Biophysical Chemistry 17 (1983) 297–300
Elsevier

CONFORMATIONAL FEATURES OF CELL-BOUND DRUGS AS DETECTED BY SELECTIVE PROTON RELAXATION RATE INVESTIGATIONS

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Received 1st December 1982
Accepted 21st January 1983

Key words: Proton relaxation rate; Procaine; Drug binding; NMR

The nonselective and selective longitudinal relaxation rates were measured for procaine protons in the presence of model lipid membranes, biological membranes and whole cells. Unlike the nonselective relaxation rates, the selective rate was shown to be particularly sensitive in detecting binding interactions with macromolecular cell constituents. It was shown that the aromatic moiety of procaine is involved in binding the cell plasma membrane.

1. Introduction

Nuclear magnetic resonance (NMR) methods have been widely used in studying interactions between a small ligand and a macromolecule, since exchange between free (F) and bound (B) environments, which is usually a fast process on the NMR time scale, results in a weighted average of relaxation parameters:

$$1/T_i = p_F/T_{iF} + p_B/T_{iB} \quad (i = 1,2)\ (p_F + p_B) = 1 \qquad (1)$$

However, the necessity of using low concentrations of the macromolecule ($p_B \ll 1$) has limited the investigations to paramagnetic systems where the dipolar and scalar interactions between nuclear and electron spins give rise to very short relaxation times T_{iB}. The applications of exploiting paramagnetic metal probes and spin labels have been thoroughly reviewed elsewhere [1–4].

The possibility of making a similar investigation in diamagnetic systems has been recently reevaluated, since measuring the selective proton relaxation rates [5] has been shown to be a very sensitive method for the delineation of enzyme-substrate interactions [6].

The aim of the present report is to demonstrate that the selective proton relaxation rate measurements can be suitably applied in studying the interaction of small 'NMR-visible' drugs with whole cells. Procaine was chosen due to the fact that its anesthetic and antiviral actions have been widely investigated [7–11].

2. Materials and methods

Procaine (from Sigma) solutions were obtained either in ^2H_2O or in physiological deuterated solution. Human group O erythrocytes were separated from freshly drawn blood containing 0.1 M sodium citrate, washed in isotonic phosphate buffer, centrifuged at 2000 rpm for 15 min, and suspended in deuterated phosphate buffer. Erythrocyte ghosts were prepared using the method of Dodge et al. [12]. HEp-2 cells (Human Laryngeal Epidermoid carcinoma), grown in Eagle's minimal essential medium, were dispersed by trypsin, centrifuged at 1000 rpm for 20 min at 4°C and suspended in deuterated phosphate buffer. Dipalmitoylphosphatidylcholine was obtained from Sigma and used without further purification. Multilamellar vesicles were prepared by the method of Bangham et al. [13]. Unilamellar vesicles were made by sonication of multilamellar vesicles as

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described elsewhere [14,15].

The NMR experiments were performed with a Varian XL-200 NMR spectrometer. The nonselective longitudinal relaxation rates were measured using a $(\pi - \tau - \pi/2 - t)_n$ pulse sequence. The selective longitudinal relaxation rates were measured in the initial rate approximation [5] by giving a selective π pulse obtained by switching the decoupler on at the selected frequency for a long time (about 20 ms). After time τ, a nonselective $\pi/2$ pulse was given to detect the longitudinal magnetization.

3. Results and discussion

The 200 MHz NMR spectrum of procaine is shown in fig. 1. The proton relaxation rate data are summarized in table 1. When the dipolar ^1H-^1H interaction is the dominant relaxation mechanism, the nonselective (R^{ns}) and selective (R^s) proton longitudinal relaxation rates of any proton i are given by [16]:

$$R_i^{ns} = \sum_{i \neq j} \rho_{ij} + \sum_{i \neq j} \sigma_{ij} \qquad (2)$$

Table 1

Relaxation parameters for procaine (4 mg/ml) in ^2H_2O at pH 7.0

The assignments are referred to fig. 1.

Resonance	R^{ns} (s^{-1})	R^s (s^{-1})	R^{ns}/R^s
a	0.81	0.80	1.01
b	1.19	1.26	0.94
c	1.32	1.27	1.04
e	0.48	0.33	1.45
f	0.40	0.28	1.43

$$R_i^s = \sum_{i \neq j} \rho_{ij} \qquad (3)$$

where ρ_{ij} and σ_{ij} are the direct relaxation rate and the cross-relaxation term for a proton pair, respectively:

$$\rho_{ij} = \frac{1}{10} \frac{\hbar^2 \gamma_H^4}{r_{ij}^6} \left\{ \frac{4\tau_c}{1+(\omega_o \tau_c)^2} + \frac{6\tau_c}{1+(2\omega_o \tau_c)^2} + \tau_c \right\} \qquad (4)$$

$$\sigma_{ij} = \frac{1}{10} \frac{\hbar^2 \gamma_H^4}{r_{ij}^6} \left\{ \frac{6\tau_c}{1+(2\omega_o \tau_c)^2} - \tau_c \right\} \qquad (5)$$

