Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 927-932

BINDING OF Ca^{2+} AND Mg^{2+} TO PHOSPHATIDYLSERINE VESICLES: DIFFERENT EFFECTS ON P-31 NMR SHIFTS AND RELAXATION TIMES

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Received April 23,1979

SUMMARY. Phosphorus-31 NMR lines corresponding to inner and outer surfaces of sonicated phosphatidylserine vesicles can be distinguished by the effects of added Ca^{2+} or Mg^{2+} at low bulk concentrations (millimolar or less). The changes in chemical shift and relaxation times indicate that Ca^{2+} binds directly to the PS phosphate, neutralizing at least a portion of the negative charge and restricting the motion of this group. Mg^{2+} ion also binds to the head group, but apparently not as strongly as Ca^{2+}, nor is the mobility of the headgroup affected as much.

INTRODUCTION

Calcium ion plays a key role in many processes involving model and biological membranes.[1,2] Binding, differential scanning calorimetry and x-ray diffraction studies[3,4] show that Ca^{2+} binds strongly and specifically to the headgroups of PS vesicles, forming a more tightly packed and highly ordered structure than do other cations such as Mg^{2+} and Na^{+}. The P-31 NMR results[5] obtained with equimolar PS-Ca^{2+} precipitated systems are consistent with the results cited above[1-4]: the P-31 NMR line is too broad to be observed, as would be the case if the Ca^{2+}-PS-phosphate complex had a rigid structure. Binding of Ca^{2+} to phophatidylcholine (PC) vesicles at Ca^{2+} concentrations several orders of magnitude greater than used in this study has also been reported,[6,7] but no change in the P-31 relaxation times was observed.[6] For the acidic lipid, phosphatidylglycerol (PG),[8] Ca^{2+} and Mg^{2+} gave essentially equivalent effects on the P-31 NMR. On the basis of conductivity measurements, it has been concluded[9] that Ca^{2+} and Mg^{2+} bind in a

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‡Abbreviations used: PS-phosphatidylserine; PC-phosphatidylcholine; PG-phosphatidylglycerol; EDTA-ethylenediamine tetraacetic acid.

0006-291X/79/110927-06$01.00/0

similar way to PS. We present here P-31 NMR results which show that Ca^{2+} binds strongly to the PS headgroup of sonicated vesicles at Ca^{2+} concentrations below that which causes aggregation or precipitation; Mg^{2+} also binds, but apparently less strongly, and with different effects on head group mobility. These differences between Ca^{2+} and Mg^{2+} correlate with their relative ability to produce fusion in PS vesicles.[1,2]

MATERIALS AND METHODS. Dispersions of sonicated unilamellar PS vesicles (15 to 30 mM in lipid) were prepared as described elsewhere[1] in a 90% H_2O-10% D_2O buffer containing 0.1M NaCl, 2 mM L-histidine, 2 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid and 0.1 mM EDTA, adjusted to pH 7.4. Equilibrium dialysis was used to introduce Ca^{2+} or Mg^{2+} at a specified bulk concentration in the sample solution; since the volume of dialysate was several hundred times greater than that of the sample being dialyzed (1.5 ml) and since the dialysate solution was changed several times during the course of a four-hour dialysis, the concentration of free Mg^{2+} or Ca^{2+} in the sample solution could be taken as that in the dialysate. Samples were run in a nitrogen atmosphere. Phosphorus-31 FT-NMR spectra were taken at 40.5 MHz in quadrature phase detection mode on a Varian XL-100, NTC TT-100 NMR spectrometer at 34±1C. Spin-lattice relaxation time measurements employed a standard 180-τ-90 inversion recovery sequence[10] with at least 15 τ values and a delay between acquisitions of 10 s. Overlapping inner and outer lines were separated for the case of Ca^{2+} additions by subtraction of a scaled inversion recovery spectrum at a τ value corresponding to a null for the outer line. Line widths were determined from a fit of the separated lines to a Lorentzian line-shape function. Proton decoupling with square wave modulation was used.

