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Original article

Nuclear magnetic resonance for studying recognition processes between anandamide and cannabinoid receptors

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Abstract

The understanding of the molecular basis of cannabinoid activity has greatly improved since the discovery of CB1 and CB2 receptors. In this paper, the ligand binding processes between the endogenous cannabimimetic ligand, anandamide (AEA), and the cannabinoid receptors from different parts of rat brain were studied by nuclear magnetic resonance spectroscopy. The NMR approach is based on the comparison of selective (R_1^{SE}) and non-selective (R_1^{NS}) proton spin-lattice relaxation rates of the ligand in the presence and absence of macromolecular receptors, as well as R_1^{NS} and R_1^{SE} temperature dependency analysis. From these studies, the ligand–receptor binding strength was evaluated on the basis of the calculation of the "*affinity index*". The derivation of the "*affinity index*" from chemical equilibrium kinetics for all systems allowed the comparison of the ability of anandamide to interact with cannabinoid receptors present in different brain sectors.

Keywords: NMR; Proton spin-lattice relaxation rate; Affinity index; Anandamide; Cannabinoid receptors

1. Introduction

For centuries marijuana, derived from the *Cannabis sativa*, has been used for its medicinal as well as its psycotropic effects [1]. Trans-Δ-tetrahydrocannabinol (THC) is the major active psychotropic component of cannabis [2]. Central nervous system responses to THC and synthetic cannabimimetic drugs include the therapeutically beneficial analgesia, attenuation of nausea and vomiting in cancer chemotherapy, appetite stimulation in wasting syndromes, and reduction of intestinal motility [3]. These effects are mediated by receptors, two of which have been identified as the CB1 and CB2 receptors [4]. In 1992, the first cannabinoid receptor, CB1 was cloned and classified as a member of the family of G-protein-coupled receptors [5]. The CB1 cannabinoid receptors have been found in high abundance in brain neurons, with highest levels expressed in basal ganglia, cerebellum, hippocampus [6]. Considerably lower expression was found in pheripheral tissues, as testis, uterus, vascular tissue, lungs. Following agonist binding, CB1 receptors couple to the inhibition of adenylate cyclase, inhibition of N- and Q-type voltage-operated calcium channels, and stimulation of inwardly rectifying and A type potassium channels. A second cannabinoid receptor, CB2, was cloned in 1993. The CB2 receptor was found in the periphery and mainly in tissues of the immune system, coupled to inhibition of adenylate cyclase, but did not appear to couple to ion channel regulation [7,8].

The first endogenous cannabinoid was isolated from porcine brain by Devane et al. and was found to be an unsaturated fatty acid ethanolamide, cis-5,8,11,14 eicosatetraenoylethanolamine (AEA or anandamide).

In human brain, AEA has been found mainly in the hippocampus, in the thalamus, in the cerebellum and in the striatum, but it is also found in the periphery, precisely in the spleen and heart [9].

Anandamide activates CB1 receptors more efficaciously than CB2 receptors [6], exhibiting higher affinity for the can-

Abbreviations: AEA, cis-5,8,11,14 eicosatetraenoylethanolamine or anandamide; $[A]_L^T$, affinity index (mg^{-1} dm^3 s^{-1}); R_1^{NS}, non-selective proton spin-lattice relaxation rates; R_1^{SE}, selective proton spin-lattice relaxation rates.

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nabinoid CB1 receptor (K_i CB1 = 89 ± 10 nM) than for the CB2 receptor (K_i CB2 = 371 ± 102 nM) [10].

More recently, 2-arachidonylglycerol (2-AG), isolated from intestinal tissue [11], has been found to be a second endogenous CB ligand, but at more modest potencies and efficacies compared to anandamide, and may be more selective for CB2 receptors under certain conditions [12].

AEA, as other endocannabinoids, is not stored in vesicles, but phospholipid molecules found within the membrane phospholipid pool serve as precursor and storage depots for anandamide release; phosphatidylethanolamine is enzymatically converted to N-arachidonylphosphatidylethanolamine, the storage form of anandamide [13]. Due to its hydrophobic properties, AEA seems to act on the same or neighboring cells and termination of its endocannabinoid activity appears to be a two-step process that involves carrier-mediated re-uptake of AEA across the plasma membrane, followed by enzymatic degradation by the fatty acid amide hydrolase (FAAH) [14].

