Effects of Calcium and Neomycin on Phase Behavior of Phospholipid Bilayers

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Abstract □ Calcium ion (Ca^{2+}, 5 m*M*) caused a large upward shift in the transition temperature (T_c) of dipalmitoyl phosphatidylglycerol liposomes, apparently interacting with external and internal lipid in the bilayer. Neomycin (1 m*M*) caused only a small shift, apparently not penetrating the internal lipid domains. When liposomes were first incubated with Ca^{2+} followed by neomycin, the shift in T_c indicated displacement of Ca^{2+} by the drug. Liposomes of dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine did not interact significantly with either cation. In mixed dipalmitoyl phosphatidylcholine–dipalmitoyl phosphatidylglycerol and distearoyl phosphatidylcholine–dipalmitoyl phosphatidylglycerol liposomes, addition of Ca^{2+} or neomycin resulted in phase separation.

Keyphrases □ Calcium—effect on phase behavior of phospholipid bilayers, liposomes, neomycin □ Neomycin—effect on phase behavior of phospholipid bilayers, liposomes, calcium □ Liposomes—effects of calcium and neomycin on phase behavior of phospholipid bilayers □ Phase behavior—effects of calcium and neomycin on phospholipid bilayers, liposomes

Acidic phospholipids have been suggested as the membrane sites responsible for interactions with basic antibiotics in bacteria (1) and mammalian cells (2, 3). In the case of aminoglycoside antibiotics, such interactions have been discussed as mechanisms underlying their nephro- (2) and ototoxicity (3). The aminoglycosides also exert intracellular effects in bacteria and in afflicted eukaryotic cells. The mechanism of cell penetration is not clear. Internalization of aminoglycosides into lysosomes has been reported for the renal cortex (4), but not for other organs. Entry *via* a basic amino acid transport system (5) may be an alternate pathway.

Studies with monomolecular films (6) and liposomes (7) have shown that neomycin and other aminoglycosides affect Ca^{2+} binding to phospholipids, and that the degree of calcium displacement is strongly dependent on the head group of the anionic lipid. Differential scanning calorimetry is a valuable tool in determining physical changes of bilayers resulting from ion or drug interactions with liposomes, *e.g.*, gel-to-liquid phase transitions, phase separation, or membrane fusion (8–11). The present study investigates the actions of Ca^{2+} and neomycin, a highly toxic aminoglycoside, on thermotropic properties of synthetic negatively charged and neutral liposomes with well-defined transition temperatures.

EXPERIMENTAL

Liposomes were prepared from dipalmitoyl L-α-phosphatidylcholine (I), distearoyl L-α-phosphatidylcholine (II), and the ammonium salt of dipalmitoyl L-α-phosphatidyl-DL-glycerol (III)[1]. These lipids showed no impurities by TLC and by analysis of pretransition and main transition peaks of their liposomes.

Lipid samples (15 μmoles) dissolved in chloroform were dried under a nitrogen stream, and residual solvent was removed *in vacuo*. The dried lipid was suspended by vortexing in 10 m*M* tris buffer containing 100 m*M* NaCl and 0.1 m*M* EDTA (ethylenediaminetetraacetic acid), final pH 7.0. The resultant phospholipid concentration was 30 m*M*. Compounds I, III, and I–III (60:40 mole %) were suspended at 42°, II and II–III (60:40 mole %) at 58°. A 60:40 mole % ratio was selected, since at least 40 mole % negatively charged lipid was required to produce clearly defined thermal changes in the presence of Ca^{2+} at concentrations within the physiological range. Aliquots of the liposomal suspensions were incubated with 5 m*M* $CaCl_2$ or 1 m*M* neomycin for 60 min at 25° or 42° or, in the case of II-containing vesicles, at 58°.

Thermograms were obtained with a scanning calorimeter[2]. Aliquots of liposomal suspension (15 μl, containing 0.5–1 μmole of lipid) were transferred to volatile sample pans and properly sealed. Reference sample pans were prepared with the same amount of buffer. Inclusion of Ca^{2+} or neomycin or Ca^{2+}–neomycin mixtures in the reference sample pans did not affect the thermograms. Each sample was scanned between 5° and 70° at a rate of 5°/min and a range of 2 mcal/sec. Indium and water were used as calibration standards.

