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A NUCLEAR MAGNETIC RESONANCE STUDY OF THE INTERACTION OF L-EPINEPHRINE WITH PHOSPHOLIPID VESICLES

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SUMMARY

Proton magnetic resonance has been used to measure the spin–spin relaxation times, T_2, of dispersions of phospholipid vesicles containing L-epinephrine. A decrease in T_2, as evidenced by a differential line broadening of the L-epinephrine resonance signals, is observed when the phospholipid is phosphatidylserine but is not observed with phosphatidylcholine or phosphatidylethanolamine. From these results the interaction is concluded to be predominantly ionic, and the stability constant for the L-epinephrine-phosphatidylserine complex has been estimated to be about $1 \cdot 10^3$ M^{-1}, assuming a 1:1 stoichiometry. The differential line broadening pattern also has provided information concerning the motional characteristics of the various groups of L-epinephrine in the complex.

INTRODUCTION

The hormone L-epinephrine (adrenaline) is known to stimulate the activity of the plasma membrane-bound enzyme adenyl cyclase[1]. The enhanced activity of adenyl cyclase resulting from this stimulation gives rise to an increased production of cyclic adenosine 3′,5′-monophosphate which in turn regulates the activity of a number of enzymes. Thus, this system provides a control mechanism whereby a relatively simple molecule such as L-epinephrine can regulate the activity of a number of enzymes[2]. In spite of the considerable importance of and current interest in the hormone–adenyl cyclase system, little information is available concerning the nature of the hormone–membrane interaction.

Phospholipids constitute a major proportion of most cell membranes and phospholipid bilayers have been used extensively as models for cellular membranes[3]. Sonicated aqueous dispersions of phospholipids spontaneously form closed concentric bilayers of phospholipid called liposomes[3]. Although in the intact membrane–adenyl cyclase system the hormone may, in fact, interact with a "receptor" protein located on the membrane, at present no direct experimental evidence is available with regard to the site of hormone binding. A study of the interaction of L-epinephrine with phospholipids (which, like proteins, contain charged and hydrophobic regions within the same molecule) may prove helpful in understanding the mode of interaction of the hormone with the intact system. Furthermore, hormone–phospholipid interactions may be important in the control mechanism of the adenyl cyclase system.

In this investigation, nuclear magnetic resonance (NMR) has been used to investigate the interaction of L-epinephrine with phospholipid vesicles. Spin–spin relaxation times for the L-epinephrine resonances have been evaluated from line width measurements. From the concentration and temperature dependence of these relaxation times the nature of the L-epinephrine–phospholipid interaction has been ascertained and the stability of the L-epinephrine–phosphatidylserine complex has been estimated.

EXPERIMENTAL METHODS

Materials

Chromatographically pure L-α-lecithin (phosphatidylcholine) isolated from chicken egg yolks, and phosphatidylethanolamine and phosphatidyl-L-serine from bovine brain were purchased from General Biochemicals and were used without further purification. Analysis of the phospholipids using previously described procedures[4] indicated only minor neutral lipid contamination. The solid compounds were stored at −10° and stock solutions of the phospholipids were prepared immediately before use.

The L-epinephrine was purchased from Sigma Chemical Co. and was used without further purification. Analysis by paper chromatography using a solvent system of phenol saturated with water and sulfur dioxide revealed no detectable impurities after spraying with a $K_3Fe(CN)_6$ visualization reagent[5]. Due to slow air oxidation of L-epinephrine to adrenochrome, stock solutions of L-epinephrine were prepared immediately before use.

The phospholipids and L-epinephrine were lyopholized twice from ^2H_2O to free these compounds of most of their exchangeable hydrogens. The ^2H_2O was purchased from International Chemical and Nuclear Corporation (99.75 %) and Mallinckrodt (99.8 %).

All other reagents used in this study were of commercial analytical grade.

Rat liver plasma membranes were prepared by the method of MARINETTI *et al.*[1] and RAY[6] and were suspended in ^2H_2O after repeated centrifugations and washings with ^2H_2O.

