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INTERACTION OF CALCIUM AND NEOMYCIN WITH ANIONIC PHOSPHOLIPID-LECITHIN LIPOSOMES

A DIFFERENTIAL SCANNING CALORIMETRY STUDY

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Abstract—The interactions of calcium and neomycin with liposomes of various anionic phospholipids plus lecithin were studied by differential scanning calorimetry. Phosphatidylinositol bisphosphate differed from other acidic phospholipids in its interactions with both calcium and neomycin. Calcium, at concentrations as low as 1 mM, induced the appearance of a second transition peak in phosphatidylinositol bisphosphate-enriched liposomes only. Neomycin acted antagonistically and precluded this phase separation. In addition, neomycin lowered the phase transition temperature of phosphatidylinositol bisphosphate-lecithin liposomes while it raised the transition temperature of all other anionic phospholipid-lecithin liposomes tested. This fluidizing effect of neomycin and the antagonism to calcium may induce critical alterations of properties of biological membranes. The study supports and extends our previous findings and conclusions that phosphatidylinositol bisphosphate may play a crucial role in the expression of aminoglycoside toxicity.

The ototoxicity (and possibly also the nephrotoxicity) of aminoglycosides may be based on specific membrane actions of these drugs. A number of studies on cochlear and renal lipid metabolism have provided evidence for anionic phospholipids as binding sites for aminoglycosides [1, 2]. Our previous work on phospholipid–aminoglycoside interactions *in vivo* [3] and *in vitro* [4, 5] has specifically implicated the polyphosphoinositides, notably phosphatidylinositol bisphosphate, in such a role.

Physicochemical investigations of aminoglycoside/lipid interactions have shown a complex pattern. Binding between the polycationic drugs and isolated phospholipids was demonstrated by a variety of techniques, but the binding affinity of the drugs did not correlate with their established toxicity [2] and neither did their ability to displace calcium from these lipids. On the other hand, increases of surface pressure of monomolecular lipid films containing phosphatidylinositol bisphosphate induced by a number of aminoglycosides demonstrated a significant correlation with ototoxicity [6]. The uniqueness of the aminoglycoside–phosphatidylinositol bisphosphate interaction may result from a specific charge orientation in the molecules leading to the formation of a complex with area-determining characteristics in monolayers [7]. It is not known, however, what impact this interaction may have on membrane characteristics such as fluidity and permeability.

Differential scanning calorimetry of liposomes is an established procedure to investigate membrane fluidity [8, 9]. We have therefore employed this procedure to study further the interactions of the aminoglycoside, neomycin, with various anionic phospholipids in order to determine the effects on physicochemical properties of bilayers.

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MATERIALS AND METHODS

Distearoyl L-α-phosphatidylcholine (DSPC), L-α-phosphatidylserine (PS), phosphatidylinositol (PI), and neomycin sulfate (Nm), reagent grade, were purchased from the Sigma Chemical Co., St. Louis, MO. Phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2) were isolated from Sigma brain extracts by chromatography on immobilized neomycin [10]. Lipids were stored in the dark under nitrogen at −20°; PIP and PIP_2 were dried and the other lipids were in chloroform–methanol (95:5, v/v). The purity of the lipids was greater than 95% as determined by TLC. All other chemicals were of reagent grade and the water was thrice distilled.

Preparation of liposomes. An aliquot of the chloroform–methanol (95:5, v/v) solution of a lipid mixture was transferred to a 10-ml round-bottom flask. The solvents were evaporated under nitrogen while the flask was rotated by hand to aid the formation of a uniformly thin lipid layer at its bottom. Residual solvent was removed by storing the flask overnight under vacuum. The dried lipid was then suspended in 0.05 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.0, $\mu = 0.2$) by vortexing, and suspensions were centrifuged at 10,000 *g*. Liposomes were resuspended in the same buffer to yield a final concentration of approximately 15 μmoles lipid/ml (determined as in Ref. 11). When examined by negative strain and freeze-fracture electron microscopy, liposomes were found to be multilamellar.

Differential scanning calorimetry (DSC). Aliquots of the vesicle suspensions were incubated at 60° with various concentrations of Ca^{2+}, Nm, or both for 2 hr to maximize penetration into the layers of the liposomes. Samples were then centrifuged, and the wet pellets were scanned in a Perkin–Elmer DSC-2C scanning calorimeter with a digitalized calorimeter output. Approximately 30 μl of sample containing 0.5 to 1 μmole lipid was placed in a hermetically sealed sample pan while the reference pan contained an equal amount of buffer. All scans shown were obtained at a heating rate of 5°/min and a range setting of 1 mcal/sec. The calorimeter was calibrated with Indium standard (Perkin–Elmer). All experiments were repeated on at least three separate preparations.