Phase-transition temperatures (T_c) were determined as the intercept between the slope of the ascending endothermic peaks and the base line. The midpoint value of transition was estimated as the maximal endothermic peak. After each run, the amount of lipid in the sample pan was determined (12). All experiments were repeated on at least three separate preparations.

RESULTS AND DISCUSSION

The thermograms for liposomes containing I or II (Fig. 1a and b) show well-defined sharp transition peaks with a broad pretransition peak. Incubation with Ca^{2+}, neomycin, or Ca^{2+} plus neomycin did not result in any changes in the thermograms. These data are in agreement with our previous monolayer (6) and liposome binding studies (7), which show no interaction between neutral phospholipids and Ca^{2+} or neomycin.

Liposomes containing III (Fig. 2a) have a clear transition peak at 41°. Incubation with Ca^{2+} (Fig. 2b) caused a 14° upward shift in T_c, in good agreement with previous observations (13). The extent of the T_c shift was related to the Ca^{2+} concentration, and 10 m*M* Ca^{2+} resulted in the loss of the transition peak up to 70°. This upward shift has been interpreted as the result of a combination of charge neutralization and specific complexation (13).

Incubation of liposomes containing III with neomycin at 25° (Fig. 2c) broadened the main transition peak of III and split it into two approximately equal components at 40° and 42°. These could represent two distinct domains of phospholipid molecules with different physical properties resulting from shallow perturbation of the bilayer primarily in the region of the phospholipid head-groups (14) and a coexistence between parent and modified phases (15). The split peak caused by neomycin thus suggests an interaction with the outer and lack of interaction

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Perkin-Elmer, Model DSC-2C.

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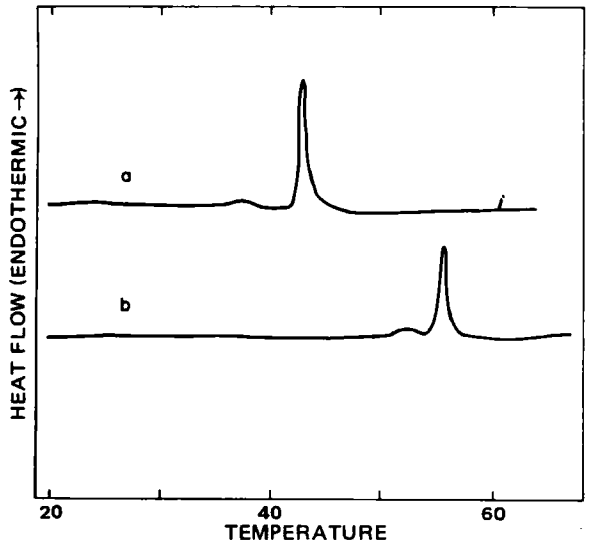

Figure 1—*Thermograms of liposomes containing I (a) and II (b). Conditions of liposome formation and incubation are described in the text.*

with the inner domains of the bilayer. Bilayers which are good permeability barriers to hydrophilic molecules above and below their T_c values (16, 17) are, however, highly permeable at their phase-transition temperature. Incubation of the liposomes with neomycin near their phase-transition temperature could conceivably alter the accessibility of the inner lipid layer. After such treatment at 42°, the split peak still remained (40° and 42°), but the 42° portion of the peak was clearly predominant (Fig. 2d). No such influence of temperature was seen with III and Ca^{2+}. This suggests that Ca^{2+} has access to the inner bilayer regions even when they are in the gel state, while the access of neomycin to the inner regions is limited by and dependent on the fluidity of the membrane. It should be pointed out, however, that the interaction of neomycin with III results in a T_c shift of only ~2°, whereas Ca^{2+} causes a much larger shift, indicating a solidifying effect (13). Thus, the resulting complexes with Ca^{2+} or neomycin have different thermal properties.

In another series of experiments, III was first incubated with Ca^{2+} at 42° and subsequently with neomycin. The resultant thermogram (Fig. 2e) shows the appearance of a distinct transition peak with a midpoint value of 43° and a very small peak at ~55°. Thus, it appears that neomycin, even at a concentration one-fifth that of Ca^{2+}, is essentially reversing the Ca^{2+} effect, *i.e.*, solidification, on the thermal properties of

