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Selective ^{1}H-NMR relaxation investigations of membrane-bound drugs in vitro

3. Calcium-entry blockers and adenosine

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Selective proton relaxation rates (SPRR) were measured for selected protons of nimodipine or diltiazem in the presence of neutrophils, allowing detection of binding to the cell membrane. Fast exchange of drug molecules between bound and free environments was shown to be the main factor determining the enhancement of SPRR, whereas viscosity effects could be neglected. The SPRR enhancement was almost completely 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 calcium-entry blockers to the neutrophil surface.

1. Introduction

Various physiological effects of adenosine seem to be mediated by membrane-bound receptors. Biochemical and pharmacological studies have led to the subdivision of adenosine receptors into the A_1 (or R_i) and the A_2 (or R_a) subtypes [1,2]. These receptors are coupled in an inhibitory (A_1) or stimulatory (A_2) manner to adenylate cyclase [1,2]. However, adenosine receptors coupled to other effector systems, such as ion channels, may exist as well [3–5]. In fact, (i) some of the actions of adenosine may not be mediated simply by effects on adenylated cyclase and (ii) in some systems (for instance, in nerve terminals) effects on adenylate cyclase activity may be difficult or impossible to demonstrate [6,7].

The general inhibitory effect of adenosine receptor agonists on neurotransmitter release suggests that adenosine might exert its effect in neurons via inhibition of calcium influx [3,5]. Similarly, in eliciting vasodilation, adenosine may act by interfering with calcium entry and availability, by the direct modulation of voltage-dependent fluxes [8,9].

It has been shown that, at the ligand-binding level, calcium antagonists of the dihydropyridine class have specific and potent effects on both adenosine receptors and uptake sites [10]. It has been also noted that the non-dihydropyridine calcium channel blockers, such as verapamil and diltiazem, display a much lower potency for both

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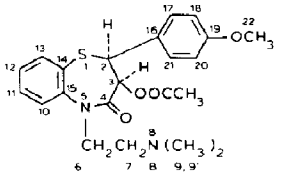
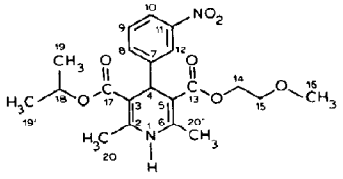


Diltiazem



Nimodipine

Fig. 1.

the receptor and uptake system [10]. These findings have suggested that adenosine sites are selectively coupled with the dihydropyridine-sensitive site.

In order to ascertain whether the sites for adenosine and those for calcium entry blockers are coupled, human neutrophils were herein considered, since adenosine (i) affects free radical formation and enzyme release from these cells [11] and (ii) it may act with a mechanism involving membrane calcium transport, as suggested by some experimental evidence [11].

Two calcium entry blockers belonging to different classes were chosen, nimodipine {(3-[(2-methoxyethoxy)carbonyl]-5-(isopropoxycarbonyl)-4-(3-nitrophenyl)-2,6-dimethyl-1,4-dihydropyridine)} and diltiazem {3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one} (fig. 1).

Among various physical techniques, ^{1}H-NMR investigations were given prominence, since NMR has the potential of delineating conformational features of ligands bound at macromolecular receptors. It was in fact determined, a few years ago, that measuring spin-lattice relaxation rates after 'selective' excitation of any ligand nuclei yields a parameter (R_1^{sel}) that is very sensitive to binding of the ligand to large macromolecules or even to whole cells [12–15]. By measuring such selective relaxation rates, binding of flunarizine to the surface of human neutrophils was previously detected [16] and antagonism between flunarizine and adenosine at the neutrophil surface was demonstrated to occur.

2. Materials and methods

Nimodipine was supplied by Janssen Pharmaceutica (Beerse, Belgium), diltiazem and adenosine being supplied by Sigma (St. Louis, MO). All substances were used without further purification. Solutions were made in ^2H_2O (99.95%; Merck, Rahway, NJ) and the p^{2}H was adjusted with either ^{2}HCl or NaO^{2}H.