The transition temperature (T_m) was defined as the temperature at the apex of the transition peak, i.e. when the excess specific heat reaches a maximum. T_m values were calculated with the Perkin–Elmer TADS, DSC Standard Software Program. This measure gave better reproducibility (±0.2° for all T_m), particularly with mixed lipid preparations, than the temperature of the onset of melting. Slower heating rates (1° and 2°/min) produced identical T_m but a slight broadening of the peak. Sharpness of the peak was also a function of the amount of lipid in the sample, but T_m values were always within ±0.2° regardless of sample size.

RESULTS

DSPC liposomes. To ensure that the thermotropic changes produced by addition of Ca^{2+} or Nm to mixed liposomes were the result of interactions with the anionic component, the effects of Ca^{2+} and neomycin on pure DSPC liposomes were first tested. The thermogram showed the main peak at 56.7° and a pretransition peak at 53.0°. Addition of Ca^{2+} or Nm (up to 10 mM) had no significant effect on the T_m of the main transition peak. This indicated that the interactions between Ca^{2+} or Nm (at concentrations of less than 10 mM) and neutral phospholipids [5, 12] are small as compared to those with acidic lipids (see below).

PS-DSPC liposomes. Ca^{2+}, up to 10 mM, induced a concentration-dependent increase of T_m of PS-DSPC liposomes (1:1 molar ratio) (Fig. 1A). Nm also increased T_m but, unlike Ca^{2+}, concentrations of Nm above 1 mM caused no significant shift (<0.2°) in T_m (Fig. 1B).

To test whether the ratio of the lipid components influenced the effect of Ca^{2+} and Nm on the mixed liposomes, mixtures of PS-DSPC in molar ratios of 1:2 and 2:1 were investigated (Table 1). Again, Ca^{2+} and Nm caused concentration-dependent increases in the T_m which for the latter agent peaked at concentrations well below 10 mM. The effect of Ca^{2+} was more pronounced in the PS-DSPC (2:1) system. Upon addition of 10 mM Ca^{2+}, an endothermic peak at the phase transition temperature of pure DSPC appeared, indicating some separation of this component from the original mixture.

Next, the effect of Nm in the present of Ca^{2+} was investigated (Table 1). When both Ca^{2+} and Nm were present at low concentration (1 mM), the increase in T_m approximated that induced by Nm alone. When Ca^{2+} and Nm were both present at 5 mM, the T_m ranged between those induced by the individual cations. At a concentration of Ca^{2+} greatly exceeding that of Nm, e.g. 10:1, the T_m was closer to that found in the presence of Ca^{2+} alone than of Nm alone.

PI-DSPC liposomes. While 1 mM Ca^{2+} increased T_m of PI-DSPC liposomes (1:1), higher concentrations of Ca^{2+} did not cause a further increase (Fig. 1C). Nm also increased T_m, and the magnitude of change was dependent on its concentration (Fig. 1D) up to 10 mM.

The addition of Ca^{2+} or Nm to PI-DSPC (1:2) and PI-DSPC (2:1) liposomes resulted in essentially similar thermotropic changes (Table 2). When Ca^{2+} and neomycin were both present at 1 mM, the increase in T_m approximated or exceeded that caused by Nm alone. When both cations were present at 5 mM, the T_m shifted to values between those obtained with Ca^{2+} and Nm alone in the case of PI-DSPC (1:1) and PI-DSPC (1:2) mixed liposomes. However, the increase in T_m for PI-DSPC (2:1) mixtures was close to that caused by Nm alone. At a ratio of Ca^{2+}:Nm of 10:1, Ca^{2+} dominated, as T_m was very close to that determined in the presence of Ca^{2+} alone.