RESULTS AND DISCUSSION

As Ca^{2+} or Mg^{2+} is added to a sample of PS vesicles, the P-31 line forms an upfield shoulder which continues to shift upfield with increasing metal ion concentration, so that eventually two lines become partially resolved (at about 0.4 mM Ca^{2+}, 1.0 mM Mg^{2+} bulk concentration). The approximate ratio of integrated intensities, lowfield line (unshifted) to upfield, is 1 to 1.6-2.2, at metal ion concentrations where the two lines are partially resolved. At 0.5 mM Ca^{2+} concentration (or greater) aggregation occurs, whereas the Mg^{2+} concentration can be increased to 2 mM without apparent signs of aggregation. Addition of excess EDTA to the sample (at about two-fold times the amount of free metal ion present) reverses the shift for both added Ca^{2+} and Mg^{2+}, so that only one line is observed, with chemical shift and relaxation times corresponding to those before metal ion is added. In Table 1 the chemical shift differences are given for various metal ion and lipid concentrations.

Inversion recovery spectra are shown in Figure 1a-c for 0.4 mM bulk Ca^{2+} concentration in Figure 1d for 2.0 mM bulk Mg^{2+}. It is evident from the Figure that the effects of Ca^{2+} and Mg^{2+} are qualitatively different: in the case of Mg^{2+}, the relaxation times of shifted and non-

TABLE 1

Phosphorus-31 Chemical Shift Differences between "Inner" and "Outer" Phosphate Groups of Phosphatidylserine Vesicles[a]

Concentration (millimolar)			Shift (ppm)[b]
Ca^{2+}	Mg^{2+}	PS	
0.1[c]	-	15	.074
0.2[c]	-	15	.14
0.4	-	15	.34
0.4	-	30	.34
0.4	-	40	.35
-	1.0	40	.40
-	2.0	40	.53
-	2.0	20	.70

a For sonicated (unilamellar) vesicles at an ambient temperature 34 ± 1 C, under experimental conditions as given in the text.

b Shift with respect to unshifted downfield line.

c Peaks not resolved; shift is that of the composite peak from the line position in the absence of Ca^{2+} or Mg^{2+}.

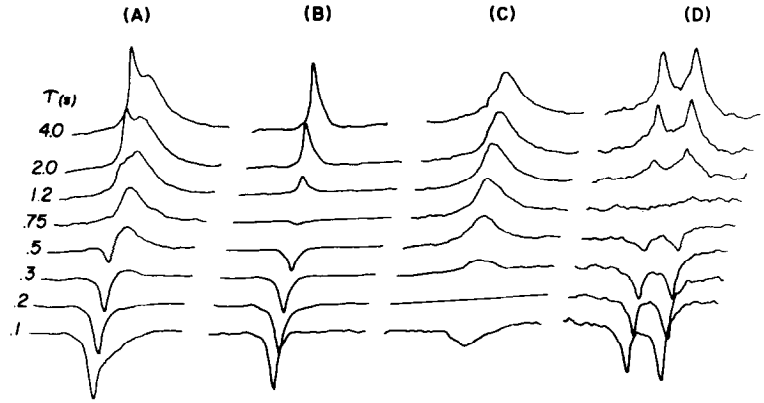

Fig. 1. Phosphorus 31 NMR Inversion Recovery (180-τ-90) Spectra of PS vesicles with bound Ca^{2+} or Mg^{2+}:
(A) "Inner" and "Outer" lines for samples 15 mM in PS, 0.4 mM Ca^{2+}.
(B) "Inner" line for 15 mM PS, 0.4 mM Ca^{2+}, obtained by subtraction of 1c (outer line) from spectra 1a at corresponding τ values.
(C) "Outer" line for 15 mM Ps, 0.4 mM Ca^{2+}, obtained by subtraction of scaled spectra at τ = 0.2s in 1a from those at other τ values in 1a.
(D) "Inner" and "Outer" lines for 30 mM PS, 2.0 mM Mg^{2+}.

