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INTERACTION OF DIVALENT CATIONS AND PROTEINS WITH PHOSPHOLIPID VESICLES

STEPHEN WAGNER, ALEC KEITH and WALLACE SNIPES

Biophysics Laboratory, The Pennsylvania State University, University Park, PA 16802 (U.S.A.)

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Summary

The broadening of spin-label absorption lines resulting from spin-exchange reactions that occur during collision with paramagnetic Ni^{2+} is diminished when Ni^{2+} binds to phospholipid vesicles. Subsequent addition of non-paramagnetic ions that compete for binding sites releases Ni^{2+} into solution and restores the line-broadening. The concentrations of various ions required to achieve this effect was used to order the ions with respect to their binding to vesicles containing phosphatidylethanolamine and phosphatidylglycerol. The relative strengths of binding for those ions studied were: $Ca^{2+} > Mg^{2+} > Zn^{2+} > Sr^{2+} > Ba^{2+}$. The spin-broadening assay was also used to study the effects of two proteins on the availability of Ni^{2+}-binding sites on the vesicles. Ribonuclease, which is thought to associate electrostatically as an extrinsic protein on the surface of vesicles, completely blocked the Ni^{2+}-binding sites at comparatively low protein concentrations. Quantitative considerations of these data suggest the possibility that Ni^{2+} may bind preferentially to phosphatidylglycerol, and that these binding sites are aggregated in the ribonuclease-containing vesicles. In contrast to ribonuclease, cytochrome *c* does not block Ni^{2+}-binding sites on the phospholipid vesicles, but rather contains sites of its own that bind Ni^{2+}, both when the protein is in solution and when it is associated with the vesicles. These results are consistent with other studies which suggest that cytochrome *c* becomes partially embedded in membrane bilayers and associates with phospholipid molecules through hydrophobic interactions.

Abbreviation: TEMPONE, 2,2,6,6-tetramethylpiperidone-*N*-oxyl.

Introduction

The interaction of divalent cations with model and cellular membranes often has a pronounced effect on the physical and/or biological properties of the membrane. The binding of Ca^{2+} and Mg^{2+} to negatively charged phospholipid vesicles increases the cooperative sol to gel phase transition temperature of the fatty acyl chains [1,2]. Ca^{2+} triggers lateral phase separations in bilayers that contain a mixture of neutral and negatively charged phospholipids [3,4], and appears to play an essential role in the fusion of negatively charged phospholipid vesicles [4,5]. Ca^{2+}, Sr^{2+}, Ba^{2+} and Mn^{2+} promote the fusion of cell membranes induced by the hemagglutinating virus of Japan [6].

To further elucidate the nature of metal ion interactions with cell components, we have developed a spin-label assay for metal ion chelation and complex formation [7]. This assay is based on the broadening of nitroxide spin-label lines caused by spin-exchange interactions that occur upon collision of the spin label with paramagnetic ions and their complexes. Its advantages in comparison to other conventional assays for metal ion coordination are that the analysis is rapid, easy to perform, does not require that preparations be optically clear, crystallizable, or chemically defined, and is sensitive enough to detect 0.4 to 1.0 μmol of coordinating ligand. These characteristics make the technique particularly suitable for the study of biological membrane systems.

In this report, we have used the spin-label assay to investigate the interactions of divalent ions with membrane vesicles. Competition experiments were used to order the strength of binding of several divalent ions to phospholipid vesicles containing phosphatidylglycerol and phosphatidylethanolamine. In addition, the effects of exogenously added proteins on the bindings of certain metal ions to membrane vesicles were studied. These results are interpreted in terms of the various mechanisms whereby different types of proteins are thought to associate with phospholipid bilayers.