Preparation of solutions

Stock solutions of the phospholipids were prepared by adding the required amount of the solid compound to ^2H_2O and sonicating the solution at 0° under a stream of dry nitrogen for 20 min using a 20 kHz Heat Systems sonifier at a power output of 55 W. If necessary the p^{2}H of the resulting solution was adjusted to 7.4 with concentrated ^{2}HCl or anhydrous Na_2O_2. (The p^{2}H of a solution was taken to be the pH reading obtained from a Radiometer Model 26 pH meter and type GK2302C combination electrode standardized with buffers in H_2O *plus* 0.4 (ref. 7).)

Stock solutions of L-epinephrine were prepared by adding the required amount of the solid compound to ^2H_2O and adding an equivalent amount of concentrated ^{2}HCl. The p^{2}H was then adjusted to 6.4 with concentrated ^{2}HCl and anhydrous Na_2O_2 (solutions of L-epinephrine greater than 0.1 M precipitate above p^{2}H 6.4). A 1.0 M KCl solution was prepared by dissolving the required amount of reagent grade KCl in ^2H_2O. Phosphate buffer containing 0.1 M total phosphate was prepared by dissolving

anhydrous Na_3PO_4 in ^2H_2O and adding sufficient anhydrous $POCl_3$ to give a final p^2H of 7.4.

Solutions were prepared for measurement by pipetting together aliquots of phospholipid, L-epinephrine, KCl and phosphate buffer so as to yield the desired concentrations. All solutions were 0.1 M in KCl and 0.01 M in total phosphate buffer. The p^2H of all solutions was 7.4.

Proton magnetic resonance measurements

Samples prepared as described above were placed in NMR tubes obtained from Willmad Glass Co., type 506-PP. The NMR spectra were obtained on a Varian Associates A-60A or HA-100-IL spectrometer. When necessary, signal averaging was performed by means of a Varian C 1024 time averaging computer. Variable temperature experiments were carried out utilizing a Varian V-4343 variable temperature controller. The temperature of the probe was determined by measuring the chemical shift of either methanol or ethylene glycol. A coaxial insert containing tetramethylsilane provided a lock signal for the HA-100-IL and a reference signal for measuring chemical shifts. Spin–spin relaxation times, T_2, were computed from the high resolution spectra recorded on the HA-100-IL by measuring the width of the resonance signal at half height, $\Delta\nu_{1/2}$, and using the relationship $T_2 = 2/\Delta\nu_{1/2}$ (sec)[8].

