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EPR study of spermine interaction with multilamellar phosphatidylcholine liposomes

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Abstract

The interaction of spermine with egg-yolk phosphatidylcholine liposomes was investigated. The EPR spin labeling technique evidenced that spermine induces modifications of some membrane functions of biological interest like water permeability and is a possible modulator of diffusion processes for charged and polar molecules. The association constant for a hypothesized complex between spermine and the phosphate group of phosphatidylcholine was evaluated by enzymatic methods.

Keywords: Liposome; Polyamine; Phosphatidylcholine; Multilamellar liposome; Spermine; EPR

1. Introduction

The naturally occurring polyamines (putrescine, spermidine and spermine) are ubiquitous metabolites of living cells and take part as biochemical regulators in several cellular processes [1]. At physiological pH polyamines are polycations known to bind to negatively charged molecules and macromolecules such as phosphate ions, nucleic acids and acidic phospholipids in membranes [2,3]. Systematic studies of the binding mechanism of spermine to mono and multilayer liposomes of different phospholipid composition have demonstrated that the binding strength depends on the acidic group type, being stronger, for example, for phosphatidate (PA) than for phosphatidylserine (PS) and phosphatidylinositol (PI) [4–8]; whereas no significant binding of polycations like spermine and gentamicin to zwitterionic lipids, e.g., phosphatidylcholine (PC), has been observed.

The specific adsorption of polyamine at the membrane surface can be explained by the Gouy-Chapman-Stern theory. In PC/PS liposomes spermine can interact with more than one negative acidic group forming higher order complexes (2:1); bridging complexes are also formed by spermine in aggregated liposomes, especially in PA [9,10].

In the present work the interaction of spermine with the zwitterionic egg-yolk phosphatidylcholine (EYPC) multilamellar liposomes has been evidenced and its association constant has been evaluated by enzymatic methods. An EPR (electron spin resonance), spin labeling technique was used to show how spermine attached to the membrane surface may affect some membrane properties, such as its permeability to water or accessibility to different ions.

As paramagnetic probes we used *n*-doxyl stearic acid spin labels (*n*-SASL, $n = 5, 7, 10, 12, 16$), with the nitroxide free radical group attached to the specified positions along the alkyl chain. In this way it is possible to monitor at atomic scale the depth dependence of membrane properties [11–15].

2. Materials and methods

2.1. Materials

Chemicals were analytical grade. *n*-doxyl stearic acid spin labels, spermine, horse radish peroxidase type II (HRP), *N*,*N*-dimethylalanine and 4-aminoantipyrine were purchased from Sigma. Egg yolk phosphatidylcholine (EYPC), with a purity approx. 99% by TLC measurements, was also purchased from Sigma.

Bovine serum amine oxidase (BSAO) was purified according to Vianello et al. [16]

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2.2. Liposome preparation

Multilamellar vesicles were prepared as reported by Kusumi et al. [17]. In particular, a mixture of EYPC (5 μmol), *n*-SASL (50 nmol) and spermine (3 μmol) in chloroform was dried with a stream of nitrogen gas and further dried under vacuum for at least 14 h. The dried lipids were suspended in 0.1 ml of Hepes 0.1 M buffer solution, pH 7.1, containing either 10 mM nickel chloride ($NiCl_2$) or 10 mM nickel iminodiacetic acid (Ni-IDA). In experiments at pH 3.5, citrate buffer 50 mM was used. At these pH values, spermine is completely protonated [18]. The lipid dispersion was warmed at about 40°C, mixed vigorously with a vortex for 30 s and then centrifuged at $12000 \times g$ for 15 min at 4°C. The loose pellets were used for EPR investigations. The samples for measurements at −140°C were prepared as above, at pH 7.1 without adding the paramagnetic broadening agents.

2.3. EPR measurements

EPR measurements were performed on a Bruker ER 200 D, 9 GHz spectrometer at microwave power range from 0.1 to 140 mW. Samples were placed in a gas permeable TPX tube 1 mm i.d. (Wilmad, NJ, USA) and centered in the resonant cavity, then deoxygenated under nitrogen flow. For saturation measurements the sample temperature was carefully controlled and kept constant at 25.1 ± 0.1°C. Low temperature experiments were made under gas flow from liquid nitrogen.

