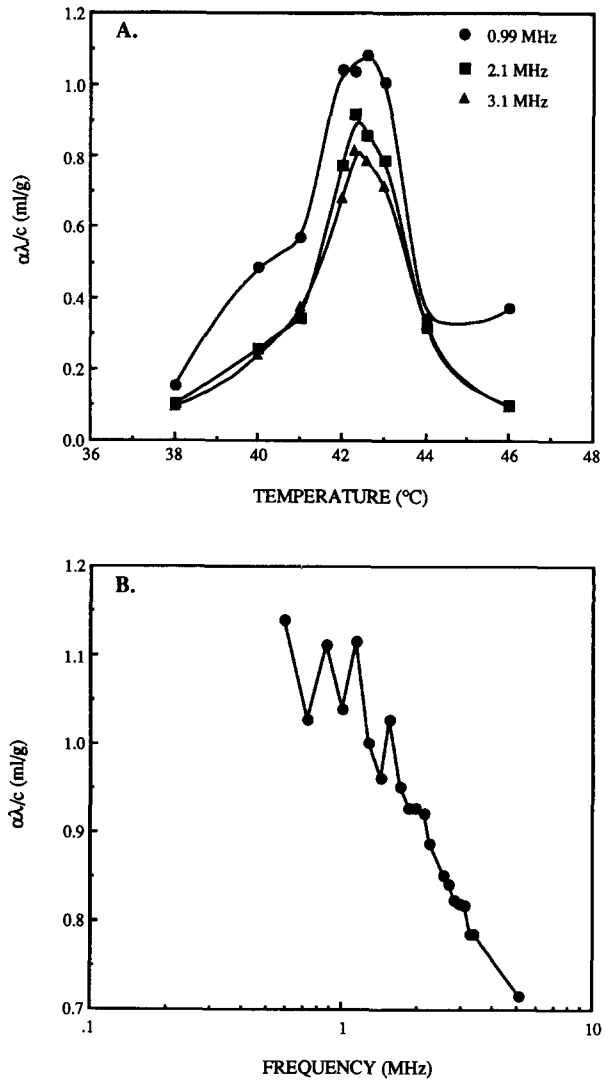


Fig. 7. (A) $\alpha\lambda/c$ versus temperature in 5 mol% A23187 LUVs in suspension. (B) $\alpha\lambda/c$ versus frequency in 5 mol% A23187 LUVs in suspension.

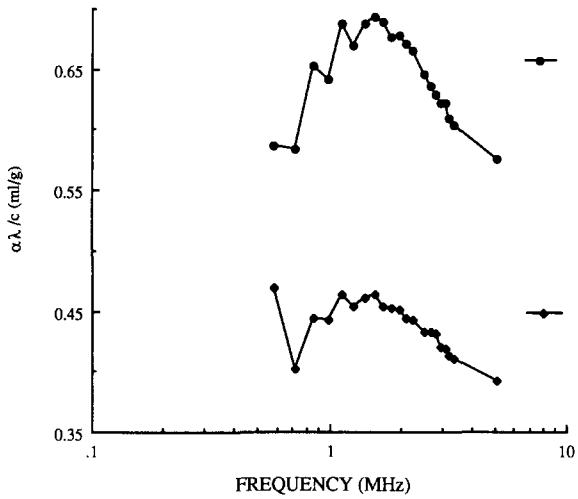


Fig. 8. $\alpha\lambda/c$ versus frequency in 2 mol% A23187 LUVs without (●) and with 0.5 mM $CaCl_2$ (◆).

Most striking in the ultrasound experiments was $\alpha\lambda/c$ as a function of frequency at T_m (see Figs. 4B–7B). 1 and 2 mol% A23187 LUVs had an $\alpha\lambda_{max}$ at 1.55 MHz compared to 2.1 MHz for standard LUVs. For 5 mol% A23187 LUVs, no single maximum in $\alpha\lambda/c$ was found in the operating frequency range as $\alpha\lambda/c$ exhibited a number of small peaks, at 0.57 MHz, 0.87 MHz, and 1.13 MHz, respectively, which increased in amplitude with decreased frequency (see Fig. 7B). The possibility also exists that a broader relaxation exists, below the range of measurement presently available.