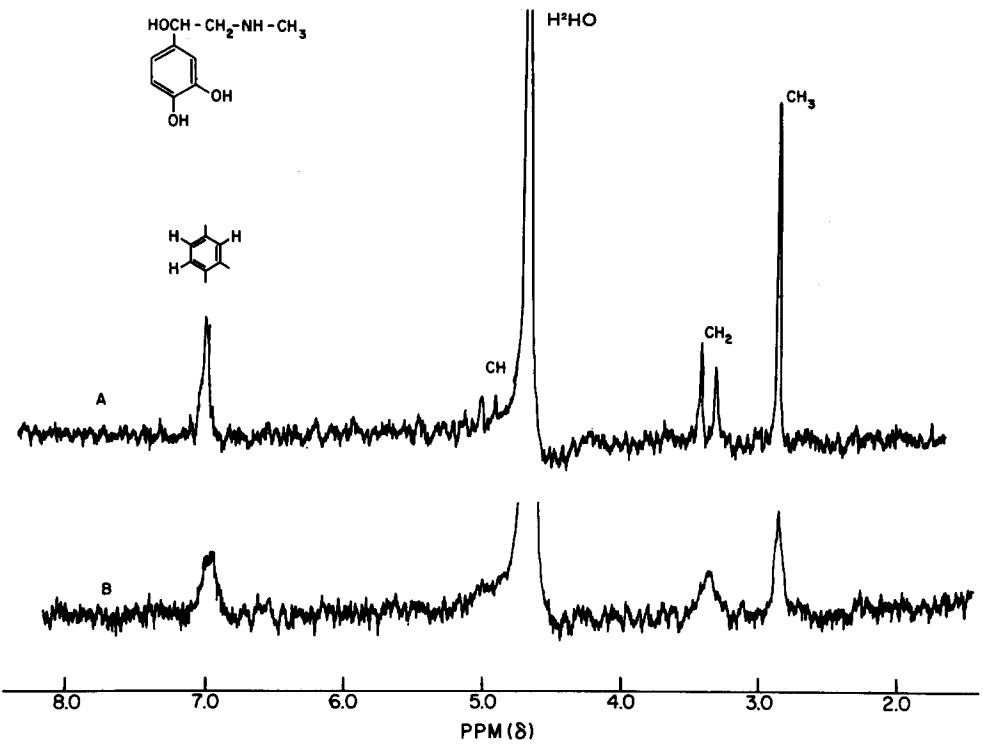

Fig. 1. The 60 MHz NMR spectra of (A) 0.05 M L-epinephrine and (B) a mixture of 0.05 M L-epinephrine and 2% phosphatidylserine. The solvent is ^2H_2O, p^2H 7.4 at 35°. The spectrum amplitude scale is the same for both spectra. The resonance signal due to phosphatidylserine in (B) occurs upfield at approx. 1.33 ppm.

RESULTS

The 60 MHz NMR spectra of free L-epinephrine and a mixture of L-epinephrine and phosphatidylserine are shown in Fig. 1 along with the peak assignments. The spectrum of L-epinephrine exhibits resonance at 2.85 ppm ($-CH_3$), 3.36 ppm ($-CH_2-$) and 7.00 ppm (aromatic protons)[9]. The triplet signal due to the methyne proton was obscured by the H^2HO resonance and could not be observed in the dilute solutions employed for this study. Under conditions for which appreciable line broadening is observed in phosphatidylserine–L-epinephrine solutions, no observable line broadening occurs in corresponding solutions containing either phosphatidylcholine or phosphatidylethanolamine. The resonances of L-epinephrine did not undergo any observable change in chemical shift in the presence of the phospholipids. However, in the case of phosphatidylserine, some mixtures gave very broad lines so that a small change in chemical shift would be difficult to detect.

To detect the presence of differential line broadening, the relative change in the spin–spin relaxation time, $1/T_2$, is plotted as a function of mole percent L-epinephrine for the resonance signals of L-epinephrine and the $-(CH_2)_n-$ signal of phosphatidylserine in Fig. 2. Rather than compare the absolute values of $1/T_2$, which reflect to a large extent the intrinsic differences in the proton relaxation of the different groups (methyl, methylene, phenyl, *etc.*), the relative change in relaxation time for each group is compared[10]. The relative relaxation time, $T_{2,0}/T_2$, is the ratio of the relaxation time of an 0.05 M solution at 30°, $T_{2,0}$, to the observed relaxation time of a mixture of L-epinephrine and phosphatidylserine of specified composition and temperature. Thus, an increasing value of $T_{2,0}/T_2$ reflects a decreasing spin–spin relaxation time and a resulting increase in line broadening. As shown in Fig. 2, the resonance signal of the methyl group is broadened to a greater extent than is the resonance line

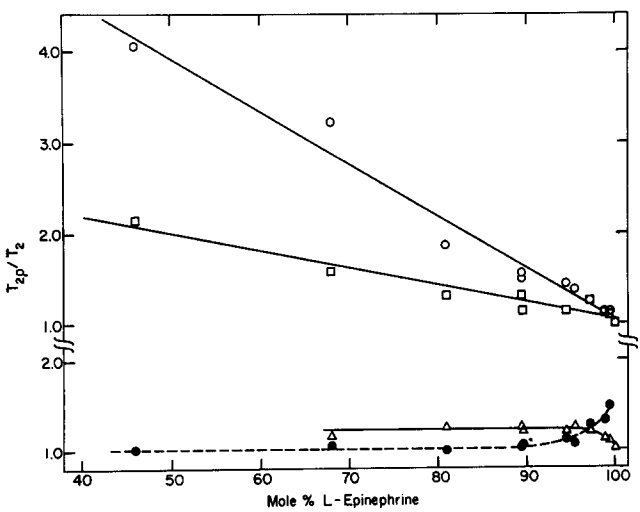

Fig. 2. Concentration dependence of the relative spin–spin relaxation times in L-epinephrine–phosphatidylserine mixtures: ○, $-CH_3$ of L-epinephrine; □, $-\varphi$ of L-epinephrine; △, $-CH_2-$ of L-epinephrine; ●, $-(CH_2)_n-$ of phosphatidylserine. The concentration of L-epinephrine ranges from 0.10 to 0.002 M and that of phosphatidylserine ranges from 2 to 0.5%. The data were obtained from spectra taken at 100 MHz, 30°. See text for definition of $T_{2,0}/T_2$.