PIP-DSPC liposomes. In liposomes of PIP-DSPC (1:1 molar ratio), Ca^{2+} caused a slight upward shift in T_m at a 1 mM concentration with no further effect at higher concentrations; Nm induced upward shifts

Table 1. Effect of Ca^{2+} and neomycin on phase transition of PS-DSPC liposomes

	Transition temperature (T_m)(°)					
	PS-DSPC (2:1 molar ratio) (T_m = 40.5°)		PS-DSPC (1:1 molar ratio) (T_m = 43.4°)		PS-DSPC (1:2 molar ratio) (T_m = 4.95°)	
Concentrations	Ca^{2+}	Nm	Ca^{2+}	Nm	Ca^{2+}	Nm
0.1 mM		43.2		45.5		51.2
1 mM	41.1	47.3	44.7	47.7	50.8	51.7
5 mM	53.7	47.3	45.7	47.5	51.1	53.6
10 mM	56.0	47.5	51.2	47.7	52.3	53.6
1 mM Ca^{2+}/1 mM Nm	47.3		48.4		52.6	
5 mM Ca^{2+}/5 mM Nm	51.7		47.9		52.7	
10 mM Ca^{2+}/1 mM Nm	58.8		51.1		52.4	

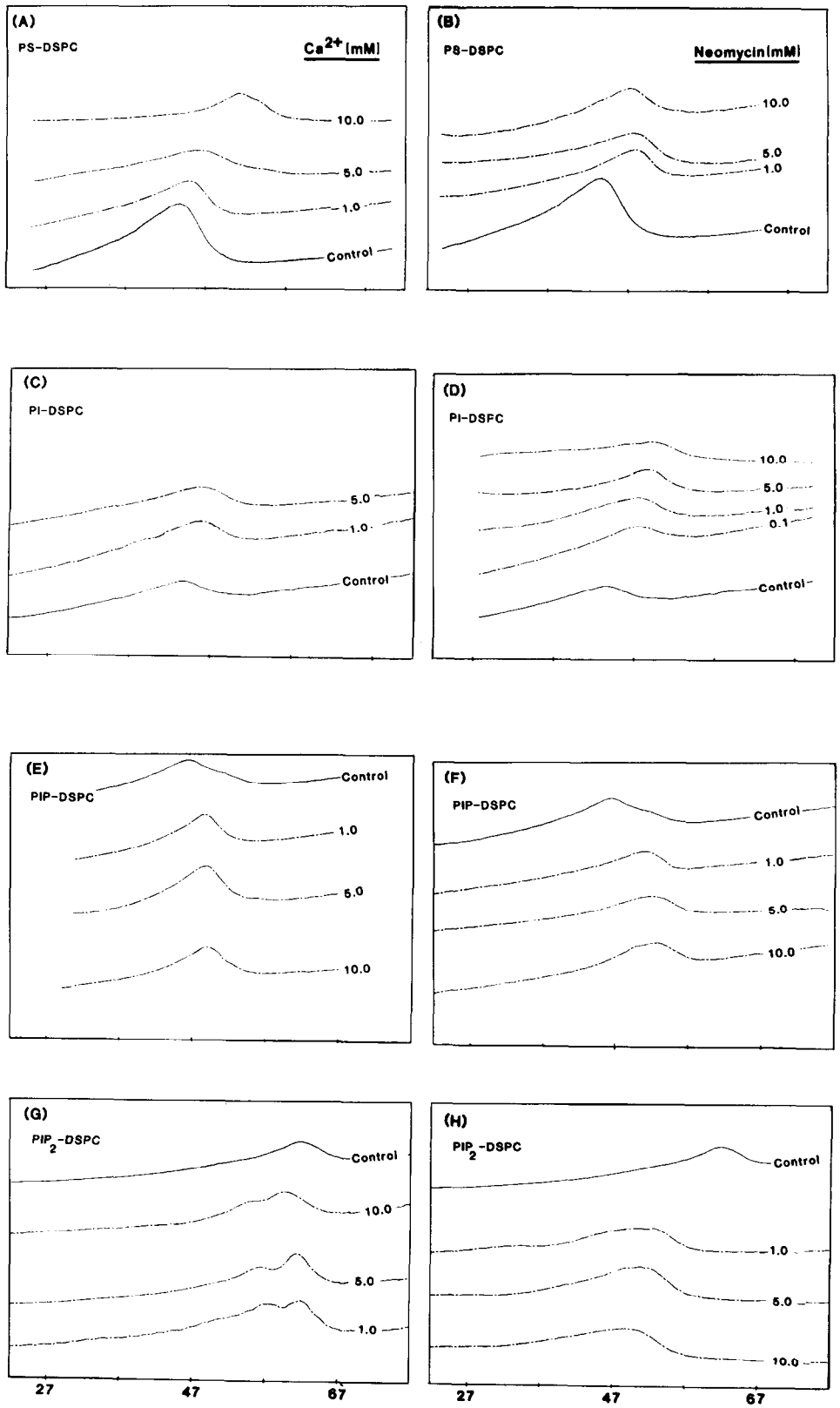

Fig. 1. Thermograms of mixed liposomes of acidic phospholipids and distearoyl L-α-phosphatidylcholine (1:1 molar ratio) incubated with various concentrations of Ca^{2+} or neomycin as described in Materials and Methods. Abbreviations: DSPC, distearoyl L-α-phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol bisphosphate; and Nm, neomycin B.

Table 2. Effect of Ca^{2+} and neomycin on phase transition of PI-DSPC liposomes

Concentrations	Transition temperature (T_m)(°)					
	PI-DSPC (2:1 molar ratio) (T_m = 38.7°)		PI-DSPC (1:1 molar ratio) (T_m = 43.9°		PI-DSPC (1:2 molar ratio) (T_m = 48.7°)	
	Ca^{2+}	Nm	Ca^{2+}	Nm	Ca^{2+}	Nm
0.1 mM		42.7		46.3		50.7
1 mM	40.4	43.6	45.0	47.4	49.3	52.0
5 mM	40.1	44.1	45.3	48.7	49.5	53.5
10 mM	41.7	45.3	45.4	49.2	49.4	54.1
1 mM Ca^{2+}/1 mM Nm	45.0		49.2		51.7	
5 mM Ca^{2+}/5 mM Nm	44.5		46.9		52.4	
10 mM Ca^{2+}/1 mM Nm	42.5		45.8		49.3	

Table 3. Effect of Ca^{2+} and neomycin on phase transition of PIP-DSPC Liposomes*

Concentrations	Transition temperature (T_m) (°)	
	Ca^{2+}	Nm
0.01 mM		46.6
0.1 mM	47.1	49.9
1 mM	48.5	50.4
5 mM	48.7	51.4
10 mM	48.9	52.1