In a separate experiment, $\alpha\lambda/c$ of 2 mol% A23187 LUVs was determined as a function of frequency at T_m, with and without $CaCl_2$ (0.5 mM) added (see Table I). The phase transition temperature shifted to 43.7°C, while $\Delta T_{1/2}$ is broadened to 2.5 C° (see Table I). However, as is seen in Fig. 8, 2 mol% A23187 LUVs with 0.5 mM $CaCl_2$, have the same $\alpha\lambda/c$ frequency dependence as obtained for 2 mol% A23187 LUVs, although $\alpha\lambda/c$ as a function of frequency is somewhat broadened by the presence of $CaCl_2$. Two small amplitude peaks in $\alpha\lambda$ as a function of frequency were observed at 0.87 MHz and 1.13 MHz which were smaller in amplitude than the peaks observed in 5 mol% A23187 LUVs although they were still centered at 0.87 MHz and 1.13 MHz. These peaks have not been observed in standard LUV suspensions or in previous experiments with LUV suspensions in which $CaCl_2$ or ^2H_2O have been added [11,12].

ESR experiments on 2 and 5 mol% A23187 LUVs

ESR determinations of the midfield line width (W_0) in the presence and absence of 2 and 5 mol% A23187 in LUVs in suspension showed no significant differences between the A23187 LUV samples and the control LUVs, in the range from 37 to 48°C. While the presence of 2 mol% 12-NS PC probably caused a small amount of spin-exchange broadening, the presence of A23187, even at levels that greatly affected the ultrasonic absorption, showed no change in the ESR spectra of 12-NS PC. This suggests that any changes must be very small or that the probe is insensitive to the presence of A23187. Also, the spin-exchange broadening itself is sensitive to changes in fluidity caused by the presence of A23187. As no changes occurred, A23187 probably did not cause changes in the fluidity of the bilayer. Also, no significant changes are observed in the distances between the spectral outer extrema in normal versus A23187 samples throughout the temperature range. Therefore the data is not shown.

Discussion

The incorporation of ionophores into the phospholipid bilayer should affect the fluidity, phospholipid dynamics and even the structure of the membrane, dependent upon the mechanism of action of the ionophore, and the region of the membrane to which the ionophore partitions. For example, Gramicidin A, a 15 amino acid peptide (M_r 1882), forms a channel by transverse association of a dimer across the phospholipid bilayer and affects the relaxational absorption of ultrasound at the phase transition [2,13]. The incorporation of gramicidin into the phospholipid bilayer of LUV suspensions causes the LUV phase transition to be broadened and decreases ΔH as a function of concentration of gramicidin [2]. Notably the relaxation frequency, as given by $\alpha\lambda_{max}$ for 5 mol% gramicidin incorporation is shifted from 2.1 MHz to 0.75 MHz [2].

The incorporation of A23187 into the phospholipid bilayer of LUVs changes the relaxation frequency of the phospholipid phase transition, suggesting a perturbation of the structural relaxation of the hydrophobic carbon side chains at T_m. At concentrations of 1 and 2 mol% A23187 in LUVs, ΔH_{cal} increases with increasing A23187 concentration, and the relaxation frequency, where $\alpha\lambda_{max}$ occurs, decreases to 1.55 MHz. This peak is presumably shifted from the 2.1 MHz relaxation frequency seen in standard LUV suspensions, as that peak is no longer observed. This suggests that the presence and/or activity of individual molecules of A23187 in LUV membranes interacted in a dynamic manner with phospholipid molecules to change the kinetics of the molecular event to which ultrasound couples. In contrast, DSC measurements show that the incorporation of A23187 only slightly perturbs the thermotropic phase transition, without any significant change in ΔH of the transition.

When 0.5 mM $CaCl_2$ was added to 2 mol% A23187 LUV suspensions, the T_m of the phase transition was increased to 43.7°C, the amplitude of $\alpha\lambda/c$ was decreased and the peak in $\alpha\lambda/c$ as a function of temperature was broadened (see Table I). However, the

frequency dependence of $\alpha\lambda/c$ at T_m was not changed (as compared to 2 mol% A23187 LUVs), suggesting that the interaction of A23187 with Ca^{2+} does not perturb the site or character of ultrasound interaction with the LUV bilayer. This data is in agreement with our previous work that showed that $CaCl_2$ added to standard LUV suspensions did not change the character of the phospholipid relaxation detected by ultrasound [11].