Whereas a specific transporter, which provides a mechanism for high-affinity uptake of endocannabinoids, has been characterized but not isolated or cloned yet, the enzyme responsible for anandamide hydrolysis has been cloned in 1996 [15] and found to be membrane associated, pH-dependent and selective.

The recognition step, which is related to the surface properties of the interacting molecules [16–18], is crucial in any biological reaction involving two or more chemical structures.

Several experimental and theoretical approaches have been developed to study the recognition processes between ligands and receptors [19–21]. Nuclear magnetic resonance techniques have been widely used for studying association equilibria in biological systems [22–24], providing the advantage of non-invasivity and non-alteration of the normal bio-functionality of the biomolecules under investigation [25]. The large number of spectral parameters that can be measured and analyzed by using NMR techniques, allowed the resolution of various chemical and biological problems, related to the analysis of the kinetics [26], as well as to the structural features of the processes [27].

The most important parameters which are able to give powerful information about ligand–receptor interaction are the chemical shift [28], relaxation rates and linewidth [29], NOE [30], together with NMR methods such as pulsed gradient diffusion [31].

In this paper, we present an approach based on NMR measurement which is capable of thoroughly investigate the interaction processes occurring between anandamide and cannabinoid brain receptors.

The NMR methodology used in this work is based on the comparison of selective ($R_1{}^{SE}$) and non-selective ($R_1{}^{NS}$) proton spin-lattice relaxation rate analysis of the anandamide (i.e. the ligand) in the presence and absence of cannabinoid receptors. Assuming fast chemical exchange between the bound and the free environments with respect to both chemical shift difference and proton relaxation rate, the formation of intermolecular adducts affects $R_1{}^{NS}$ and $R_1{}^{SE}$ to different extents, depending on the dynamical parameters (i.e. the correlation time τ_c). In particular, the slower ligand dynamics in the ligand–receptor complex mostly affects $R_1{}^{SE}$. In the presence of well resolved proton resonances, $R_1{}^{SE}$ can be easily determined in different systems. The contributions arising from the fraction of the ligand bound to the cannabinoid receptors allowed the calculation of the "*affinity index*" $[A]_L^T$, a useful parameter related to the strength of non-specific and/or specific interactions occurring within the systems [32]. The affinity index calculated from proton selective relaxation rates provides a deeper knowledge of the dynamics of the AEA–receptor interaction process, and offers several advantages. It is calculated as the slope of a straight line passing trough the origin and is less affected by intrinsic errors than the intercept calculation. It represents the global affinity between the ligand and the cannabinoid receptors, and its calculation does not require an a priori knowledge of the number of ligand coordination sites present at the macromolecule surface or their specific kinetics constant values. In particular, this methodology allows the comparison of the strength of the interaction processes involving the same ligand and different cannabinoid receptors.

The aim of this paper was to use a spectroscopic method, described previously in [33,34] in order to evaluate the strength of the interaction between AEA and cannabinoid receptors. The knowledge of the equilibrium constants K_i for the binding of CB1 receptors with anandamide, served to check the validity of the proposed NMR methodology.

Different interactions were analyzed using three brain samples, the whole brain, the cerebral cortex and the cerebellum. The knowledge of the distribution of CB1 receptors in the brain was useful in the analysis of the molecular recognition phenomena between a specific ligand (AEA) and different samples obtained from three zones of the brain.

2. Theory

The relaxation process assumes an exponential decay with a rate constant R_1. For multispin interactions that occur in complex systems of biomolecules, the "non-selective" spin-lattice relaxation rate $R_1{}^{NS}$ of an i nucleus interacting with neighboring j nuclei is described in Eq. (1). The selective $R_1{}^{SE}$ obtained by excitation of the i nucleus, while the j nuclei are at thermal equilibrium [35] is described in Eq. (2) [27,36]:

$$R_1^{NS} = \frac{1}{10}\frac{\gamma_H^4\hbar^2}{r_{ij}^6}\left[\frac{3\tau_c}{1+\omega_H^2\tau_c^2}+\frac{12\tau_c}{1+4\omega_H^2\tau_c^2}\right] \quad (1)$$

$$R_1^{SE} = \frac{1}{10}\frac{\gamma_H^4\hbar^2}{r_{ij}^6}\left[\frac{3\tau_c}{1+\omega_H^2\tau_c^2}+\frac{6\tau_c}{1+4\omega_H^2\tau_c^2}+\tau_c\right] \quad (2)$$

