RECEPTORS ON THE NEUTROPHIL MEMBRANE

I. Flunarizine–Adenosine Antagonism as Detected by ^{1}H-Nuclear Magnetic Resonance Relaxation Investigations

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ABSTRACT Selective proton relaxation rates were measured for selected protons of flunarizine in the presence of neutrophils, yielding evidence of a binding interaction that involves different moieties of the drug. Fast exchange between bound and free environments was demonstrated as the main factor determining the selective relaxation rate enhancement, whereas viscosity effects were shown to be negligible. The relaxation effect was cancelled out by the presence of adenosine as a cosolute in a dose-dependent fashion, leading to the suggestion that the endogenous mediator adenosine affects binding of flunarizine to the neutrophil surface.

INTRODUCTION

It has been previously demonstrated that adenosine is able to in vitro inhibit, in a dose-dependent manner, free radical formation and enzyme release from human neutrophils when triggered by adequate stimuli (1, 2).

The hypothesis that adenosine could act through a membrane calcium transport involving mechanism was suggested by the following experimental evidence. (*a*) The inhibitory effect on the A23187-induced activation, strictly dependent on extracellular calcium entry, was completely cancelled by increasing Ca^{++} concentration in the suspending medium (1, 2). (*b*) The ^{45}Ca uptake induced by the ionophore is accordingly and significantly reduced (unpublished data). (*c*) In cases where the role of extracellular calcium role entry played minor roles, if any, such as in the formyl-methionyl-leucyl-phenylalanine (FMLP)-dependent and phorbol myristate acetate (PMA)-dependent activation, the inhibitory effect was scarcely or not at all relevant (1, 2). (*d*) The same behavior as adenosine was shown by the so-called calcium entry blocker drugs (nifedipine and flunarizine) (3, 4), according also to reports from other authors (5, 6).

The pharmacologic effect of such drugs on neutrophil as well as on other cell lines is attributed to specific sites that regulate calcium entrance. It can therefore be suggested that adenosine may act by mimicking the mechanism of calcium entry blockers and possibly compete with them at the same sites.

The aim of the present report is to support such hypothesis by demonstrating antagonism between adenosine and a calcium entry blocker at the level of the neutrophil membrane.

Flunarizine (1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl) piperazine) has been chosen for this purpose. Its mechanism of action has been shown (7) to involve the same site as verapamil, which is allosterically linked to the sites for the other two groups of calcium antagonists (nitrendipine-like and diltiazem).

The binding interaction between flunarizine and the neutrophil membrane as well as the flunarizine-adenosine antagonism were approached by measuring ^{1}H-nuclear magnetic resonance (NMR) spin-lattice relaxation rates upon selective irradiation of well resolved spin systems within the flunarizine molecule. The rationale for such measurements is well documented in the literature (8–10) and only a brief theoretical discussion will be herein resumed, showing that the selective relaxation approach makes detection of binding to ill-defined receptors feasible.

MATERIALS AND METHODS

Chemicals

Flunarizine was supplied by Janssen Pharmaceutica (Beerse, Belgium) and was used without further purification. Solutions were made in D_2O 99.95% (Merck & Co., Inc., Rahway, NJ) and the pH was adjusted with either DCl or NaOD. Adenosine was supplied by Sigma Chemical Co. (St. Louis, MO) and was used without further purification.

Cell Preparation

Normal human venous blood, anticoagulated with preservative-free heparin (5 IU/ml) was mixed with 3 ml of high molecular weight dextran (70,000 mol wt, Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and allowed to sediment at room temperature for 60 min. The supernatant leukocyte-rich plasma was centrifuged at 250 *g* for 7 min. The pellet was

exposed to hypotonic lysis to remove red cells by the addition of 3 ml NaCl 0.2% for 75 s; restoration of isotonicity was obtained by the addition of 7 ml NaCl 1.2%. The cells were centrifuged at 250 *g* for 7 min and the pellet was resuspended in 1 ml of Hanks' balanced salt solution containing salt-poor human serum albumin at a concentration of 0.5 g/dl (HBSS/A). The final cell preparation contained 90–95% granulocytes.

NMR Measurements

The NMR measurements were performed with an XL-200 NMR spectrometer (Varian Associates, Inc., Palo Alto, CA) at the constant temperature of 303 ± 1 K. The nonselective spin-lattice relaxation rates, R^{ns}, were measured by using the inversion recovery pulse sequence $(180\text{-}\tau\text{-}90\text{-}t)_n$. The selective spin-lattice relaxation rates, R^s, were measured in the intial rate approximation (11) by giving a selective 180° pulse with the proton decoupler at the selected frequency for a relatively long time (typically 20 ms). After the variable delay a nonselective 90° pulse was given to detect the longitudinal magnetization. The R^{ns} and R^s values were obtained from a three-parameter exponential regression analysis of the recovery curve for longitudinal magnetization.