2.4. Order parameter

One important motional component in lipid bilayers is the 'wobbling' of the long molecular axis about a direction near perpendicular to the bilayer surface. Wobbling is more effective as we translate from the surface to the center of the bilayer, where the flexibility of the alkyl chains is larger. The increasing motional freedom is reflected in the EPR spectra of SASL's which change from the quasi rigid shape of the 5-SASL to the fluid-like shape of 16-SASL at the hydrocarbon end. The marked effect of the motion can be monitored by experimental parameters (Fig. 1) like the maximum splitting $2A'_{\parallel}$ or the order parameter S, as defined by: $S = 0.5407 \times (3(A'_{\parallel} - A'_{\perp})/(A'_{\parallel} + 2A'_{\perp}))$ [12,19,20].

2.5. Hydrophobicity

The values of the hyperfine splitting parameters of nitroxide groups increase with the polarity of the solvent. A direct insight into the hydrophobic barrier in membranes can be obtained by measuring the hyperfine splitting of *n*-SASL and displaying the polarity profile along the lipid chain. In a frozen sample at liquid nitrogen temperature the splitting between the outermost lines, $2A_{max}$, is nearly equal to $2A_{zz}$ and is used as an experimental parameter for monitoring the water penetration in membranes [11,12,15].

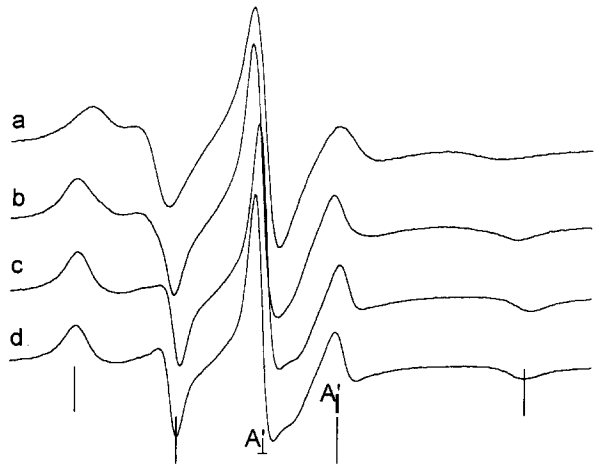

Fig. 1. EPR spectrum of 5-SASL in EYPC liposomes. (a) pH 3.5; (b) pH 7.1; (c) pH 8.1; (d) spectrum of EYPC liposomes plus spermine: the spectra at different pH are fully superimposable. The peak positions for calculating S are indicated. The central peak height is used for saturation measurements.

2.6. Accessibility

The accessibility of spin-labeled stearic acids in liposomes to paramagnetic agents can give information about the membrane permeability to different ions or uncharged molecules. When a nitroxide is exposed to collisions with a fast relaxing paramagnetic species such as molecular oxygen or transition metal ions, Heisenberg exchange occurs [13]. As a result, the spin-lattice (T_1) relaxation time of the nitroxide is shortened. The effective relaxation rate is linearly dependent on the concentration of the fast relaxing agent [r] through a constant k which is a measure of the nitroxide accessibility.

Continuous wave (CW) saturation experiments are sensitive to the product of both relaxation times: spin-lattice (T_1) and spin-spin (T_2), and the following equation holds [13,21]:

$$1/T_1T_2 = (1/T_1T_2)^\circ + (k/T_2^\circ) \cdot [r] \quad (1)$$

where the index ° refers to the relaxation times for the nitroxide in the absence of the fast relaxing agent.

For the systems of interest, T_2 is much shorter than T_1 and remains essentially unchanged by the presence of relaxing agents.

Eq. (1) can be rewritten as:

$$k' = 1/T_1T_2 - 1/(T_1T_2)^\circ \quad (2)$$

where:

$$k' = (k/T_2^\circ) \cdot [r] \quad (3)$$

can be used to monitor the local concentration of the relaxing agent, provided the motional conditions which affect k are not modified.

