MOLECULAR PHARMACOLOGY, 20:124–127

^{1}H Nuclear Magnetic Resonance Study on Transbilayer Permeation of Chlorpromazine in Lecithin Vesicles

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Received December 9, 1980; Accepted March 11, 1981

SUMMARY

KITAMURA, K., H. KANO, K. YONEYAMA, AND K. HOZUMI. ^{1}H nuclear magnetic resonance study on transbilayer permeation of chlorpromazine in lecithin vesicles. *Mol. Pharmacol.* **20**:124–127 (1981).

Permeation of chlorpromazine across lecithin bilayers has been investigated by ^{1}H NMR spectroscopy of lecithin vesicle solutions containing the shift reagents Pr^{3+} in the inside and Eu^{3+} in the outside aqueous phase of these vesicles, which were prepared by ultrasonication and ultrafiltration techniques. The ^{1}H NMR spectrum of this sample showed that the choline methyl signal of the lecithin was split into two peaks, one signal arising from the internal monolayer shifted downfield by entrapped Pr^{3+} and the other to the external monolayer shifted to upfield by Eu^{3+}. Addition of chlorpromazine hydrochloride to the sample reduced the lanthanide-induced shifts of both the internal and external choline methyl signals, due to the displacement of the lanthanide ions at the external and internal surfaces of the vesicle by the interaction of chlorpromazine with polar headgroups at both surfaces. The chlorpromazine interaction with the internal surfaces serves as a test of the transbilayer permeation of chlorpromazine. The addition of chlorpromazine also induced an upfield shift in the signal of the C-2 methylenes adjacent to the carbonyl groups in the fatty acid chains of the lecithin. This indicates that chlorpromazine molecules interacting with the external and internal surfaces insert their phenothiazine rings into the lecithin monolayers to such an extent that the C-2 methylene in the fatty acid chain could interact with the plane of the phenothiazine ring, the high field shift thus being induced by a magnetic anisotropy of the ring current.

INTRODUCTION

Interaction of chlorpromazine with lecithin vesicles (single bilayers) or liposomes (multibilayers) has been investigated by electron spin resonance (1); ^{1}H (2), ^{13}C (3, 4), and ^{31}P (4) NMR; and calorimetric analyses (4). The results of these studies revealed that the chlorpromazine interacts with polar headgroups of the membrane surface (1–4), displacing the divalent ion Mn^{2+} (2) and the trivalent ions Eu^{3+} (2) or Yb^{3+} (3) from the surface. It was also suggested that the phenothiazine ring of the chlorpromazine was located near the carbonyl groups of fatty acid chains in the lecithin membrane (3, 4). The calorimetric study indicated that the chlorpromazine lowers the phase transition temperature of dipalmitoyl phosphatidylcholine (4). In spite of these studies, no information has been obtained as to whether the chlorpromazine permeates across the lecithin bilayer and interacts with the internal membrane surface as it does with the external surface.

In this paper, we report evidence for the permeation of chlorpromazine through the lecithin bilayer by means of ^{1}H NMR spectroscopy. In ^{1}H NMR, the presence of the lanthanide ions Pr^{3+} or Eu^{3+} in a sample induces a downfield or upfield shift, respectively, in the signal of the nuclei to which they have access (2, 5). If lecithin vesicles containing Pr^{3+} in the internal aqueous phase can be suspended in an aqueous solution of Eu^{3+}, the choline methyl signal of the internal surface is shifted downfield by entrapped Pr^{3+} and that of the external surface upfield by Eu^{3+}, so that the behavior of the internal choline methyl signal can be observed independently from that of the external choline methyl signal. Such a vesicle solution could be prepared by ultrasonication followed by ultrafiltration techniques. If the chlorpromazine permeates across the lecithin bilayer and interacts with the internal membrane surface displacing Pr^{3+}, the Pr^{3+}-induced downfield shift of the internal choline methyl signal should be reduced by an addition of the chlorpromazine to the sample solution.

METHODS

Preparation of vesicles. Egg yolk lecithin purchased from Sigma Chemical Company (St. Louis, Mo.) was purified by column chromatography (1). To approximately 200 mg of dried lecithin was added 4 ml of D_2O containing 6 mM Pr^{3+} and the mixture was dispersed by

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a shaking mixer (Labo Mixer MS-8; Iuchi, Tokyo, Japan). It was then ultrasonicated in an ice-water bath under nitrogen stream for 15 min to form vesicles (in practice, a 3-min sonication with a 3-min intermission was repeated 5 times). The vesicle solution was centrifuged at 3500 rpm for 15 min to eliminate any sediment stripped from the sonication tip.