Materials and Methods

Spin-labels. The water-soluble spin label, TEMPONE (2,2,6,6-tetramethylpiperidone-*N*-oxyl), was synthesized by using the procedure of Rosantsev [8]. The fully deuterated compound, [^{2}H]TEMPONE, was synthesized from deuterated acetone and ammonia. Deuteration reduces the minimum linewidth of TEMPONE in dilute solutions by diminishing dipolar interactions of the unpaired electron with protons on the spin-label molecule. The deuterated spin label was used in the chelation assay because the narrower minimum linewidth of its electron spin resonance (ESR) spectrum improves the sensitivity for detecting line-broadening due to electron-spin exchange. Another isotopic form of the spin label, [^{15}N]TEMPONE, was used as a reference sample to monitor spectrometer sensitivity. The synthesis and spectral characteristics of [^{15}N]TEMPONE have been reported [9].

ESR measurements and linewidth determinations. ESR spectra were recorded at X-band microwave frequencies with a JEOL spectrometer, model JES-ME-1X. Sample temperature was controlled to 25 ± 1°C with a temper-

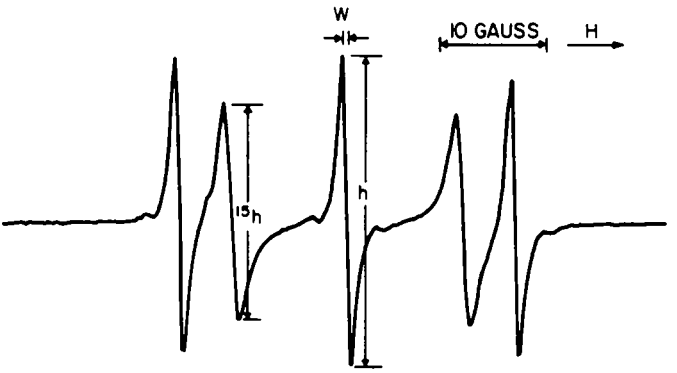

Fig. 1. First-derivative ESR spectrum of a composite sample of [^{2}H]TEMPONE and the [^{15}N]TEMPONE standard. The spectral parameters used in calculating W_{ex} from Eqn. 1 are shown.

ature-control unit constructed in our laboratory. Linewidth determinations were made by measuring lineheights rather than linewidths on a first-derivative ESR spectrum, and relating the two via the equation, $A = kW^2h$, where A is the area underneath an absorption line, W and h are the width and height, respectively, taken peak-to-peak on a first-derivative spectrum, and k is a constant. In comparing different spectra, it is essential to know the spectrometer gain and sensitivity to a high degree of accuracy. To monitor these parameters, a reference sample of [^{15}N]TEMPONE in a small capillary was placed in the cavity along with the experimental sample and served as an internal standard. Fig. 1 shows a composite spectrum of the reference and a sample of [^{2}H]-TEMPONE, and indicates the parameters used in linewidth determinations. As described previously [7], the linewidth (W_{ex}) for an experimental sample under conditions where spin-exchange broadening is present can be expressed in terms of the initial linewidth (W_{in}) for the same spin label in the absence of a broadening agent by the equation:

$$W_{ex} = \left[\frac{h_{in}}{h_{ex}} \cdot \frac{^{15}h_{ex}}{^{15}h_{in}}\right]^{1/2} W_{in} \qquad (1)$$

In this expression, h_{ex} and h_{in} are the midfield lineheights of the sample under investigation in the presence and absence, respectively, of added line-broadening agent. $^{15}h_{ex}$ and $^{15}h_{in}$ refer to the lineheight of the [^{15}N]TEMPONE reference for the corresponding composite spectra. We have found that this procedure allows the determination of values for W_{ex} that are reproducible to an accuracy of ±5%.

Vesicle preparation. The vesicles used in these studies were prepared from the phospholipids of *Pseudomonas* BAL-31. The phospholipids of this organism are composed of approx. 75% phosphatidylethanolamine and 23% phosphatidylglycerol, with only traces of other species [10]. The predominant fatty acyl chains [11] are palmitoleate (52%), palmitate (20%) and oleate (13%). Lipids were extracted by using the procedure of Folch et al. [12], dried under nitrogen, weighed, and taken up as vesicles in aqueous solution by vortexing.