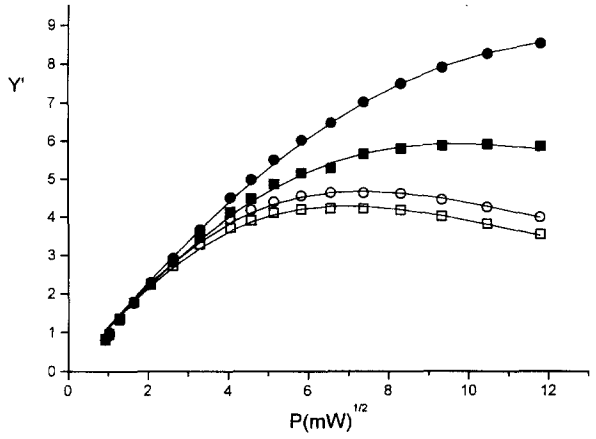

Fig. 2. Central peak height (Y' in arbitrary units) of the EPR spectrum plotted versus the microwave power (mW) square root. The saturation curves of 5-SASL in EYPC liposomes are obtained in presence (●) and in absence (○) of Ni^{2+}. Curves for the same conditions when spermine is added to liposomes are also shown (■, □). The continuous lines are the fittings from Eq. (4).

Assuming a homogeneous Lorentian lineshape, the Y' peak to peak amplitude of the derivative spectrum is given by an expression of the form:

$$Y' = A\frac{\sqrt{P}}{(1 + c \cdot P)^{3/2}} \quad (4)$$

where P is the microwave power, A is a normalising factor and c is a parameter proportional to the T_1T_2 product.

The plot of Y' versus $\sqrt{P}$ represents the saturation curve for a given spin label (Fig. 2) from which the adjustable parameters A and c can be obtained by the best-fit procedures. In the discussion of experimental results we will use the accessibility parameter as defined by Eq. (2) with c in the place of the product T_1T_2.

2.7. Determination of the association constant

The analytical method originates from the specific action of the enzyme amine oxidase from bovine serum which catalyzes the oxidative deamination of spermine [22] according to the following scheme:

$$R - CH_2NH_2 + O_2 + H_2O \rightarrow R - CHO + NH_3 + H_2O_2$$

In a coupled enzymatic reaction, catalyzed by peroxidase from a horse radish, the produced hydrogen peroxide oxidizes a reduced colourless dye (*N,N*-dimethylalanine and 4-aminoantipyrine) to a spectrophotometrically detectable compound with high molar absorbance ($\epsilon = 1.2 \cdot 10^4$ M^{-1} cm^{-1}, referred to the H_2O_2 concentration, at 555 nm). Spermine is stoichiometrically oxidized and quantitatively monitored by the dye conversion into the coloured compound [23]. The method is characterized by sensitivity better than 0.5 μM.

In particular, after centrifugation of the liposomes dispersion prepared, as previously described, at different spermine concentration, an aliquot (30 μl) of the supernatant was added to a buffered solution (pH 7.2) of 4 mM *N,N*-dimethylalanine and 3 mM 4-aminoantipyrine containing 0.5 nmol of BSAO and 1 nmol of HRP. The spermine concentration was calculated from the absorbance value, recorded after 5 min of incubation at 37°C, and corrected by the blank. The concentration of the linked polyamine is then the difference between the concentrations of added and free spermine, without meaningful errors if we assume that the spermine concentration in the aqueous phase inside the vesicles is the same as in the bulk solution.

Measurements were carried out on a Beckman DU7 spectrophotometer equipped with a thermostatic quartz cell.

3. Results and discussion

3.1. EPR lineshapes and order parameter S

EPR spectra have been obtained for stearic acids, spin labeled at the 5th, 7th, 10th, 12th or 16th carbon position, incorporated in EYPC liposomes, with and without spermine, as described in sample preparation.

The line shapes of 5-SASL (Fig. 1) depend on the pH and are clearly modified in presence of spermine. The effect is still observed in the spectra of 7- and 10-SASL, but obscured by the tendency to overlap of the parallel and perpendicular components in the spectra of 12 and 16-SASL.