By Eqs. (1) and (2), in fast molecular conditions typical of the free ligand, $R_1{}^{NS} > R_1{}^{SE}$. When a ligand is bound to a macromolecule with slow macromolecular reorientation, ($\omega_0\tau_c >> 1$), there is a substantial contribution to the selective spin-lattice relaxation rate from the bound ligand. This occurs under the following conditions, $R_1{}^{SE} > R_1{}^{NS}$.

The spin-lattice relaxation rate of a ligand under conditions of fast chemical exchange between the free and bound states is

described by:

$$R_{1\exp=}X_B R_{1B} + X_F R_{1F} \quad (3)$$

where $R_{1\exp}$ is the relaxation rate of the ligand in the presence of the receptor, R_{1B} and R_{1F} are the relaxation rates of the pure bound and free environments, and X_B and X_F are the molar fractions of the ligand in bound and free conditions.

If we consider the ligand–macromolecule equilibrium:

$$M + L \rightleftharpoons ML \quad (4)$$

assuming $[L] >> [M_0]$, it has been shown that:

$$\Delta R = \frac{K R_{1B}}{1 + K[L]}[M_0] \quad (5)$$

where $\Delta R = R_{1\exp} - R_{1F}$, K is the thermodynamic equilibrium constant, and $[M_0]$ is the initial receptor concentration.

As suggested by Eq. (5), plot ΔR vs. $[M_0]$ would have a straight line through the origin, with slope:

$$[A]_L^T = \left(\frac{K R_{1B}}{1 + K[L]}\right) \quad (6)$$

which was defined as "*affinity index*" (mol^{-1} dm^3 s^{-1}) [32].

The affinity index is a constant if temperature and ligand concentrations are specified, as suggested by the T and L subscripts in the affinity index symbol. In our case the affinity index is reported as (mg^{-1} dm^3 s^{-1}). In fact, in this experiment the receptor concentration is shown as mg of total protein present in the tissue [37]. It is not possible to know the molecular weight of the proteins.

The recognition process between small ligands and biomacromolecule surfaces can be studied by nuclear magnetic resonance spectroscopy if the following conditions hold:

- The ligand must experience a fast chemical exchange between the free and bound environments with respect to the NMR timescale. In these conditions the NMR parameters have a weighted means between the values assumed in each environment.
- The total bound ligand must be small compared to that of the free ligand.
- The observed NMR parameters (i.e. in this case, the proton spin-lattice relaxation rates), must be heavily affected by the presence of the receptors.

3. Results and discussion

The anandamide structure was reported in Fig. 1. The anandamide proton spectrum was previously assigned by Bonechi et al. [38]. In order to verify the existence of interaction processes between anandamide and cannabinoid receptors, obtained from three different cerebral samples, NH non-selective and selective proton spin-lattice relaxation rates were measured as a function of protein receptor concentration.

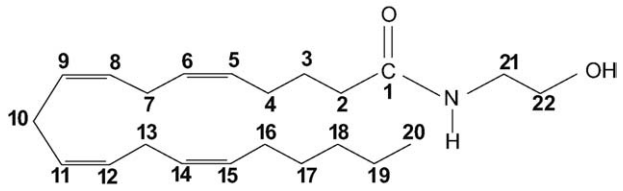

Fig. 1. Structure and numbering of cis-5,8,11,14 eicosatetraenoylethanolamine (anandamide or AEA).

In particular, this study focused on the interactions between the AEA solution and:

- whole brain receptors;
- cerebellum receptors;
- cerebral cortex receptors.

Fig. 2 shows the plot of R_1^{SE} and R_1^{NS} of anandamide in relation to whole brain receptor concentration. In the absence of the proteins, the NH non-selective spin-lattice relaxation rate is larger than the selective relaxation rate, while increasing receptor concentration R_1^{SE} becomes greater than R_1^{NS}. In particular, R_1^{SE} was subject to a strong increase, while R_1^{NS} remained constant in the presence of receptor. These results led to the hypothesis that interaction processes occurred between anandamide and total brain receptor.