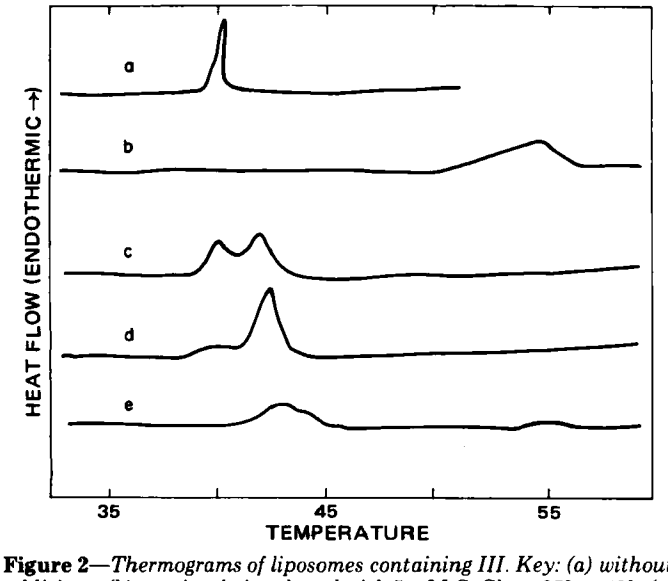

Figure 2—*Thermograms of liposomes containing III. Key: (a) without additions; (b) previously incubated with 5 mM $CaCl_2$ at 25° or 42°; (c) previously incubated with 1 mM neomycin at 25°; (d) previously incubated with 1 mM neomycin at 42°; (e) previously incubated with 5 mM $CaCl_2$ followed by 1 mM neomycin at 42°.*

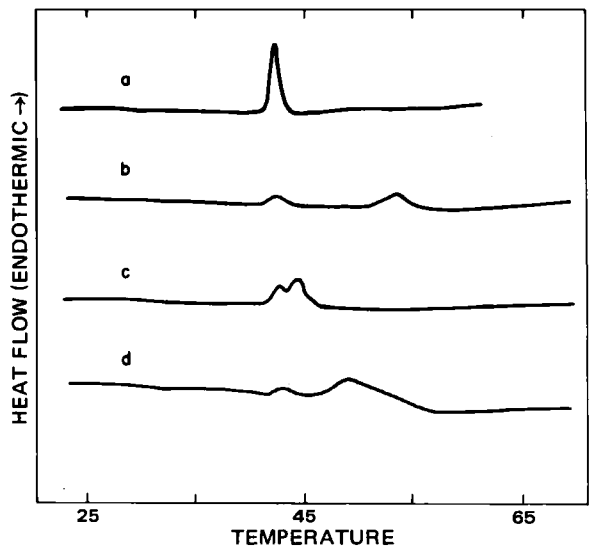

Figure 3—*Thermograms of mixed I–III liposomes (3:2 molar ratio). Key: (a) without additions; (b) incubated with 5 mM $CaCl_2$ at 42°; (c) incubated with 1 mM neomycin at 42°; (d) incubated with 5 mM $CaCl_2$ followed by 1 mM neomycin at 42°.*

the bilayers, indicating displacement of Ca^{2+} by neomycin.

Mixed liposomes containing I–III, 60:40 mole %, show a clear transition peak with a T_c value of 41.5° (Fig. 3a). This value is in agreement with that of Findlay and Barton (18), who pointed out that the almost identical T_c values for pure I, pure III, and I–III mixtures indicate complete miscibility of the components in the bilayer. In this mixed-lipid system, Ca^{2+} caused phase separation (Fig. 3b) with a small, but clearly defined peak at 41° and a major peak at 53°. Since the Ca^{2+}–III ratio is high and since there is free lateral movement of the lipid within the bilayer, the 41° peak should represent a population very rich in I and the 53° peak either a Ca^{2+}–III or a Ca^{2+}–I–III complex. The addition of neomycin to liposomes composed of I–III (Fig. 3c) resulted in a split peak centering at ~42° and 44°, which is shifted upwards ~2° from the split peak observed on incubation of liposomes composed of pure III with neomycin. This is possibly due to the incorporation of I into the neomycin–III complex.

Addition of neomycin to mixed I–III liposomes, preincubated with Ca^{2+} (Fig. 3d), produced a small transition peak at ~42.5° and a larger one at ~49°. Whereas neomycin clearly displaced Ca^{2+} from liposomes containing pure III (Fig. 2e), it appears that the presence of a neutral phospholipid in the bilayer reduced this capability. The appearance of a new peak at 49° may indicate that steric interactions in the Ca^{2+}–I–III complex interfere with neomycin binding.