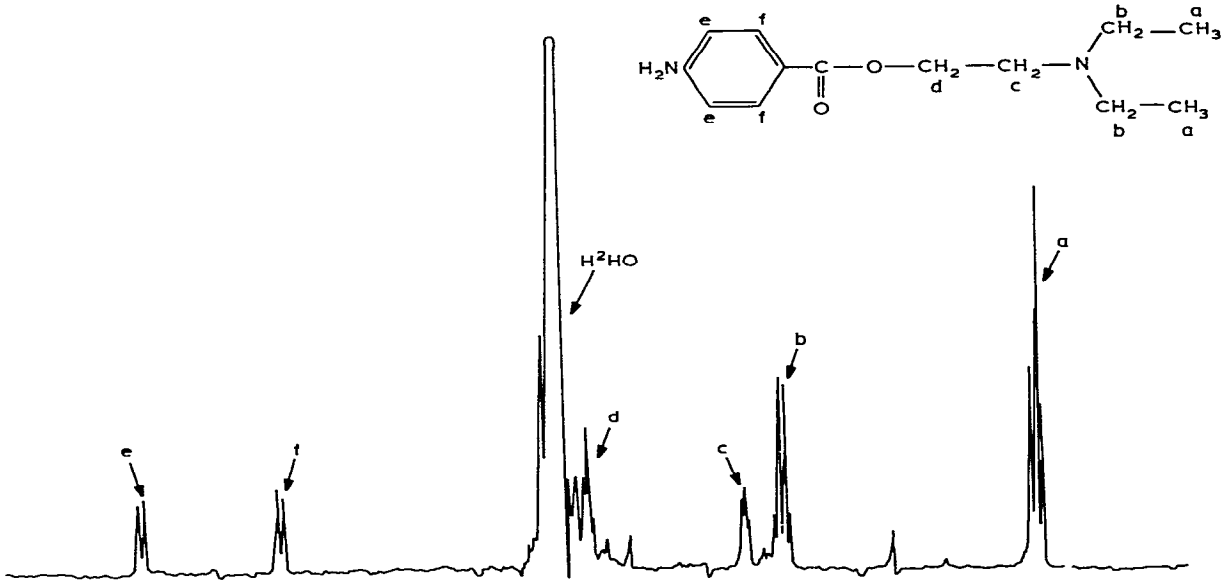

Fig. 1. 200 MHz proton NMR spectrum of procaine (4 mg/ml) in ^2H_2O at pH 7.0; $T = 295$ K.

Table 2

Nonselective and selective longutidinal relaxation rates of aromatic protons of procaine (4 mg/ml) in ^2H_2O at pH 7.0 after the addition of 0.1 ml of different biological preparations

The assignments are reported in fig. 1. $T = 295$ K.

Biological sample added	Proton e			Proton f		
	R^{ns} (s^{-1})	R^{s} (s^{-1})	R^{ns}/R^{s}	R^{ns} (s^{-1})	R^{s} (s^{-1})	R^{ns}/R^{s}
–	0.48	0.33	1.45	0.40	0.28	1.43
Erythrocytes (4%)	0.51	0.64	0.80	0.44	0.56	0.79
Erythrocyte ghost (4%)	0.52	0.63	0.82	0.46	0.57	0.81
HEp-2 cells	0.53	0.68	0.78	0.42	0.52	0.81
Dipalmitoylphosphatidyl-choline vesicles	0.49	0.56	0.87	0.42	0.51	0.82

where r_{ij} is the interproton distance, ω_0 the proton Larmor frequency and τ_c the motional correlation time. As a consequence, the ratio R^{ns}/R^{s} is a function of $\omega_0\tau_c$ [6]. Using the data in table 1 the following considerations are possible:

(i) As $R^{ns} \simeq R^{s}$ for methyl and methylene protons, the relaxation mechanism of these is the intramolecular dipolar interaction.

(ii) As $R^{ns}/R^{s} \simeq 1.5$ for the aromatic protons, the dipolar interaction between *ortho* and *meta* protons provides the relaxation mechanism and the extreme narrowing limit holds. In this case an upper limit can be estimated for the motional correlation time: $\tau_c < 7.7 \times 10^{-10}$ s.