Ultrafiltration. A 10-ml portion of the lecithin vesicle solution was placed in an ultrafiltration cell (MC-1; Bio-Engineering, Tokyo, Japan) and filtrated through the membrane (Diafilter A-50T, of which fractional molecular weight is 50,000; Bio-Engineering) with stirring under a pressure of 2 kg/cm^2 with nitrogen. In order to test leakage of lecithin through the membrane, 10 ml of 1% lecithin vesicle-water solution was prepared and filtrated through the membrane to concentrate to 2 ml. The filtrate was evaporated to dryness under reduced pressure. Only a trace (<1 mg) was found by weighing so that the leakage of lecithin was confirmed to be negligible.

Replacement of Pr^{3+} in the outside aqueous phase of vesicles by Eu^{3+}. To 3 ml of a 5% lecithin vesicle-D_2O sample containing 6 mM Pr^{3+} both inside and outside of the vesicles was added 7 ml of 6 mM Eu^{3+}-D_2O. Since Pr^{3+} and Eu^{3+} ions do not penetrate the lecithin bilayer, the outside aqueous phase contained 3/10 × 6 mM Pr^{3+} and 7/10 × 6 mM Eu^{3+}, whereas the inside phase remained unchanged from 6 mM Pr^{3+}. The solution was then concentrated to one-fourth volume by ultrafiltration to restore the initial 5% vesicle concentration. The same process of dilution with the Eu^{3+}-D_2O and concentration by ultrafiltration was repeated four times, until the Pr^{3+} in the outside aqueous phase was practically all replaced by Eu^{3+}.

^1H NMR measurement. Spectra were measured at 100 MHz in the field sweep mode by a Varian HA-100D spectrometer locked on the HDO signal in the samples. Sample temperature was approximately 28°. The methylene proton signal of the fatty acid chains of lecithin was used as an internal reference.

RESULTS AND DISCUSSION

The ^1H NMR spectrum of the lecithin vesicles prepared in D_2O containing 6 mM Pr^{3+} is shown in Fig. 1A. Since this sample contained Pr^{3+} in both inside and outside aqueous phases of the vesicle, choline methyl signals of the internal and external lecithin monolayers were both shifted downfield, appearing as a single peak by overlapping. Then the Pr^{3+} in the outside aqueous phase was replaced by Eu^{3+} in the manner described above. Fig. 1B shows the ^1H NMR spectrum of the vesicle sample thus obtained, containing Pr^{3+} inside and Eu^{3+} outside of the vesicles. The external choline methyl signal was shifted to higher field by Eu^{3+} and appeared at 1.79 ppm, while the Pr^{3+}-shifted internal choline methyl signal was detected at 2.18 ppm, which appeared somewhat larger than those expected from the ratio to the external choline methyl signal, 1:1.8 (5), because of overlapping with some glycerol protons (it resolved in Fig. 1D). Addition of a small amount of Gd^{3+} to the sample solution confirmed the assignment of these split choline methyl signals, since it has been shown that Gd^{3+} broadens the signal of the nuclei to which it approaches (3, 6). Fig. 1C