RESULTS AND DISCUSSION

The spin-lattice relaxation rates, as usually measured by inverting with a strong radiofrequency 180° pulse all the resonances and sampling the recovery of longitudinal magnetization components, are contributed by all the possible $^1H—^1H$ dipole–dipole interactions and by other eventual relaxation mechanisms (such as dipolar interactions with nuclei other than ^1H, scalar coupling to fast relaxing nuclei, etc.). Such nonselective relaxation rates can be therefore expressed by the following equation:

$$R_i^{ns} = R_i + \sum_{i \neq j} \sigma_{ij} \tag{1}$$

where

$$R_i = \sum_{i \neq j} \rho_{ij} + \rho_i^*, \tag{2}$$

where ρ_{ij} and σ_{ij} are the direct, and cross-relaxation terms respectively for the *i-j* dipolar interaction and ρ_i^* accounts for "other" relaxation mechanisms. In terms of the single-, double-, and zero-quantum relaxation transition probabilities, W_1, W_2, and W_0, among the four energy levels of any two-spin system, it may be written (12)

$$\rho_{ij} = 2W_1 + W_2 + W_0 \tag{3}$$

$$\sigma_{ij} = W_2 - W_0. \tag{4}$$

It follows that, for any spin pair, the contribution of W_0 to relaxation is cancelled out from the relaxation expressions. Since W_0 is the only term almost linearly affected by the motional correlation time modulating the *i-j* interaction, while W_1 and W_2 pass through a maximum at $\omega\tau_c = 1$ and $\omega\tau_c = 0.5$, respectively, and decrease afterwards, the nonselective relaxation rate will be very small for slowly reorienting molecules.

If the alternative method of irradiation is used that inverts with soft selective 180° pulses only narrow range of frequencies of any given resonance, all the σ terms in Eq. 1 are zero and such selective relaxation rate will contain a contribution by W_0, almost linearly increasing with slowing down of motions.

Therefore it can be concluded that investigations of binding interactions of a small molecule with some macromolecular receptor are suitably approached by detecting receptor-induced changes in the selective relaxation rate of any given spin system within the small molecule.

The main drawbacks of such approach are that: (*a*) slowing down of motions caused by binding to a macromolecule must be distinguished from viscosity changes that, in principle, would yield the same effects, and (*b*) the exchange rate of the small molecule between free and bound environments has to be relatively fast, since the fraction of bound molecules must be kept quite small in NMR experiments.

The ^1H-NMR spectrum of flunarizine is reported in Fig. 1 where the assignments are referred to the formula above the spectrum. The equivalence between protons in the two fluorophenyl rings, as well as between the two ortho and the two meta protons in the other aromatic ring, suggest that the degrees of internal motion underwent by the rings is as fast as to average the chemical environment of nuclear spins.

The whole relaxation data are summarized in Table I. Analysis of the ratios *F* between nonselective and selective relaxation rates provides an estimate of the relative weight of proton–proton dipole–dipole interaction on the relaxation mechanism or, alternatively, in the presence of 100% dipolar relaxation, information on the rate of reorientational motions (extreme narrowing or spin diffusion limit), being 1.5 the theoretical value expected for dipolar only relaxation in the extreme narrowing region.

The *F*'s in Table I are all somewhat smaller than 1.5, with the exception of H_{11}, H_{15}, and H_{16}. It should be however observed that the H_{11}, H_{15} doublet and the H_{16} singlet (Fig. 1) are the only well resolved resonances in the spectrum. We can therefore reasonably assume that the relatively small *F*'s, measured for the other resonances, arise from partial irradiation of nearby protons, that even the most carefully prepared soft pulse cannot avoid. We can conclude that dipolar relaxation is very likely to be the unique relaxation mechanism and that molecular motions are fast in the NMR time scale, as already anticipated by the equivalence of chemical environments, as detected by chemical shifts. It can also be considered that H_{11}, H_{15}, or H_{16} are the best suited resonances for exploiting the selective irradiation method in both binding and antagonism studies.

The effects of 6×10^4 neutrophils/ml on the proton relaxation rates are described in Table II. As expected, while non-selective relaxation rates are affected by not more than 10%, selective relaxation rates undergo very large enhancements of ~50%, yielding much smaller *F* values compared with the flunarizine solution. Since viscosity changes are not likely to be responsible of the