In 5 mol% A23187 LUVs in suspension, $\alpha\lambda/c$ as a function of temperature and especially frequency is changed significantly, suggesting a peak in $\alpha\lambda/c$ below the low frequency limit of the acoustic interferometer (Fig. 7B) and showing a strong perturbation of the ultrasonic interaction with the phospholipid bilayer. However, at such high A23187 concentrations, the A23187 itself may absorb ultrasound significantly. At low frequencies (0.58–1.27 MHz) $\alpha\lambda/c$ as a function of temperature is above the normal baseline of 0.06 ml/g even at 38.0°C and peaks at a plateau of 1.0 C° in width near T_m, above which temperature it decreases in amplitude to a 0.37 ml/g baseline, and remains there to 46.0°C (see Fig. 7A). At frequencies above 1.55 MHz, $\alpha\lambda/c$ versus temperature is qualitatively like that of standard LUV suspensions, except for the slight increase in $\alpha\lambda/c$ and T_m, and the decreased cooperativity of the transition. Below 1.55 MHz a number of small $\alpha\lambda/c$ peaks are observed at T_m. These peaks may not be significant given the increased error at the low frequency region of measurement. However, as these peaks have not been observed with other liposomes preparations and are consistent and increasing with A23187 concentration, the data would suggest that the A23187 itself (at high concentrations), or A23187 complexes, absorb ultrasound in a different manner (at low frequencies) than does the phospholipid membrane.

In contrast to the large changes in the ultrasonic absorption coefficient in A23187 LUVs, the ESR data for 2 and 5 mol% A23187 LUVs show no significant perturbation of the hydrophobic region of the bilayer by A23187 over the temperature range of 37–48°C, specifically at C-12, using the nitroxide probe, 12-NS PC. This result would be expected if the relaxation that ultrasound detects is a structural relaxation related to a group of phospholipids. In this case, the A23187 would perturb the group relaxation without necessarily changing the mobility or fluidity of single phospholipid molecules. The lack of any change is in fact observed with the ESR probe, 12-NS PC.

It is reasonable to conclude from these results that A23187, incorporated into LUV suspensions, partitions into the hydrophobic portion of the lipid bilayer, and is functional in the transport of Ca^{2+} ions across the bilayer as a mobile carrier ionophore (see Figs. 3A and 3B). As the incorporation of A23187 into LUV membranes reduces the relaxation frequency from 2.1 MHz to 1.55 MHz at 1 and 2 mol% A23187, it also suggests that A23187 perturbs the membrane in the region where ultrasound interacts with the LUV membrane, in the hydrophobic fatty acyl chain region of the bilayer. A23187 may slow the conformational changes of side chains, or more possibly, perturbs changes in structural organization of the side chains that occur at T_m. The fact that Ca^{2+} addition did not change the relaxation time, although it did perturb T_m and the $\Delta T_{1/2}$ of the transition, suggests that the action of A23187 in transporting Ca^{2+} across the membrane does not affect the character of its presence in the bilayer or that any such effect occurs on a time scale not detectable with this system.

Though the specific event to which ultrasound couples is not known, from these and other experiments, it is suggested that the event is related to the conformation or packing of the fatty acyl side chains of the phospholipids in the bilayer. Whether the event is related to a specific conformational state of single phospholipid side chains or the combined structural organization of these side chains in the lipid membrane will possibly only be ascertained by the use of specifically modified phospholipids in ultrasound and ESR experimentation. The presence of small peaks in $\alpha\lambda$ as a function of frequency at T_m in LUVs with increasing portions of A23187 suggests other dynamic phenomena the ultrasound may be detecting. Such peaks might be speculated to be due to the presence of alternate A23187 configurations, A23187 complexes or even A23187-phospholipid complexes, superimposed upon the absorption of the perturbed phospholipid bilayer. Further experimentation with ESR and acoustic interferometry at a lower frequency range may allow more complete information about this system to be drawn.

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