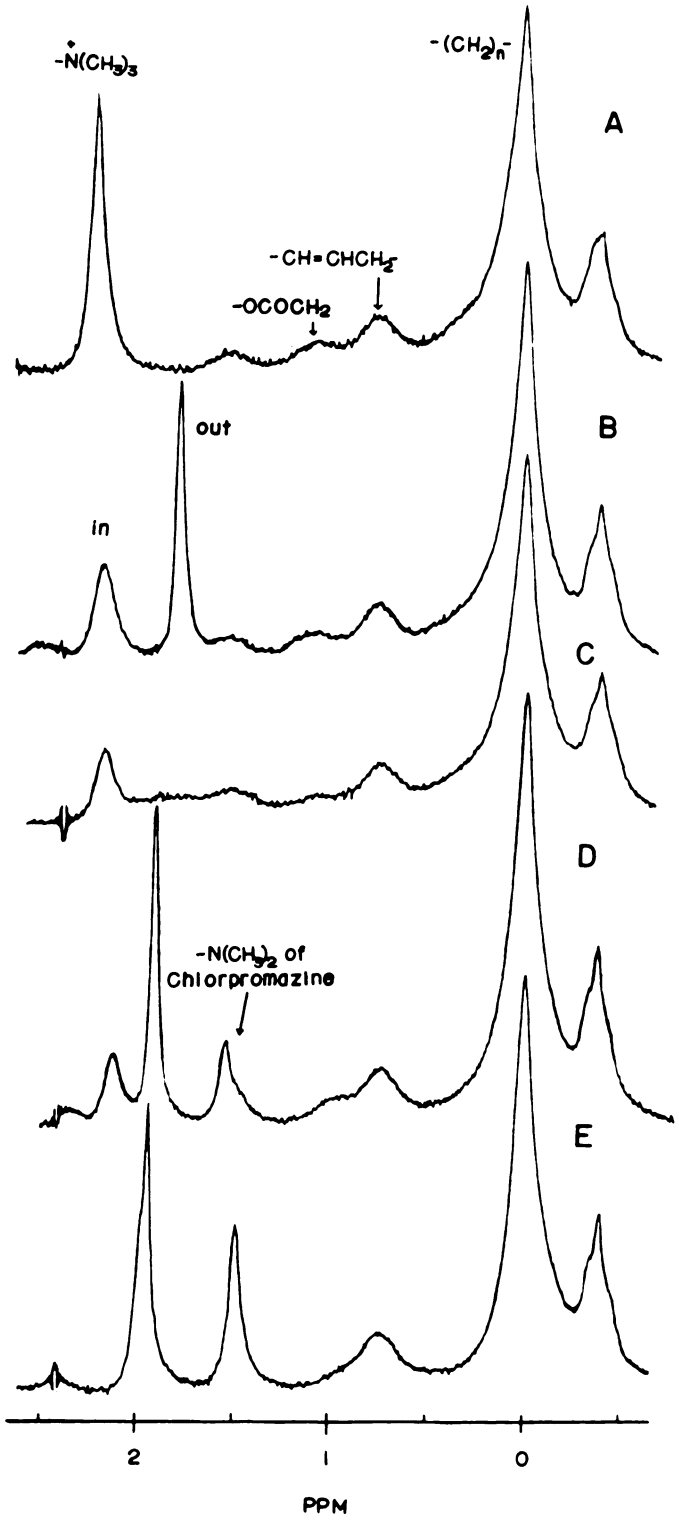

FIG. 1. *^1H NMR spectra of lecithin vesicles in D_2O*

A. Lecithin vesicles containing 6 mM Pr^{3+} both inside and outside of the vesicle.

B. Lecithin vesicles containing 6 mM Pr^{3+} inside and 6 mM Eu^{3+} outside of the vesicle.

C. A small amount of Gd^{3+} was added to the same sample as B.

D. 21 mM chlorpromazine was added to the same sample as B.

E. 45 mM chlorpromazine was added to the same sample as B.

For details, see Methods.

shows the disappearance of the upfield-shifted external choline methyl signal resulting from the Gd^{3+}-induced broadening.

When the chlorpromazine hydrochloride was added to the vesicle solution containing Pr^{3+} inside and Eu^{3+} outside of the vesicle, the Eu^{3+}-induced, upfield-shifted choline methyl signal was moved to downfield as a result of the interaction of chlorpromazine with the external membrane surface as previously reported (2), whereas the Pr^{3+}-induced, downfield-shifted internal choline methyl signal was also moved at the same time, but upfield. A typical spectrum was illustrated in Fig. 1D. Further addition of the drug resulted in a further downfield shift of the external choline methyl signal and an upfield shift of the internal choline methyl signal, respectively, so that an overlapping of the two choline methyl signals finally formed a single peak with a slight shoulder at 45 mM chlorpromazine concentration, as shown in Fig. 1E. This result indicates that the chlorpromazine permeates through the lecithin bilayer and interacts also with the polar headgroups of the internal surface, causing the displacement of Pr^{3+}. The lanthanide-induced shift changes of the internal and external choline methyl signals in relation to the amount of chlorpromazine added are plotted in Fig. 2.