Normal human venous blood, anticoagulated with preservative-free heparin (5 IU/ml), was mixed with 3 ml of high molecular weight dextran (70000 Da, Pharmacia, Uppsala, Sweden) and allowed to sediment at room temperature for 60 min. The supernatant leukocyte-rich plasma was centrifuged at $250 \times g$ for 7 min. The pellet was then subjected to hypotonic lysis to remove red cells by the addition of 3 ml of 0.2% NaCl for 75 s; restoration of isotonicity was obtained by the addition of 7 ml of 1.2% NaCl. The cells were centrifuged at $250 \times g$ 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.

The NMR measurements were performed with a VXR-200 NMR spectrometer (Varian Associates, Palo Alto, CA) at a constant temperature of 303 ± 1 K. The nonselective spin-lattice relaxation rates, R_1^{nsel}, were determined by using the inversion recovery pulse sequence $(180\text{–}\tau\text{–}90\text{–}t)_n$. The selective spin-lattice relaxation rates, R_1^{sel}, were measured in the initial rate approximation [17] by giving a selective 180° pulse with the proton decoupler at the selected frequency for a relatively long time (typically 20–30 ms). After the variable delay, a nonselective 90° pulse was applied to detect the longitudinal magnetization. The R_1^{nsel}

and R_1^{sel} values were obtained from a three-parameter exponential regression analysis of the recovery curve for longitudinal magnetization.

3. Results and discussion

The selective relaxation rate of any proton i is given by [12,17,18]:

$$R_1^{sel} = \sum_{i \neq j} \rho_{ij} + \rho_i^* \quad (1)$$

where ρ_{ij} denotes the direct relaxation term accounting for dipole-dipole interaction between the excited proton and any other nearby proton and the summation is extended to all the interacting i-j proton pairs; ρ_i^* accounts for relaxation mechanisms other than the dipolar type. As a function of the motional correlation time, R_1^{sel} increases almost linearly with the slowing down of molecular motions. This is the reason why, in contrast with the usual nonselective spin-lattice relaxation rate (R_1^{nsel}), R_1^{sel} makes it possible to detect binding to large macromolecules.

In a free ligand in solution, measuring R_1^{sel} and R_1^{nsel} is just a way of determining the efficiency of the dipolar relaxation mechanism. From this point of view, the data in table 1 strongly suggest that the dipolar interaction provides the main relaxation mechanism for most protons of both diltiazem and nimodipine. It is in fact predicted from the theory that R_1^{nsel}/R_1^{sel} should measure 1.5 for a pure dipolar relaxation mechanism, provided molecular motions occur within the extreme narrowing region ($\omega^2\tau^2 \ll 1$, ω being the Larmor frequency and τ the motional correlation time) [17].

Table 1

Nonselective and selective ^{1}H-NMR spin-lattice relaxation rates and F ratios ($F = R_1^{nsel}/R_1^{sel}$) for some protons of diltiazem (DLZ) and nimodipine (NMD) (0.1 mol dm^{-3} in ^2H_2O) at p^{2}H = 7.6 and T = 303 K

Substance	Proton	R_1^{nsel} (s^{-1})	R_1^{sel} (s^{-1})	F
NMD	H_8	1.60	1.16	1.38
	H_{10}	0.90	0.67	1.35
	H_4	1.18	0.86	1.37
	H_{18}	0.95	0.68	1.39
DLZ	H_6	4.31	3.10	1.39
	$H_{6'}$	3.69	2.62	1.41
	$H_{17,21}$	1.59	1.15	1.38
	$H_{18,20}$	1.46	1.03	1.42

Table 2

Enhancement of proton relaxation rates ($\Delta R = _{cell} - R_{blank}$) measured for selected protons of nimodipine (NMD) and diltiazem (DLZ) (0.1 mol dm^{-3} in ^2H_2O) at p^{2}H 7.6 and T = 303 K in the presence of 6×10^4 neutrophils/ml

Substance	Proton	ΔR_1^{nsel} (s^{-1})	ΔR_1^{sel} (s^{-1})
NMD	H_{10}	0.12	0.47
	H_4	0.08	0.43
DLZ	H_6	0.21	1.42
	$H_{6'}$	0.16	0.81
	$H_{17,21}$	0.07	0.57