The relaxation parameters of the aromatic protons were therefore taken in order to demonstrate the interaction of procaine with cellular constituents. The data, summarized in table 2, were obtained under different experimental conditions as reported. The nonselective and selective relaxa-

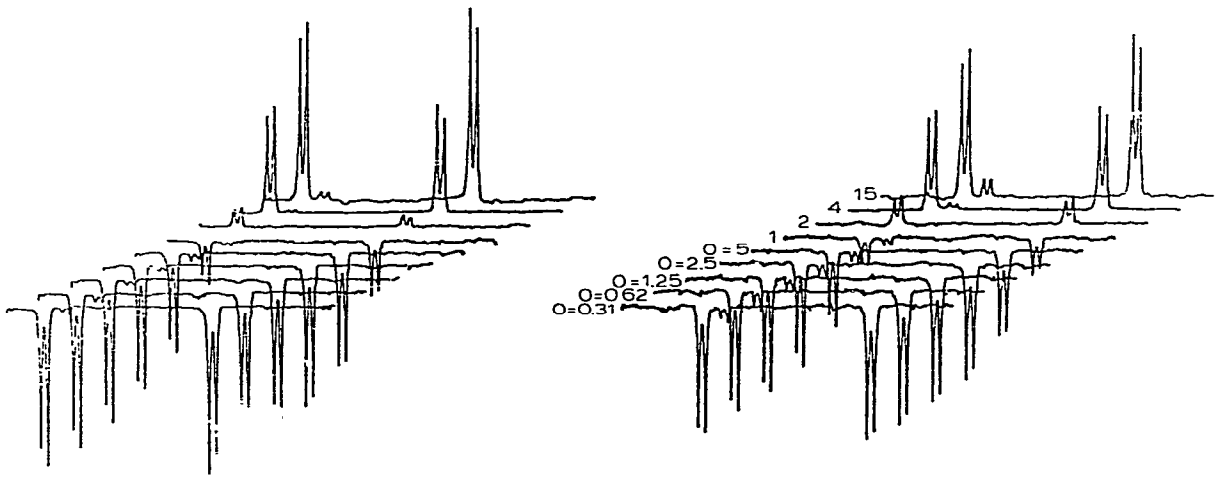

Fig. 2. Nonselective partially relaxed spectra of the aromatic protons of procaine (4 mg/ml) in ^2H_2O at pH 7.0 before (left) and after (right) the addition of 0.1 ml of human erythrocytes (4%) in deuterated physiological buffer. $T = 295$ K.

tion rate measurements obtained in the absence and presence of erythrocytes are shown in figs. 2 and 3, respectively. An analysis of these data shows that R^{ns} is affected very little by the presence of macromolecules (a 10% change in R^{ns} is around the limit of experimental error). However, the changes in R^{s} and hence in R^{ns}/R^{s} indicate slowing down of molecular motions.

Binding of a fraction of procaine molecules, rather than a viscosity effect, can account for the observed effect. In fact, a value of R^{ns}/R^{s} in the range 0.6–0.8 is consistent with $\tau_c \simeq 1 \times 10^{-9}$ s which is more than one order of magnitude longer than that of procaine in aqueous solution. On the other hand, exchange of procaine between environments characterized by very different motional freedom and, hence, by very different selective relaxation rates can easily account for the 90–100% change in R^{s}. Moreover, similar enhancements of R^{s} were obtained with whole cells, membranes and model phospholipid bilayers, that is to say under different conditions of viscosity.

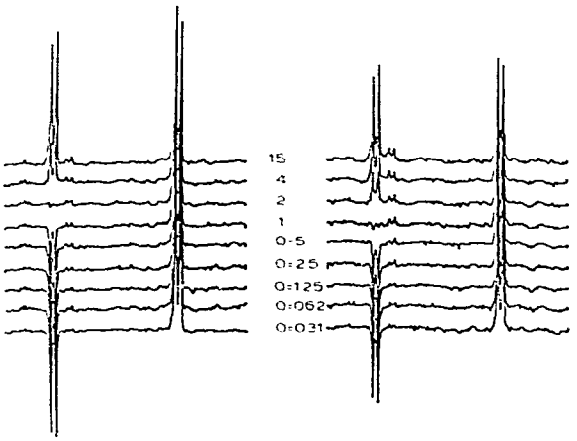

Fig. 3. Selective partially relaxed spectra of one of the aromatic protons of procaine (4 mg/ml) in ^2H_2O at pH 7.0 before (left) and after (right) the addition of 0.1 ml of human erythrocytes 4% in deuterated physiological buffer.

It can be concluded that the method of selective relaxation rate makes it possible to investigate binding of relatively small drugs to macromolecular cell constituents and even to whole cells; the considerable effect of the binding on R^{s} can be used for the delineation of binding sites and of conformation properties in the bound environment. In the case of procaine, binding to the cell plasma membrane can be easily detected by measuring R^{s} of aromatic protons which appear to be involved in the interaction. It is worth underlining that, in interacting with both erythrocytes and erythrocyte ghosts, procaine undergoes some hydrolysis, which can be followed in the NMR spectrum since the products of reaction display diverse chemical shift parameters (see the small doublet of *p*-aminobenzoic acid in figs. 2 and 3).

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