On the other hand, there may be a possibility that transbilayer permeation of lanthanide ions themselves might be induced by chlorpromazine, resulting in a mutual mixing of the internal Pr^{3+} and the external Eu^{3+}. In such a case, the same amount of Pr^{3+} and Eu^{3+} should be exchanged between the entrapped aqueous phase and the outer bulk aqueous phase through the bilayer, so that most of the internal Pr^{3+} will be replaced readily by Eu^{3+}, since volume of the entrapped aqueous phase is much smaller than that of the bulk aqueous phase. Thus, the reduction of the lanthanide-induced shift by chlorpromazine should be larger in the internal choline methyl signal than in the external choline methyl signal, but this is not consistent with the experimental result. Accordingly, it cannot be considered that the transbilayer permeation of the lanthanide ions themselves is so induced by chlorpromazine as to cause the reduction of the lanthanide-induced shifts.

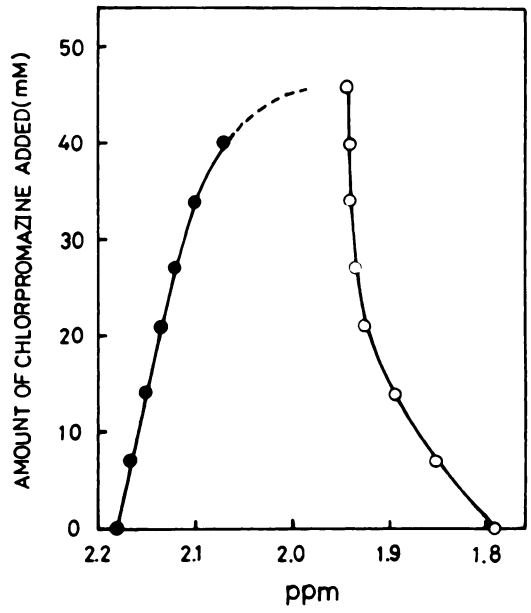

FIG. 2. *Effect of chlorpromazine on the lanthanide-induced shifts of internal* (●) *and external* (○) *choline methyl signals*

The spectra were measured within 10 min after the additon of chlorpromazine and no further spectral change was observed even after 24 hr or more, indicating that the permeation process was relatively fast. The lecithin vesicles with chlorpromazine attained equilibrium readily and were stable at any molar ratio studied.

It has been reported that fusion of vesicles produced signal broadening to the entire ^1H NMR spectrum of the vesicles (7). Since no broadening was observed in any of the signals in Fig. 1D and E (after the addition of chlorpromazine), compared with Fig. 1B (before the addition of chlorpromazine), it was obvious that fusion of the vesicles was not induced by the chlorpromazine.

The two curves in Fig. 2 indicate different features for the external and internal choline methyls. At a relatively low concentration of chlorpromazine (< 20 mM), the shift changes induced by the chlorpromazine were greater for the external choline methyl signal than for the internal choline methyl signal, and, at the higher concentrations of chlorpromazine (> 25 mM), Eu^{3+} on the external surface was almost completely displaced, whereas a fair amount of the Pr^{3+} on the internal surface still remained bound. Although no satisfactory interpretation of this phenomenon could be obtained, it must be noted that the chlorpromazine must permeate through the hydrophobic region of bilayer to be able to interact with the internal surface.

As mentioned above, some preliminary information on the localization of chlorpromazine in the lecithin bilayer has already been obtained (3, 4). Further spectral evidence relevant to this problem was obtained here. In Fig. 1A, a signal from the C-2 methylene adjacent to the carbonyl carbon in the fatty acid chain was observed as a broad line at 1.10 ppm. Since it was known that the C-2 methylene signal appears at the same position in the absence of Pr^{3+}, the C-2 methylene would not be affected by Pr^{3+}, at least at a concentration of 6 mM. This implies that the C-2 methylene was sufficiently distant from the Pr^{3+} binding site. By stepwise addition of chlorpromazine to the vesicle solution, the C-2 methylene signal was gradually shifted upfield and, at 45 mM chlorpromazine concentration, it was overlapped with another signal located at 0.77 ppm (Fig. 1E), which was assigned to the methylene groups adjacent to olefinic carbons in the fatty acid chain (8). This result indicates that the chlorpromazine molecules interacting with the external and internal surfaces insert their phenothiazine rings into the lecithin monolayers to such an extent that the C-2 methylene in the fatty acid chain could take its place upon the plane of the phenothiazine ring, so that high-field shift could be induced by a magnetic anisotropy of the ring current.

ACKNOWLEDGMENT

The authors thank Dr. Y. Kuroda of Kyoto University for use of the HA-100D NMR spectrometer.

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