1 mM Ca^{2+}/1 mM Nm	51.8	
5 mM Ca^{2+}/5 mM Nm	50.5	
10 mM Ca^{2+}/1 mM Nm	49.1	

* PIP-DSPC (1:1 molar ratio); T_m = 46.1°.

in T_m proportional to its concentration up to 10 mM (Fig 1, panels E and F).

The effect of Nm dominated that of Ca^{2+} when these ions were present in 1 mM or 5 mM concentrations each. The effect of Ca^{2+} predominated only when Ca^{2+} was added to a 10:1 excess (Table 3).

PIP_2-DSPC liposomes. The thermogram of the PIP_2-DSPC mixed liposomes (1:1 molar ratio) in the absence of Ca^{2+} (Fig. 1G) or Nm (Fig. 1H) was unlike that of the other anionic lipid-DSPC systems. The phase transition temperature was greater than that of either of its components (PIP_2 = <10°; DSPC = 56.7°).

All concentrations of Ca^{2+} tested resulted in the appearance of a double peak (Fig. 1G). The T_m of the first peak corresponded approximately to that of the untreated PIP_2-DSPC liposomes, and the other T_m value approximately to that of pure DSPC. The addition of neomycin (Fig. 1H) induced concentration-dependent downward shifts of the T_m. The effect of Nm dominated that of Ca^{2+} when both cations were present in equimolar (1 or 5 mM) concentrations (Table 4). Only in the presence of high Ca^{2+} (10 mM) and low Nm (1 mM) concentrations did the T_m approximate that observed with Ca^{2+} alone. However, no phase separation was seen in this case.

Preparation of pure PIP and PIP_2 is time consuming and costly. Since the effects of Ca^{2+} and Nm on the phase behavior of other mixed liposomes were essentially independent of the molar ratio, we initially limited experiments with PIP-DSPC and PIP_2-DSPC liposomes to 1:1 molar ratios. However, a few experiments were run with PIP_2-DSPC (2:1 molar ratio) liposomes. In both cases, the results were consistent: T_m was higher than those of the individual components and Ca^{2+} or Nm reduced T_m (Table 4).