The vesicle preparations contained approx. 100 mg phospholipid per ml solution.

Protein-containing vesicles were prepared by including either cytochrome *c* (Sigma Chemical Co., St. Louis, MO) or ribonuclease (Nutritional Biochemicals Corp., Cleveland, OH) in the solution used to take up the dried phospholipids. The vesicles were pelleted by centrifuging at 30 000 rev./min for 2 h in an SW41 Ti rotor. The amount of liposomal-associated protein was determined by measuring the protein remaining in the supernatant after centrifuging.

Results

Competitive binding of divalent ions to phospholipid vesicles

We have observed that the degree to which Ni^{2+} broadens spin-label lines is markedly dependent on the state of chelation or complexation of the ion [6,12]. This effect of ion binding on spin-exchange broadening was used to monitor the interaction of Ni^{2+} with phospholipid vesicles and the subsequent reduction of this interaction upon addition of varying concentrations of competing ions. Table I gives data for the linewidth of [^{2}H]TEMPONE under various conditions that illustrate the nature of the assay. In aqueous solution at 2.0 mM, the linewidth of this spin label is approx. 0.40 G. This value defines the initial linewidth, W_{in}, in Eqn. 1 and is used in determining values of W_{ex} for the additional preparations. $NiCl_2$ at 18 mM broadens the [^{2}H]TEMPONE line to approximately 2.48 G. The fully hydrated Ni^{2+} is more effective at line broadening than any chelate or complex of nickel that we have studied. The complete hydration of Ni^{2+} is a slow process, requiring several hours to more than a day to come to completion, and care was taken to use solutions of $NiCl_2$ prepared well in advance.

The addition of phosphatidylethanolamine/phosphatidylglycerol vesicles to the preparation of [^{2}H]TEMPONE and $NiCl_2$ reduces the extent of line-broadening to 1.60 G. We interpret this result as evidence that Ni^{2+} is bound to the phospholipid vesicles and is therefore less efficient at spin-exchange reactions compared to the free ion. This could be due to restricted diffusion of the ion, as well as a reduced probability of spin exchange per collision with [^{2}H]TEMPONE for the ion in the bound state. The phospholipid concentra-

TABLE I

ESR LINEWIDTH OF 2 mM [^{2}H]TEMPONE IN AQUEOUS SOLUTIONS WITH AND WITHOUT Ni^{2+}, PHOSPHOLIPID VESICLES AND Ba^{2+}

Linewidth was measured for [^{2}H]TEMPONE alone as the peak-to-peak separation on expanded first-derivative spectra. Calculated for all other preparations from Eqn. 1, using lineheight measurements. The linewidth was independent of the order in which the various components were added to the system.

[^{2}H]TEMPONE (mM)	$NiCl_2$ (mM)	Phospholipids (mg/ml)	$BaCl_2$ (mM)	Linewidth * (G)
2	—	—	—	0.40
2	18	—	—	2.48
2	18	100	—	1.60
2	18	100	100	2.44

tion of 100 mg/ml corresponds to approx. 130 mM for the phospholipids from *Pseudomonas* BAL-31. Thus, if both phosphatidylethanolamine and phosphatidylglycerol are capable of binding Ni^{2+}, there is an excess of ligands for the ions. The vesicles contain 23% of the negatively charged phosphatidylglycerol, and this species may bind Ni^{2+} more strongly. For the preparations used for the data of Table I, phosphatidylglycerol is present at about 32 mM. It should be pointed out, in making these mol ratio comparisons, that these vesicles do not appear to exclude Ni^{2+} so that the ion-phospholipid interactions exist at both the outer and inner surfaces of the bilayer. The evidence that Ni^{2+} is not excluded from the vesicles comes from the absence of any unbroadened component of [^{2}H]TEMPONE in the ESR spectra. Since the TEMPONE molecule can readily penetrate lipid bilayers, those molecules inside the vesicles would give rise to a spectral component with a linewidth of 0.4 G if Ni^{2+} were excluded from the vesicle interior. In some systems that exclude Ni^{2+}, a sharp, narrow spectrum is seen superimposed on the broadened TEMPONE component. No such component was observed for the vesicles used in this study.