Table 1
Values of the order parameter S in EYPC liposomes for different positions of the spin label in the hydrophobic region of the bilayer

Spin label position	Order parameter S					
	pH 7.1		pH 3.5		pH 8.1	
n	a	b	a	b	a	b
5	0.610 + 0.003 [a]	0.615 + 0.005	0.53	0.62	0.62	0.62
7	0.53 +0.01	0.553 + 0.002	0.50	0.55	0.56	0.56
10	0.41 +0.01	0.443 + 0.008	0.41	0.44	0.43	0.43
12	0.28 +0.01	0.328 + 0.003	0.27	0.32	0.31	0.32

Liposomes were prepared at three pH values and S values obtained from liposomes without (a) and with spermine (b) are compared.
[a] σ_{n-1} from a minimum number of four spectra.

The values of the order parameter S for n-SASL's in EYPC liposomes are reported in Table 1. The S value for the 16-SASL is not reported for the almost fluid-like nature of its spectrum.

The line shapes of SASL's at pH 7.1 are explained by the superposition of two spectral components with slightly different hyperfine splitting. The spectrum structure is also evidenced by higher temperature measurements (35°C), where the two components are clearly resolved (data not shown).

The result is interpreted in terms of two anchoring sites in the polar region of EYPC bilayers for the carboxyl group of stearic acids [24]. The two sites are related to the possible forms of the carboxyl group: the unprotonated -COO^- group is anchored in the positively charged choline region, and the protonated -COOH group is anchored in the phosphate region by hydrogen bonds. The relative weight of the two components is, of course, dependent on the pH of the solution.

The pK_a of the carboxyl group in PC membranes is about 7 [25]; at pH 8.1 -COO^- is the prevalent form and the EPR spectrum displays only a little contribution from the protonated component, whilst, at pH 3.5, carboxyl is almost fully protonated. The two forms, and the sites they occupy, are well distinguished by their order parameter values (Table 1).

It must be stressed that at pH 3.5 more than 90% of the phosphate groups of phosphatidylcholine, whose pK_a lies around 1 [26], are still deprotonated and the whole molecule maintains its zwitterionic character.

At pH 7.1 both the carboxyl forms are present and produce a clear inhomogeneous broadening of the spectrum, whilst the S values still lie very near to the values displayed by the almost pure unprotonated form (pH 8.1).

When spermine is added to liposomes the spectrum at pH 8.1 loses the residual -COOH component, but, as evidenced by S values, is substantially unaffected by spermine. The spectrum at pH 7.1 loses its broadened shape and a slight change of S values is observed. This difference may simply be explained by the loss of the composite structure of the spectrum which, in the absence of spermine, led to an error in the calculating of S from the peak positions. The spectra at pH 7.1 and 3.5 become identical to the spectra at pH 8.1.

From the above experimental observations it is evident that spermine causes the complete annihilation of the spectral component relative to -COOH, whilst the -COO^- group is left unperturbed.

One possible explanation of this effect is that the -COOH polar groups of the stearic acid form hydrogen bonds with oxygen of the phosphate groups of PC. The bonding is broken by the stronger ion–ion interaction of the negatively charged phosphate group with the positively charged spermine, whose capability of forming complexes with phosphate has been previously reported [3], and the stearic acid is forced to leave its anchoring point. Because, as regards the EPR spectra in the presence of spermine, there is no difference between the anchoring points of the -COOH and -COO^- groups, it can be suggested that -COOH group bonds to the positive choline group by ion–dipole interactions.