In the presence of receptors at the surface of neutrophils (6×10^4 cells/ml), chemical exchange between free and bound states must be taken into account. If the rate of such an exchange process is faster than the spin-lattice relaxation rate in the bound state, what is measured is merely a weighted average between the rates in the two environments:

$$R_1^{nsel}(obs) = p_f R_{1f}^{nsel} + p_b R_{1b}^{nsel} \quad (2)$$

$$R_1^{sel}(obs) = p_f R_{1f}^{sel} + p_b R_{1b}^{sel} \quad (3)$$

where p_f and p_b are the fractions of free and bound ligand, respectively. Under the conditions of our experiments, 1 ml of solution contains 6×10^4 neutrophils and 0.1 mmol of calcium entry blocker; eqs 2 and 3 can then be suitably rewritten as ($p_b \ll 1$, $p_f \simeq 1$):

$$R_1^{nsel}(obs) - R_{1f}^{nsel} = \Delta R_1^{nsel} = p_b R_{1b}^{nsel} \quad (4)$$

$$R_1^{sel}(obs) - R_{1f}^{sel} = \Delta R_1^{sel} = p_b R_{1b}^{sel} \quad (5)$$

Since the same p_b occurs in eqs 4 and 5 and since R_{1b}^{sel} can be reasonably expected to be much faster than R_{1b}^{nsel} [12], binding to neutrophils was the determining factor, as shown in table 2, causing considerable enhancement of R_1^{sel} while not severely affecting R_1^{nsel}.

Table 3

Selective relaxation rate enhancements measured for selected protons of diltiazem (DLZ) and nimodipine (NMD) (0.1 mol dm^{-3} in ^2H_2O at $p^2H = 7.6$ and $T = 303$ K) in the presence of 6×10^4 neutrophils/ml at varying concentrations of adenosine

Substance	Proton	[Adenosine] (mM)	ΔR^{sel} (s^{-1})
DLZ	H_6	–	0.82
		0.1	0.68
		1.0	0.51
		10.0	0.19
	$H_{17,21}$	–	0.57
		0.1	0.46
		1.0	0.28
		10.0	0.11
NMD	H_{10}	–	0.47
		0.1	0.41
		1.0	0.36
		10.0	0.12
	H_4	–	0.43
		0.1	0.38
		1.0	0.27
		10.0	0.10

As changes in viscosity of the medium are not likely to determine the observed selective relaxation enhancement (vide infra), it can be concluded that both ligands bind to the neutrophil cell surface, giving rise to the slowing down of the molecular motions of a small fraction of ligand molecules that undergo chemical exchange with the bulk.

Eq. 5 was considered for the studies of antagonism between adenosine and either nimodipine or diltiazem. The selective relaxation rate enhancement, ΔR_1^{sel}, was in fact measured at varying concentration of adenosine in the range 0.1–10 mmol dm^{-3} as shown in table 3. It is apparent that ΔR_1^{sel} decreases with increasing concentration of adenosine. Since R_{1b}^{sel} is not likely to change upon addition of any cosolute, the decrease in ΔR_1^{sel} must be due to a decrease in p_b, i.e., to displacement of calcium-entry blocker molecules from the bound state. It is also apparent that additions of adenosine change ΔR_1^{sel} while not severely affecting the viscosity of the medium. Viscosity effects can therefore be disregarded in interpreting spin-lattice relaxation rates. Moreover, the fact that ΔR_1^{sel} is mainly determined by parameters of the bound environment renders any eventual interaction between free ligands irrelevant for the nuclear relaxation analysis.

It can be concluded that binding of the two calcium-entry blockers to the surface of neutrophils was detected by the enhancement of selective relaxation rates. In the same way, it was shown that the presence of adenosine, as cosolute, results in the displacement of either nimodipine or diltiazem molecules from the bound site, i.e., that adenosine acts as an antagonist of the two calcium-entry blockers. Displacement of bound ligand occurred in a concentration-dependent fashion.

Interpretation of the effects of the endogenous mediator adenosine on the interactions of calcium-entry blockers at the neutrophil surface may be quite relevant from the physiological point of view. No direct evidence has so far been obtained that competition exists in that region for the same sites, since what has been detected by NMR could also be accounted for in terms of adenosine and calcium-entry blocker acting at nearby or coupled sites. Anyway, since adenosine and calcium-entry blockers behave similarly in several preparations, specifically in neutrophils, and adenosine inhibits Ca^{2+} current in different cell lines, a role for adenosine as endogenous calcium-entry blocker may also be suggested in the light of the present NMR results.

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