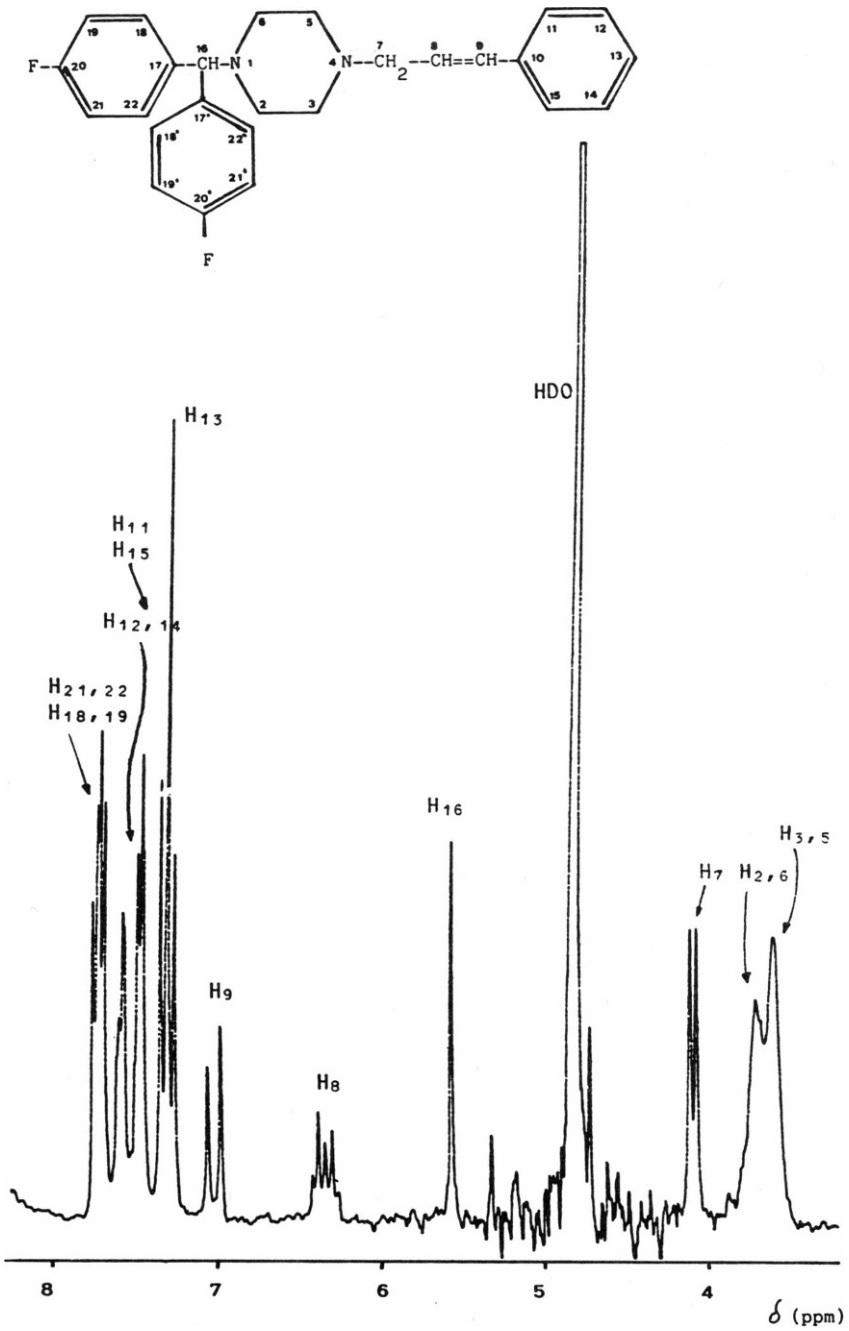

FIGURE 1 Proton NMR spectrum at 200 MHz of flunarizine 0.1 mol dm^{-3} in D_2O at pH = 7.0 and T = 303 K. The assignments are referred to the formula above the spectrum.

observed effect, as it will be considered hereafter, what selective relaxation measurements detect can only be slowing down of molecular motions of a given fraction of flunarizine molecules chemically exchanging with the much more abundant fraction of bulk flunarizine molecules. Slowing down of molecular motions is in turn arising from binding to the neutrophil cell surface. Questions remain open about the mechanism of binding and the binding constant but what is worth stressing here is that, whatever the cell receptor and the mechanism of interaction, binding of flunarizine at the neutrophil surface can be unequivocally stated and related to a spectroscopic property relatively easy to be handled.

The data in Table II can be roughly interpreted in terms of a binding interaction that involves, almost at the same extent, the phenyl, the 4-fluorophenyl, and the piperazine moieties of the flunarizine molecule, since different proton–proton vectors implying such moieties are affected quite similarly by the presence of neutrophils, as manifest in the observed F values. Further experiments are in

TABLE I
NONSELECTIVE (R^{ns}) AND SELECTIVE (R^s) RELAXATION RATES AND F RATIOS ($F = R^{ns}/R^s$) FOR PROTONS OF FLUNARIZINE 0.1 mol dm^{-3} IN D_2O AT pH = 7.0 AND T = 303 K

Proton	R^{ns}	R^s	F
	s^{-1}	s^{-1}	
H_7	4.76	3.57	1.33
H_8	2.37	1.77	1.34
H_9	2.01	1.51	1.33
H_{11},H_{15}	1.20	0.82	1.47
H_{12},H_{14}	1.33	1.01	1.32
H_{13}	0.81	0.62_5	1.29
H_{16}	3.03	2.05	1.48
H_{18},H_{22}	1.10	0.84	1.31
H_{19},H_{21}	0.76	0.56_5	1.34

Errors were ranging between 2 and 5%.

progress that will allow a delineation of the binding site and an estimate of the association constant.