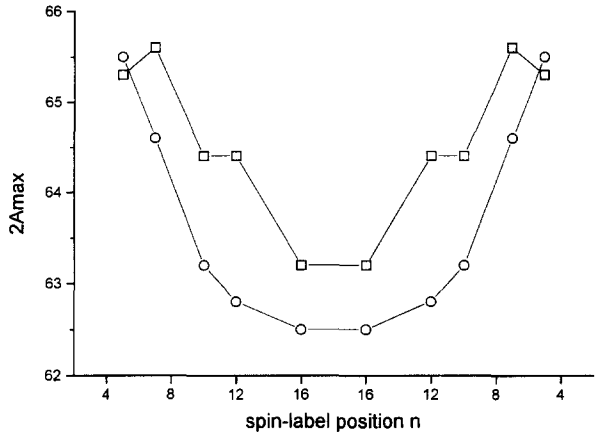

Fig. 3. Hydrophobicity profile. The values of $2A_{max}$ (Tesla·10^{-4}) are measured for different ($n = 5,7,10,12,16$) nitroxide positions in EYPC bilayers with (□) and without (○) spermine. Experiments were carried out at the temperature $T < -140$°C.

The same behaviour is alternatively explained by the complete deprotonation of the carboxyl groups of SASL's by spermine: the effect could be ascribed to the strong charge density, created by the four positive charges of spermine at the bilayer surface, which alters the local pH. Preliminary measurements at different spermine content show that, as expected, the relative weights of -COOH and -COO^- depend on the spermine concentration; in particular, the spectra of 5-SASL in liposomes prepared with 1 μmol of spermine at a bulk pH 3.5 are nearly equal to spectra of liposomes at pH 7.1 and prepared without spermine. From this point of view 1 μmol of spermine should produce an apparent pH shift of about +3 units. From rough application of the Gouy-Chapmann theory, such a shift cannot be excluded and the strong perturbation of the local pH could be a relevant effect of spermine on membrane properties.

3.2. Hydrophobicity and permeability

The plot of $2A_{max}$ measured at −140°C versus the carbon position number n (Fig. 3) is a good representation of the well known hydrophobic barrier in biological membranes [11]: lower values of $2A_{max}$ correspond to increased hydrophobicity and, in other words, to reduced water penetration. Hydrophobicity is lowered to some extent by the spermine bound to the bilayer surface; the effect is common to all position in the chain, indicating that water penetration extends up to the highly hydrophobic region at the center of the bilayer.

Spermine was also found to affect permeability of EYPC liposomes to ions and small polar molecules. We

monitored permeability to Ni^{2+} and to Ni iminodiacetic acid (Ni-IDA) using the accessibility parameter k' as defined in Eq. (2): results are plotted in Fig. 4. The accessibility profile of the charged ion Ni^{2+} roughly repeats the hydrophobicity profile, indicating that Ni^{2+} diffusion is limited to the water phase. In the presence of spermine the Ni^{2+} accessibility is strongly reduced, particularly near the surface. As there is no evidence for the reduction of water penetration from hydrophobicity data, we must ascribe the effect to an electrostatic barrier, assuming a positive charged sheet formed by the polycationic spermine bound to the bilayer surface. The situation is very different for Ni-IDA, a polar, neutral molecule. In pure EYPC, according to the k plot, Ni-IDA, like Ni^{2+}, does not enter significantly the hydrophobic region, while its accessibility profile is strongly modified when spermine is linked to EYPC liposomes, showing a clear accumulation, independent of the water content, inside the membrane. At this stage, the anomalous behaviour of Ni-IDA cannot be easily explained; we want just to note that similar data are reported by Snel [21], who observed a Ni-IDA partitioning between water and lipid phase and a consequent accumulation of the complex in DMPG liposomes, which present a net negative charge at the surface.