TABLE 2

P-31 NMR Relaxation Rates for PS Vesicles in the Presence of Ca^{2+}, Mg^{2+}

Concentration (mM)			$1/T_1$[a] (s^{-1})		$1/T_2$[b] (s^{-1})	
PS	Ca^{2+}	Mg^{2+}	inner	outer	inner	outer
15	0	0	- 0.529[c] -		- 30[c] -	
15	0.40	0	0.83	3.3	22	71
30	0.40	0	0.85	2.8	30	71
30	0	2.0	0.85	0.93	$3x10^1$[d]	$3x10^1$[d]

a Determined as indicated in text from a non-linear least squares analysis of 15 inversion recovery points: inner and outer lines were separated by subtraction of a scaled inversion recovery spectrum at the null point for the outer line (see Fig. 1a-c).

b Determined from a least squares fit of line shape to a Lorentzian line-shape function.

c Inner and outer lines not resolved in absence of Ca^{2+} or Mg^{2+}.

d Linewidths and $1/T_2$ values not determined accurately because of overlap of inner and outer lines.

shifted lines are essentially equal, whereas for Ca^{2+} both T_1 and T_2 of the upfield line are considerably shorter than those of the non-shifted line. Values of the relaxation rates are given in Table 2.

The line which shifts to higher field corresponds, presumably, to phosphate groups on the outer surface of PS vesicles. The ratio of integrated intensities cited above is in rough agreement with other estimates[6,11] of the inside/outside ratio for a variety of sonicated phospholipid preparations. Also, it has been shown that PS vesicles remain impermeable to Na^+ (and, presumably, other ions) and do not fuse or aggregate at the Ca^{2+} or Mg^+ concentrations used in these experiments,[1,4] consistent with the above interpretation and our results. The contribution of slow or intermediate exchange effects to the spin-spin relaxation rates of the outer line for added Ca^{2+} cannot be rigorously excluded on the basis of data presented here. However, one might expect such exchange effects possibly to broaden the "inner" line and to give the inside-outside shift difference a strongly non-linear dependence on Ca^{2+} and PS concentration, which effects are not observed.

The upfield direction of the shift on Ca^{2+} (or Mg^{2+}) complexation is consistent with partial neutralization of the phosphate group; for O-phosphoserine, the P-31 line shifts upfield by about 4 ppm for a pH change of 8.6 to 3.9.[12] It should also be noted that the bound di-

valent metal ion, M^{2+}, displaces some bound Na^+. In the absence of M^{2+}, with 0.1 M Na^+ present in bulk solution, approximately 60% of the external PS binding sites are associated with relatively tightly bound Na^+.[13] Thus the magnitude of a "charge neutralization" shift depends on whether bound Na^+ or M^{2+} are associated with PS carboxylate or phosphate groups and the stochiometry of the complex. From Table 1 one can infer that 0.4 mM Ca^{2+} produces about the same chemical shift difference as that extrapolated for 0.8 mM Mg^{2+}. The values[3,14] of the ratio, bound divalent cation/total PS, are roughly the same, 0.3, at these respective Ca^{2+} and Mg^{2+} concentrations (0.4 and 0.8 mM), in agreement with the equality of shift differences.

The greatly increased relaxation rates of phosphate complexed to Ca^{2+} suggest that the correlation times for the random motions important in the relaxation mechanisms* have also increased, as would be the case if the Ca^{2+}-phosphate complex were more rigid than uncomplexed phosphate. Such greater rigidity argues for a bidentate or multidentate structure for the complex, with either two phosphates or a phosphate and a carboxylate acting as the ligating groups to Ca^{2+}. The difference between our results and those found for the interaction of PG and Ca^{2+},[8] suggests that the additional binding of Ca^{2+} in PS is by carboxylate groups. The spin-lattice relaxation rate of outer phosphorus also increases for added Mg^{2+}, but the effect is not nearly as great as for Ca^{2+}. The spin-lattice relaxation rates of the inner lines are, for both added Ca^{2+} and Mg^{2+}, greater than that of the single line observed in the absence of these ions. This result may reflect an indirect effect of bound metal ion, or the true relaxation rate of the inner line, unmasked by shift of the outer.

ACKNOWLEDGEMENTS

Support of this work by the National Institutes of Health (Grant No. GM 18921 to D.P. and Grant No. GM 23850 to S.N.) and the National Science Foundation (Grant No. CHE 7506183-A02 to the Department of Chemistry, State University of New York at Buffalo, for the NMR instrumentation used) is gratefully acknowledged.

*It should be emphasized that the mechanisms, types of random motion and correlation times will not be the same for P-31 spin-spin and spin-lattice relaxation. At the fields used in this work spin-spin relaxation will involve a mixture of dipolar and chemical shift anisotropy mechanisms, whereas spin-lattice relaxation will involve only a dipolar mechanism.[15]

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