Similar results were obtained for the other two samples obtained from cerebral cortex and cerebellum. The plot of R_1^{SE} and R_1^{NS} of anandamide in relation to cerebral cortex and cerebellum receptor concentration are reported in Fig. 3 A, B, respectively. In both cases the selective relaxation rate increases with increasing concentration of the receptors indicating the presence of a drug–receptor interaction. Moreover, these experimental results show that, in the case of AEA–cerebellum receptor interaction, R_1^{SE} assumes greater values

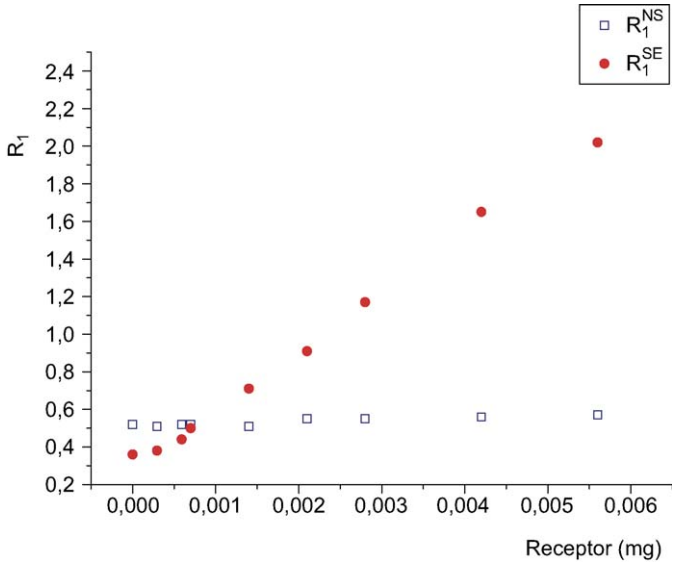

Fig. 2. Selective and non-selective proton relaxation rate (s^{-1}) of the NH proton of anandamide in relation to whole brain receptor concentration.

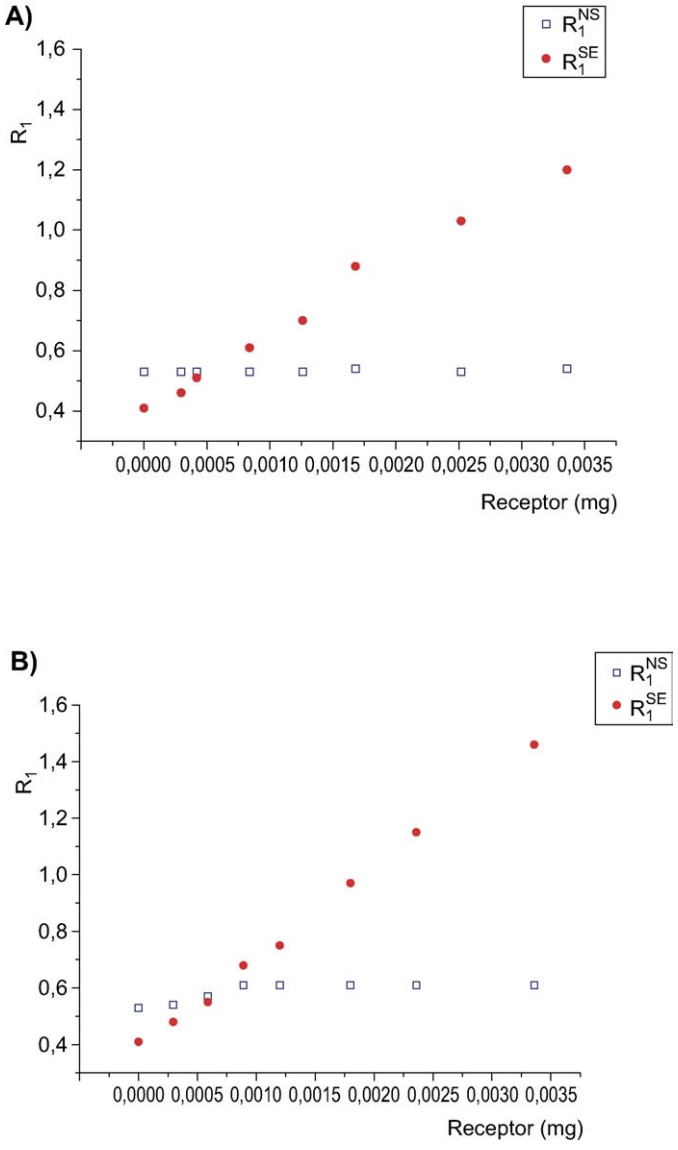

Fig. 3. A) Selective and non-selective proton relaxation rate (s^{-1}) of the NH proton of anandamide in relation to cerebral cortex receptor concentration. B) Selective and non-selective proton relaxation rate (s^{-1}) of the NH proton of anandamide in relation to cerebellum receptor concentrations.