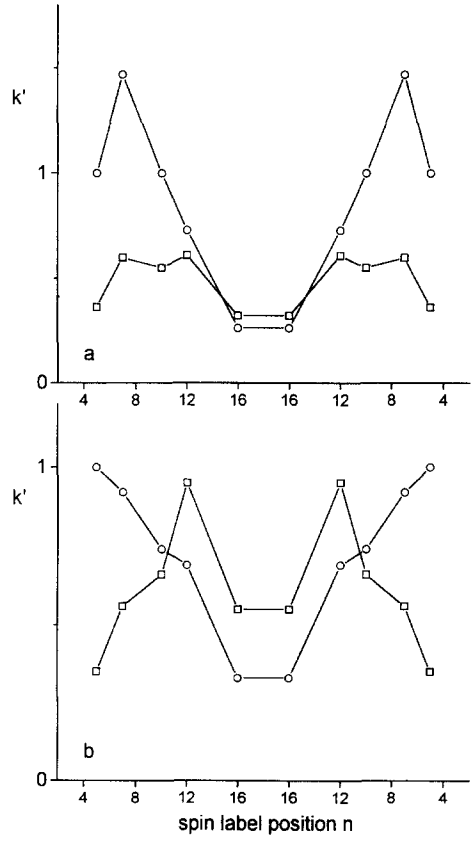

Fig. 4. Accessibility profile of n-SASL to differently charged paramagnetic agents. (a) Ni^{2+}; (b) Ni IDA. The constant k' is plotted versus the nitroxide position (n) in EYPC bilayers with (□) and without (○) spermine. The values of k' are in arbitrary units. Values of two plots must be not compared.

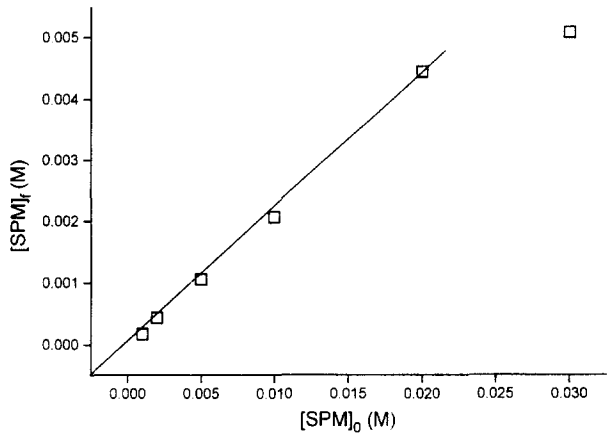

Fig. 5. Plot of the free spermine concentration $[SPM]_f$ in the supernatant versus the total spermine concentration $[SPM]_0$.

From this point of view Ni-IDA is not a good probe for discriminating between the aqueous and lipid phase [21], but is perhaps worth further investigating because its partitioning seems to depend strongly on the surface net charge, which makes it a possible probe for induced surface modifications.

3.3. Association constant

We further investigated the binding of spermine to multilamellar EYPC liposomes by measuring the concentration of free spermine in the supernatant of the centrifuged samples containing different amounts of polyamine.

Amine oxidase from bovine serum is not active on phosphate-linked spermine [3] and then, in a system with both linked and free spermine, BSAO catalizes only the oxidative deamination of the free one, allowing its accurate determination. From the plot (Fig. 5) of the free spermine concentration as a function of the initial spermine concentration $[SPM]_0$, a straight line was obtained for $[SPM]_0$ values in the range $1 \div 20$ mM. If we assume that the concentrations of free spermine $[SPM]_f$, phospholipid-bound spermine [SPM · EYPC] and phospholipid [EYPC] are in equilibrium with an 1:1 ratio between the zwitterionic EYPC and the four positive charges of SPM [2,8], i.e., SPM + EYPC ⇔ SPM · EYPC, an association constant of $91 \pm 12\ M^{-1}$ is found for our experimental conditions. This result is valid for samples with spermine contents lower than half of phospholipids amounts, i.e., $[SPM]_0 < 0.5 \cdot [EYPC]$. For higher concentrations, the association constant shifts towards higher values.

4. Conclusions

The aim of this work was to demonstrate an interaction, that was not evidenced, between spermine and zwitterionic phospholipids. The experimental results show that the

dynamical and saturation parameters of the EPR spectra of *n*-SASL in EYPC liposomes are modified by spermine, and indicate that spermine binds most likely to negatively charged phosphate groups in a possible competition with the internal electrostatic interaction between phosphate and a sterically hindered choline group. The association constant for the hypothesized reaction [SPM] + [PC] ⇔ [SPMPC] has been evaluated. EPR measurements have also shown that spermine induces modifications of some membrane functions of biological interest like water permeability, and is a possible modulator of diffusion processes for charged and polar molecules.

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