of the aromatic protons. The broadening of the methylene protons is difficult to interpret since the resonance signal, originally a doublet in free epinephrine, collapses to a singlet in the presence of phosphatidylserine. Thus, the relaxation times reported in Fig. 2 for the methylene resonance were calculated from the total width of the doublet at half signal height. Since no chemical shifts are observed, this procedure permits comparison of the relative relaxation time of the methylene group. Once the doublet has completely collapsed (at approx. 97 mole % L-epinephrine), little or no additional broadening occurs, and the relative broadening of the methylene protons is less than that of the methyl or aromatic protons. The relaxation time of the methylene protons of phosphatidylserine is seen to remain constant until a large molar excess of L-epinephrine is added at which time the phospholipid resonance signal is observed to broaden slightly.

The temperature dependence of the relative relaxation times from 5° through 80° is shown in Fig. 3. Whereas the methylene protons appear to be relatively insensitive to temperature, those of the methyl group exhibit a strong temperature dependence and the aromatic protons exhibit a temperature dependence intermediate between those of the methyl and the methylene groups. A temperature variation experiment performed on a 0.02 M solution of free L-epinephrine resulted in a smooth gradual decrease in $T_{2,0}/T_2$ from 1.0 at 5° to 0.6 at 80°.

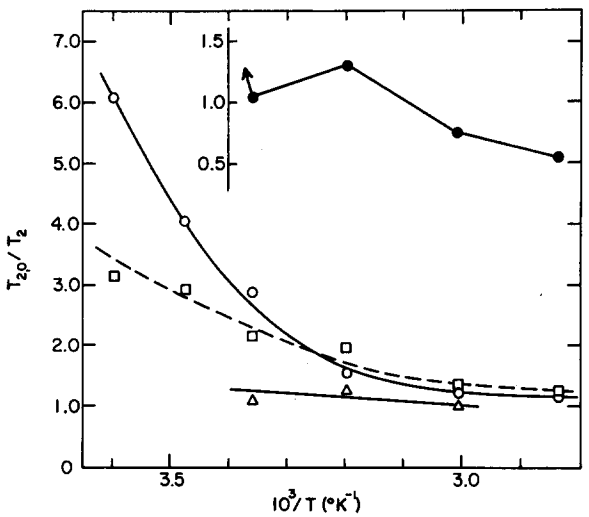

Fig. 3. Temperature dependence of the spin–spin relaxation times of an L-epinephrine–phosphatidylserine mixture: ○, $-CH_3$ of L-epinephrine; □, $-\varphi$ of L-epinephrine; △, $-CH_2-$ of L-epinephrine; ●, $-(CH_2)_n-$ of phosphatidylserine. The concentration of L-epinephrine is 0.02 M; the concentration of phosphatidylserine is 2 % ($2.35 \cdot 10^{-3}$ M). The date were obtained from spectra taken at 100 MHz. See text for definition of $T_{2,0}/T_2$.

DISCUSSION

The application of NMR spectroscopy to the study of intermolecular complex formation has been discussed elsewhere[8,10]. The formation of complexes can often be detected by observing the resulting changes in either the chemical shifts or the

relaxation times of the resonance signals in the NMR spectra of the interacting species. Chemical shifts are due to a change in the charge density at a given proton resulting from complex formation. However, quite often the protons in a complex may not experience appreciable changes in charge density but complex formation may still be detectable by broadening of the resonance lines resulting from a shortening of the spin–spin relaxation times. This shortening is primarily a reflection of the restriction of rotational freedom of certain groups stabilized by the interaction.