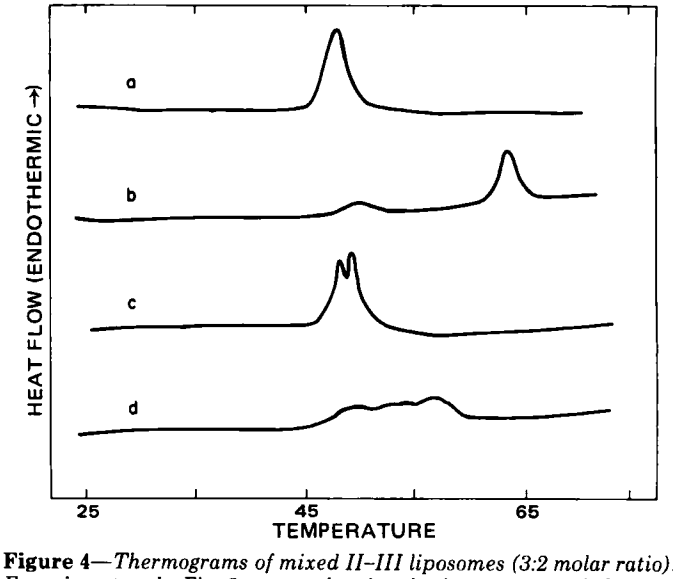

Figure 4—*Thermograms of mixed II–III liposomes (3:2 molar ratio). Experiment as in Fig. 3 except that incubations were carried out at 58°.*

The thermogram for liposomes containing II–III (60:40 mole %) shows a transition peak with a T_c value of 48° (Fig. 4a). This value is intermediate to the T_c values of pure II (57°) and pure III (41.5°), indicating good bilayer mixing. The thermograms after incubation with either Ca^{2+} or neomycin, or both (Fig. 4b, c, and d), are similar to those of liposomes composed of I–III, suggesting that the chain length of the neutral lipid does not drastically influence the overall nature of these interactions.

The displacement of calcium, which has been described for the interaction of the drug with bacteria (19), the neuromuscular junction (20), and inner ear tissues (21), may interfere with normal calcium-dependent physiological functions. The results of this study indicate that these effects may be due, in part, to resultant changes in the thermotropic properties, *i.e.*, degree of fluidity, of the lipid bilayer induced by neomycin.

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Quantitation of Ketoconazole in Biological Fluids Using High-Performance Liquid Chromatography

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Abstract □ A rapid, specific procedure is described for the quantitation of ketoconazole in biological fluids using high-performance liquid chromatography (HPLC). The procedure involves sample preparation using a reverse-phase C-18 cartridge prior to chromatography and quantitation using peak height ratios (UV absorbance detection, 231 nm) of ketoconazole to the internal standard, phenothiazine. A sensitivity of 0.2 μg/ml was achieved using a 0.5-ml sample. The mean recovery was 86.2%, and overall coefficient of variation of the procedure was 7.1%. This procedure has been used to determine ketoconazole levels in human serum, plasma, CSF, and synovial fluid. A comparison with a microbiological assay is presented, and adaptability of this procedure to quantitation by fluorescence to increase the sensitivity fivefold is discussed.

Keyphrases □ Ketoconazole—quantitation in biological fluids, high-performance liquid chromatography, humans □ High-performance liquid chromatography—quantitation of ketoconazole in biological fluids, humans

Ketoconazole, *cis*-1-acetyl-4-[4[[2-(2,4-dichlorophenyl)-2- (1*H*-imidazol-1-ylmethyl) -1,3-dioxolan-4 - yl] methoxy]phenyl]piperazine, an antifungal agent used to treat a wide variety of superficial and systemic mycoses (1–3), has the advantage over other imidazole derivatives of producing adequate, sustained blood levels following oral administration. Several microbiological assays (4–6) and three high-performance liquid chromatographic (HPLC) methods (7–9) have been described for quantitation of ketoconazole in biological fluids. HPLC techniques have the advantage of direct concentration measurement since the microbiological assays quantitate ketoconazole concentration indirectly as antifungal activity against a test organism. Microbiological procedures lack specificity for ketoconazole; thus, falsely elevated levels may be produced by active metabolites or other concurrently administered antifungal agents.

A rapid and reproducible HPLC method is described which is suitable for quantitation of ketoconazole in biological fluids both for routine monitoring (sensitivity to 0.2 μg/ml using UV detection) or for pharmacokinetic studies

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