Antagonism between flunarizine and adenosine was investigated by measuring the selective relaxation rate enhancement ΔR^s at varying concentration of adenosine in the range 0.1–10 mmol dm^{-3}, as shown in Table III. It can be easily observed that the addition of adenosine reduces the R^s enhancement, thus increasing the F value, in a way that is somehow dependent on adenosine concentration. The most straightforward interpretation of such findings is in terms of the equilibrium between free and bound flunarizine molecules rapidly exchanging in the NMR time scale. This being the case, the following equation can be written:

$$R^s_{obs} = p_f R^s_f + p_b R^s_b, \quad (5)$$

where p_f and p_b are the fractions of free and bound flunarizine molecules characterized by selective relaxation rates R^s_f and R^s_b, respectively. Since in our conditions (1 ml of solution contains 6×10^4 neutrophils and 0.1 mmol of flunarizine) $p_b \ll 1$ and $p_f \simeq 1$, Eq. 5 may be rewritten as:

$$R^s_{obs} - R^s_f = \Delta R^s = p_b R^s_b. \quad (6)$$

R^s_b represents, as already stated, the selective relaxation rate in the bound environment, as determined by magnetic interactions and molecular motions at the bound site, and it

TABLE II
ENHANCEMENT OF PROTON RELAXATION RATES ($\Delta R = R_{cell} - R_{blank}$) AND F RATIOS MEASURED FOR SELECTED PROTONS OF FLUNARIZINE 0.1 mol dm^{-3} IN D_2O AT pH = 7.0 AND T = 303 K IN THE PRESENCE OF 6×10^4 NEUTROPHILS/ml

Proton	ΔR^{ns}	ΔR^s	F
	s^{-1}	s^{-1}	
H_{11},H_{15}	0.15	0.44	1.07
H_{16}	0.39	1.11	1.08

TABLE III
SELECTIVE RELAXATION RATE ENHANCEMENTS AND F RATIOS FOR SELECTED PROTONS OF FLUNARIZINE 0.1 mol dm^{-3} IN D_2O AT pH = 7.0 AND T = 303 K IN THE PRESENCE OF 6×10^4 NEUTROPHILS/ml AT VARYING CONCENTRATIONS OF ADENOSINE

Proton	[Adenosine]	ΔR^s	F
	mM	s^{-1}	
H_{11},H_{15}	—	0.44	1.07
	0.1	0.38	1.10
	1.0	0.27	1.22
	10.0	0.16	1.31
H_{16}	—	1.11	1.08
	0.1	0.97	1.09
	1.0	0.63	1.18
	10.0	0.22	1.29

is not likely to change upon addition of other cosolutes; as a consequence the decrease in ΔR^s upon addition of adenosine is due to a decrease in p_b, that is to say to displacement of flunarizine molecules from the bound environment.

We can state the following: (*a*) since additions of adenosine, though not appreciably changing the viscosity of the medium, yield changes in ΔR^s, viscosity cannot be considered as the main source of the observed effects, which, therefore, can only be due to a "true" binding interaction; and (*b*) since ΔR^s is mainly determined by parameters of the bound site, eventual interactions at level of the free molecules are not likely to provide a suitable way of altering the observed ΔR^s. To further support this last consideration proton NMR relaxation rates and chemical shifts of flunarizine were measured as a function of concentration of adenosine in the absence of neutrophils and no significant effects could be detected.

CONCLUSIONS

We can summarize our findings by concluding that (*a*) a binding interaction is detected by measuring nuclear relaxation rates of selected protons of flunarizine in the presence of neutrophils; (*b*) the binding, though not thoroughly delineated, involves different moieties of flunarizine at almost the same extent; (*c*) the presence of adenosine as a cosolute causes displacement of flunarizine molecules from the bound site in a concentration-dependent fashion.

From the physiological point of view it is important to underline that the endogenous mediator adenosine affects binding of flunarizine to the neutrophil surface. Such an effect could be due to competition at the same site as for calcium entry blockers or at a nearby site. Since adenosine and calcium channel antagonists act similarly in several preparations, specifically in neutrophils, and slow Ca^{++} current is competitively inhibited by adenosine in different cell lines (13, 14), a role for adenosine as endogenous calcium entry blocker can be suggested.

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