Several mechanisms could lead to broadening of the epinephrine magnetic resonance lines in L-epinephrine–phosphatidylserine solutions. In principle, line broadening could result from bulk viscosity changes, magnetic inhomogeneity, self-interaction of the hormone or intermolecular complex formation. The first two possibilities are clearly ruled out since very sharp resonance lines are observed in solutions containing phosphatidylcholine and phosphatidylethanolamine. Furthermore, the observed concentration and temperature dependencies of the relaxation times (Figs. 2 and 3) are not consistent with these general broadening mechanisms. Since no concentration dependent line broadening was observed in solutions of free epinephrine, self-interaction of the hormone can also be ruled out. Thus, the observed line broadening can be attributed to the formation of a phosphatidylserine–L-epinephrine complex.

The experimental evidence indicates a rapid exchange of L-epinephrine occurs between the free and the complex species. If the rate of exchange were slow, the sharp peaks of the free species would be superimposed on the broad peaks of the complex since no chemical shifts are detected. If the rate of exchange were of the order of the reciprocal relaxation times the broadening should show a dependence on the absolute concentrations of the L-epinephrine and phosphatidylserine[8]. The observed line broadening is dependent on the mole fraction of epinephrine (Fig. 2), but not on the absolute concentrations.

At a p^{2}H of 7.4, the L-epinephrine is protonated and is positively charged, phosphatidylserine is negatively charged, and phosphatidylcholine and phosphatidylethanolamine are neutral zwitterions[11]. The absence of a detectable interaction of L-epinephrine with the neutrally charged phospholipids is good evidence that the interaction of epinephrine and phosphatidylserine is predominately of an electrostatic nature. The methyl group attached to the nitrogen of L-epinephrine experiences the greatest broadening; therefore the epinephrine molecule appears to ionically bind to the phospholipid spherule in such a way that the rotational freedom of the methyl group is restricted. Alternatively, complex formation could cause a distortion of the electric charge on the nitrogen which could then give rise to selective broadening through scalar coupling between the nitrogen and the methyl protons. However, this phenomenon was not observed in the electrostatic interaction of epinephrine with adenine nucleotides[9] and probably is not occurring in this system. The methylene group of epinephrine appears to maintain a considerable degree of rotational freedom, whereas the phenyl protons are observed to undergo a slight broadening, possibly reflecting the participation of the phenyl hydroxyls in a hydrogen bonding interaction with the phospholipid. The phenyl hydroxyls of epinephrine are believed to be important in the epinephrine stimulation of adenyl cyclase activity[12].

The concentration dependence of the linewidth of the L-epinephrine methylene resonance signal is peculiar: the doublet collapses to a singlet in the presence of a

small amount of phosphatidylserine but then experiences no further broadening as the mole percent of L-epinephrine is decreased (Fig. 2). This collapse of the doublet may be due to an exchange reaction between the epinephrine methyne proton (=CH–) and ^2H_2O in the phosphatidylserine–L-epinephrine complex, thereby eliminating spin–spin coupling with the methylene protons. However, a definitive interpretation of this phenomenon cannot be given at the present time.

The mobility of the hydrocarbon tails of phosphatidylserine (–$(CH_2)_n$–) remains insensitive to the addition of L-epinephrine until a large molar excess of the hormone is added (Fig. 2). In the presence of a large excess of hormone, the phospholipid methylene resonance signal undergoes slight broadening. This phenomenon may result from a saturation binding of the hormone to the phospholipid spherules leading to charge neutralization within the liposome, which permits tighter packing of the phospholipid molecules within the liposome.

The above conclusions reached from the concentration dependence of the relative relaxation times are corroborated by the temperature dependence of these relaxation times (Fig. 3). As the temperature is increased, the spin–spin relaxation times approach the values observed for free epinephrine. Since the rate of exchange is rapid, this temperature effect reflects the dissociation of the complex at the higher temperatures. The temperature dependence of the phosphatidylserine methylene relaxation time is also shown in Fig. 3. At the lower temperatures the resonance signal is so broad that a reliable measurement of the relaxation time could not be made. As the temperature is increased, the signal line width decreases until at 80° a quite sharp signal is observed. This behavior has been previously observed in phospholipid dispersions[13,14] and is attributed to phase transitions which occur throughout this temperature range. The anomalous behavior at 40° is not presently understood but may be due to the existence of a phase transition at or near this temperature.