Table 4. Effect of Ca^{2+} and neomycin on phase transition of PIP_2-DSPC liposomes

Concentrations	Transition temperature (T_m)(°)			
	PIP_2-DSPC (1:1 molar ratio) (T_m = 61.5°)		PIP_2-DSPC (2:1 molar ratio) (T_m = 62.6°)	
	Ca^{2+}	Nm	Ca^{2+}	Nm
0.1 mM	61.2	61.6		
1 mM	56.8, 61.5 (2 peaks)	52.6	56.8	43.6
5 mM	56.1, 61.5 (2 peaks)	50.8	56.4	42.2
10 mM	55.0, 60.5 (2 peaks)	48.5		
1 mM Ca^{2+}/1 mM Nm	52.3		41.5	
5 mM Ca^{2+}/5 mM Nm	50.9		40.7	
10 mM Ca^{2+}/1 mM Nm	60.4			

DISCUSSION

The thermotropic behavior of the PIP_2-DSPC liposomes differed from that of the other lipids tested. First, their T_m was greater than that of either of its components, a behavior which was not seen with any of the other mixtures. While unusual, a few lipid mixtures have been demonstrated to have a T_m higher than that of either component, possibly due to the formation of hydrogen bonds between available hydroxyl and the phosphate groups [13–15]. Similar hydrogen bonding may exist in PIP_2 as this lipid is not fully ionized at neutral pH due to the high surface charge density [16]. There is also some evidence that hydrogen bonding may occur between the hydroxyl groups in the inositol molecules [14]. Intermolecular hydrogen bonding between the phosphate group in DSPC and the phosphate groups and/or the hydroxyl groups in PIP_2 molecules is then possible. The existence of such bonds should raise the transition temperature since the gel state is more stabilized by these forces than is the liquid-crystalline state.

Second, the induction of the double peak by Ca^{2+} was seen in these liposomes only. This is consistent with previous findings that Ca^{2+} has a specially strong affinity to PIP_2 [16], and suggests that molecular segregation is occurring within the vesicles [17]. Other interpretations, however, cannot be ruled out. The downward shift of T_m induced by Nm is compatible with the results obtained from monolayer studies, which suggested the formation of a strong ionic complex between Nm and PIP_2 with a concomitant increase in surface pressure [5]. Similarly, proteins which bind primarily by ionic bonds to phospholipids and increase surface pressure of monolayers produce a substantial decrease in T_m [9].

In contrast to PIP_2, the behavior of other anionic lipids follows conventional patterns. Both Ca^{2+} and Nm increased T_m of mixed PS-DSPC liposomes. Nm appeared to be more effective at low concentrations (1 mM) whereas Ca^{2+} was far more effective at higher concentrations, particularly in PS-rich liposomes. Based on electrical considerations alone, it is not surprising that Nm (+6) affects the T_m of the negatively charged liposomes more than Ca^{2+}. The prevalence of Ca^{2+} at higher concentrations can be the result of a specific binding of Ca^{2+} to PS [18, 19]. In contrast, Nm was more effective than Ca^{2+} in both PI-DSPC and PIP-DSPC liposomes. This is consistent with results by Ohki *et al.* [20], who reported little or no phase separation for Ca^{2+}-treated PI-PC systems. Although Nm was more effective than Ca^{2+} at all concentrations tested, it should be pointed out that separation into a phase very rich in DSPC (appearance of a peak corresponding to pure DSPC) was not observed for either PI-DSPC or PIP-DSPC systems even at Nm concentrations of 10 mM.

The behavior of PIP_2-enriched liposomes may be the result of a unique interaction of Ca^{2+} or Nm with this lipid. The present study alone does not allow such a conclusion, as other interpretations are possible. For instance, since liposomes are multilamellar, it is unknown what fraction of the bilayer interacts with cations and whether this fraction is affected by the composition of the liposomes. However, if we consider our previous *in vivo* [4] and *in vitro* [5, 6] studies, the present results do confirm the hypothesis that PIP_2 occupies a unique role among acidic phospholipids in its molecular interactions with Ca^{2+} and with the aminoglycosides. What are the implications of such an interaction for a biological system? The Ca^{2+}-induced phase separation in PIP_2-enriched liposomes may be indicative of an important control mechanism in membranes. For instance, it can reduce the lateral diffusion in the aggregates and the transverse movement of molecules and ions across the membrane. Nm acted antagonistically and lowered T_m. This fluidizing effect of Nm and the antagonism to calcium may critically alter permeability and other properties of biological membranes. These results then support and extend our previous findings and conclusions that PIP_2 plays a crucial role in the expression of aminoglycoside toxicity.

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