If an excess of a competing, non-paramagnetic ion is added, the broadening due to Ni^{2+} is almost completely restored. The value of W_{ex} for the preparation used for Table I is 2.44 G when 100 mM Ba^{2+} is added. Ba^{2+} alone has no effect on the [^{2}H]TEMPONE linewidth. By measuring values of W_{ex} for different concentrations of the competing ions, it is possible to obtain information about the comparative ability of various non-paramagnetic ions to restore the Ni^{2+} broadening. Fig. 2 shows a graph of such data for Ba^{2+} and Ca^{2+}. By determining the concentration of various ions required to restore half the

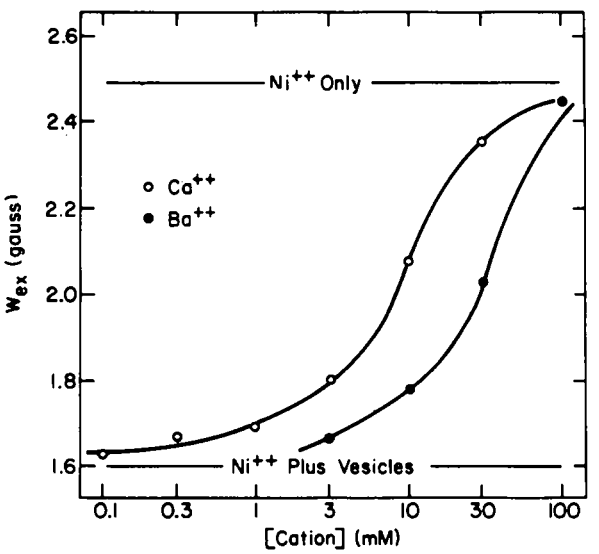

Fig. 2. Competition of Ba^{2+} and Ca^{2+} with Ni^{2+} for binding to phosphatidylethanolamine/phosphatidylglycerol vesicles. All samples contained 2 mM [^{2}H]TEMPONE, 18 mM $NiCl_2$, 100 mg/ml phospholipids, and the concentration of competing ion (as the chloride salt) shown on the horizontal axis. The value of W_{ex} with Ni^{2+} only in solution (no phospholipid) is indicated by the line at the upper portion of the graph, while the line at the lower portion gives the value of W_{ex} in the presence of Ni^{2+} and phospholipids but with no added competing ion.

TABLE II

CONCENTRATIONS OF VARIOUS NON-PARAMAGNETIC DIVALENT IONS REQUIRED TO RESTORE HALF OF THE BROADENING OF THE [^{2}H]TEMPONE LINE CAUSED BY 18 mM Ni^{2+} IN PHOSPHOLIPID VESICLE PREPARATIONS

Divalent ion	Concentration required (mM)	Normalized to 18 mM Ni^{2+}	Dissociation constant * (M)
Ca^{2+}	8.2	0.46	1.0
Mg^{2+}	8.8	0.49	1.0
Zn^{2+}	10.0	0.56	—
Ni^{2+}	—	1.00	1.2
Sr^{2+}	20.0	1.11	2.8
Ba^{2+}	30.0	1.67	3.6

* Taken from McLaughlin et al. [18].