Since the L-epinephrine–phosphatidylserine complex appears to involve rapid exchange of the L-epinephrine, an apparent binding constant can be calculated from the relaxation time data using a treatment similar to that of FISHER AND JARDETZKY[15]. The fraction of bound epinephrine molecules, α, can be computed from the expression:

$$\alpha = \frac{1/T_{2m} - 1/T_{2f}}{1/T_{2b} - 1/T_{2f}}$$

where $1/T_{2f}$ is the reciprocal spin–spin relaxation time of the free epinephrine, $1/T_{2b}$ is the reciprocal relaxation time of the complexed epinephrine, and $1/T_{2m}$ is the reciprocal relaxation time of a mixture of epinephrine and phosphatidylserine of known composition. The value of $1/T_{2f}$ can be obtained by measuring the line width of the appropriate resonance signal of free epinephrine. The value of $1/T_{2b}$ has been estimated from the intercepts of the least squares lines through the points of Fig. 2 at zero mole percent L-epinephrine. The exact shape of this curve depends on the stoichiometry and stability of the complex formed. However, over the range of concentrations accessible in this study the data appear to approximate a straight line, and the extrapolation to zero mole percent should introduce less than a factor of two error in $1/T_{2b}$. The results of calculations of K using different resonance peaks and L-epinephrine concentrations, and assuming a 1:1 interaction of the type

$$\text{L-epinephrine} + \text{phosphatidylserine} \overset{K}{\rightleftarrows} \text{L-epinephrine·phosphatidylserine}$$

TABLE I

CALCULATION OF THE STABILITY CONSTANT OF THE L-EPINEPHRINE–PHOSPHATIDYLSERINE COMPLEX

The concentration of phosphatidylserine is $2.3 \cdot 10^{-3}$ M based on a molecular weight of 850.

L-*Epinephrine* (*mole* %)	L-*Epinephrine* (*mM*)	$1/T_{2b}$ (sec^{-1})	$1/T_{2f}$ (sec^{-1})	$1/T_{2m}$ (sec^{-1})	α	K (M^{-1})
46.0*	2.0	10.9	1.6	6.6_5	0.54	920
68.0*	5.0	10.9	1.6	4.6	0.32	640
89.5*	20.0	10.9	1.6	2.6_5	0.11	820
46.0**	2.0	13.0	4.4	9.2	0.56	1030
68.0**	5.0	13.0	4.4	7.3	0.34	790

* Calculated from the epinephrine methyl proton resonance data (Fig. 2).
** Calculated from the epinephrine phenyl proton resonance data (Fig. 2).

are shown in Table I. The assumption of a 1:1 complex is not unreasonable. HAUSER *et al.*[16] found evidence for a 1:1 interaction in procaine·HCl–phosphatidylserine mixtures indicating that the hydrophilic groups of all phosphatidylserine molecules were accessible to the local anesthetic molecule. Based on a 1:1 stoichiometry, the order of magnitude of the binding constant for the epinephrine–phospholipid complex is $1 \cdot 10^3$ M^{-1}.

Recently FISCHER AND JOST[17] have studied the interaction of epinephrine with liver cells using nuclear magnetic resonances. The results obtained suggest a strong interaction of the liver cells with the phenyl ring of epinephrine, as well as with the nitrogen of the side chain. Magnetic resonance measurements we have made on suspensions of rat liver plasma membranes (0.36 mg/ml protein) containing L-epinephrine (0.05–0.002 M) did not reveal any measurable broadening of the epinephrine resonance signals. However, the concentration of binding sites on the membrane is probably quite low and the fraction of bound epinephrine may have been very small. Further experiments involving higher membrane concentrations and lower L-epinephrine concentrations are currently under way.

In conclusion, nuclear magnetic resonance measurements on solutions of phosphatidylserine containing L-epinephrine suggest an electrostatic interaction occurs between the positively charged nitrogen of epinephrine and the negatively charged hydrophilic groups of the phospholipid, with possible participation of the phenyl hydroxyls of epinephrine in hydrogen binding interactions with the phospholipid.

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