Ni^{2+} broadening, we can order the non-paramagnetic ions with regard to the strength of their interaction with the phosphatidylethanolamine/phosphatidylglycerol vesicles. These data are given in Table II, where the values are also normalized to the concentration of Ni^{2+} (18 mM) against which the non-paramagnetic ions are competing in this experiment. It should be noted, however, that the magnitude of these normalized values will depend somewhat on the concentrations of phospholipids and Ni^{2+} in the preparations, although the relative ordering of the competing ions will not change.

Protein-containing vesicles

It is reasonable to expect that the interaction of divalent ions with phospholipid vesicles occurs at the polar region where the phospholipids interface with water. Therefore, proteins that associate with phospholipid bilayers may modify the binding of an ion such as Ni^{2+}, especially if the association is of an ionic nature. We examined the effects of two proteins, ribonuclease and cytochrome *c*, on Ni^{2+} line-broadening in preparations of phosphatidylethanolamine/phosphatidylglycerol vesicles and [^{2}H]TEMPONE. These two proteins were chosen because their interactions with phospholipid vesicles have been characterized by other techniques [14—17].

Table III gives data for various preparations containing 2 mM [^{2}H]-TEMPONE and 8 mM $NiCl_2$. The linewidth of 1.25 G at this Ni^{2+} concentration is reduced to 0.83 G upon addition of phosphatidylethanolamine/phosphatidyl-

TABLE III

EFFECT OF RIBONUCLEASE ON THE BINDING OF Ni^{2+} TO PHOSPHOLIPID VESICLES

All samples contained 2 mM [^{2}H]TEMPONE.

$NiCl_2$ (mM)	Phospholipids (mg/ml)	Ribonuclease (mg/ml)	W_{ex} (G)
8	—	—	1.25
8	100	—	0.83
8	100	9.5	1.23
8	—	9.5	1.24

TABLE IV

EFFECT OF CYTOCHROME *c* ON THE BINDING OF Ni^{2+} TO PHOSPHOLIPID VESICLES AND THE COMPETITION OF Ba^{2+} FOR Ni^{2+}-BINDING SITES

All samples contained 2 mM [^{2}H]TEMPONE.

$NiCl_2$ (mM)	Phospholipids (mg/ml)	Cytochrome *c* (mg/ml)	$BaCl_2$ (mM)	W_{ex} (G)
11	—	—	—	1.65
11	100	—	—	1.16
11	100	186	—	0.55
11	—	186	—	0.60
11	100	186	100	1.07
11	—	186	100	0.64

glycerol vesicles. However, vesicles that were prepared in the presence of ribonuclease, as described in Materials and Methods, gave a value for W_{ex} of 1.23 G, which is nearly the same as that for the spin label and Ni^{2+} preparation in the absence of phospholipids. We interpret these results to mean that the ribonuclease blocks those sites to which Ni^{2+} binds on the vesicle surface and leaves the Ni^{2+} in solution. As seen in the last line of Table III, ribonuclease alone has no effect on the broadening of [^{2}H]TEMPONE lines by 8 mM Ni^{2+}.

In a similar experiment, the effects of cytochrome *c* were investigated. In this case, however, the vesicles containing the protein were even more effective in reducing Ni^{2+} line-broadening that the vesicles alone (Table IV). This enhancement appears to be due to the binding of Ni^{2+} to cytochrome *c*, since the protein alone reduces the value of W_{ex} from 1.65 to 0.60 G. We also studied the competition of Ba^{2+} for binding to the cytochrome *c*-containing vesicles and the protein alone. For the vesicle preparation, 100 mM Ba^{2+} increased the value of W_{ex} from 0.55 to 1.07 G. This is probably due in large part to competition for Ni^{2+}-binding sites on the phospholipids rather than on cytochrome *c*, since 100 mM Ba^{2+} only increased W_{ex} from 0.60 to 0.64 G in the preparation containing protein alone. The inability of Ba^{2+} to dissociate effectively Ni^{2+} from cytochrome *c* could be due to several effects, including (a) a stronger binding of Ni^{2+} in comparison to Ba^{2+}, (b) a large excess of binding sites for divalent ions on cytochrome *c*, and (c) separate binding sites for the two ions.

Discussion

The binding of divalent ions to phospholipid vesicles has been studied by several physical techniques [18—20]. In a study of the electrophoretic mobility of phosphatidylcholine vesicles in the presence of various divalent cations, McLaughlin et al. [18] deduced dissociation constants for the ions based on certain assumptions. The results in the last column of Table II were derived by assuming that each phospholipid molecule occupies an area of 60 Å^2 in the bilayer and that the ions bind to the phospholipids with a stoichiometry of 1 : 1. In the present study, our ordering of the binding of these same ions to phosphatidylethanolamine/phosphatidylglycerol vesicles is in good agreement

with that of McLaughlin et al. [18], even though the stoichiometry in our preparations is different. Based on total phospholipids, the ratio of phospholipid per Ni^{2+} for the data of Table I is approx. 6.5 : 1. Based on the negatively charged phosphatidylglycerol species, the stoichiometry is about 1.5 : 1. At present, there has been no study to establish the comparative binding of divalent cations to negative and zwitterionic species in mixed vesicle systems, and no further quantitative treatment of our data can be made.

Papahadjopoulos and his coworkers have characterized the effects of several proteins, including ribonuclease and cytochrome *c*, on the thermal phase transitions of phospholipids. They found that ribonuclease does not alter the transition temperature for dipalmitoyl phosphatidylserine vesicles, but causes an increase in the heat of transition [17]. This is consistent with an electrostatic binding of the protein to the surface of the negatively charged vesicles. Other evidence consistent with a surface binding of ribonuclease to phospholipid interfaces is that the protein does not expand the surface area of phospholipid monolayers at the air/water interface [17], and only minimally increases the permeability of phospholipid vesicles [15,17]. Our results with the spin-broadening assay show that phosphatidylethanolamine/phosphatidylglycerol vesicles containing 8.8% ribonuclease by weight have essentially all the Ni^{2+}-binding sites blocked, again consistent with extrinsic, electrostatic binding of the protein to the vesicle surface.

There is further information that can be derived from our data, regarding the distribution of the Ni^{2+}-binding sites on the vesicle surface. If it is assumed that phospholipid and ribonuclease molecules have effective cross-sectional areas of 60 and 800 $Å^2$, respectively, then 8.8% ribonuclease should cover only about 7% of the vesicle surface. This suggests the possibility that the Ni^{2+}-binding sites are aggregated in the protein-containing vesicles to allow for such a large degree of masking of these sites by the protein. One possibility is that Ni^{2+} binding is preferentially to the negative phosphatidylglycerol molecules, and that ribonuclease induces a lateral phase separation of phosphatidylglycerol into domains that are then masked by the protein. It may also be that vesicle aggregation, which occurs to some extent with ribonuclease-containing vesicles, is partly responsible for the exclusion of Ni^{2+} from the vesicle surface.

In contrast to ribonuclease, cytochrome *c* has been found to decrease the thermal phase-transition temperature for hydrocarbon chains in phospholipid vesicles [14,17], to expand the surface area of phospholipid monolayers [17] and to increase the efflux of Na^+ through acidic phospholipid vesicles [15—17]. These data collectively suggest that cytochrome *c* is partially embedded in the bilayer of vesicles and interacts hydrophobically with phospholipids. Our results, which show that cytochrome *c* does not appear to block the Ni^{2+}-binding sites on the phospholipid vesicles, are consistent with this model. Furthermore, since the protein itself binds Ni^{2+} when either alone in solution or when incorporated into vesicles, the Ni^{2+}-binding sites on the protein are most likely on a portion of the molecule that is not embedded in the bilayer.

In summary, we have presented several examples of the usefulness of the spin-broadening assay for the study of metal ion interactions in phospholipid

vesicles. We anticipate that this approach may prove of value in studies with more complex systems, including intact cells.

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