with respect to the AEA–cerebral cortex receptor system, indicating a different extent of the two binding processes.

The general conditions capable of identifying the existence of an interaction between a ligand and a macromolecule can be summarized as:

- ligand proton relaxation rate must change from the condition in which $R_1^{NS} > R_1^{SE}$ (free ligand) to $R_1^{NS} < R_1^{SE}$, in the presence of the macromolecule;
- temperature dependency analysis must show an increase of R_1^{NS} with increasing temperature (R_1^{SE} does not appear to be diagnostic since it always decreases with increasing temperature).

In systems that contain molecules with high molecular weights, it is possible that an increase in viscosity due to the presence of the macromolecule, such as receptors, could cause a slow down in the dynamics of the ligand even in the absence of an interaction with the receptor. If this occurs, the increase in the proton selective spin-lattice relaxation rate (i.e. the change from $\omega_0\tau_c << 1$ to $\omega_0\tau_c >> 1$ motion conditions) may not be due to the occurrence of a ligand–receptor recognition process, while R_1^{NS} remains unaffected since it assumes similar values under both fast and slow-motion conditions. A temperature dependent analysis of R_1^{NS} and R_1^{SE} of AEA in the presence of the three different receptors, distinguished the effects due to the ligand–receptor interaction from those caused by a change in viscosity. In systems with high viscosity, the ligand molecules are subject to a slow reorientational motion, and while R_1^{SE} still decreases, R_1^{NS} increases with increasing temperature. The R_1^{NS} and R_1^{SE} values of anandamide, in the absence and presence of receptors were analyzed to understand the behavior of relaxation rate with temperature. Table 1 reports R_1^{SE} and R_1^{NS} values of AEA in the absence and presence of the three different types of receptors. The selective and non-selective relaxation rates decrease with increasing temperature. These results suggest that the increase in the receptor concentration in solution did not cause a relevant change in the viscosity of the solution. This provides the evidence that the increase observed in R_1^{SE} values was due to the formation of anandamide–receptor complexes.

In order to compare the strength of the interaction between AEA and whole brain, cerebral cortex and cerebellum receptors, the *"affinity indexes"* for the three systems were calculated from the slope of the straight lines obtained by linear regression analysis of $\Delta R_1^{SE}(\Delta R_1^{SE} = R_{1exp} - R_{1F})$ versus receptor concentration (Fig. 4). The affinity indexes for the AEA–whole brain receptor, AEA–cerebral cortex receptor and AEA–cerebellum receptor were found to be 292.78 ± 3.25, 238.01 ± 2.50 and 313.16 ± 2.74 mg^{-1} dm^3 s^{-1}, respectively. These values suggest that the drug interacts with the protein receptorial system at different strengths. As expected, data show that the AEA binds pre-

Table 1
NH anandamide (1.4×10^{-2} M) selective and non-selective relaxation rate values in relation to temperature in the absence and presence of receptor. Relaxation rate (R_1) are reported in s^{-1}. The estimated error was 5%

	AEA				AEA			
	Without receptor		Whole brain receptor (1.5×10^{-3} mg)		Cerebral cortex receptor (1.5×10^{-3} mg)		Cerebellum receptor (1.5×10^{-3} mg)	
Temperature (K)	R_1^{NS}	R_1^{SE}	R_1^{NS}	R_1^{SE}	R_1^{NS}	R_1^{SE}	R_1^{NS}	R_1^{SE}
298	0.52	0.36	0.53	0.61	0.54	0.88	0.61	0.97
303	0.45	0.31	0.45	0.47	0.46	0.51	0.56	0.63
308	0.37	0.25	0.40	0.30	0.35	0.40	0.47	0.52

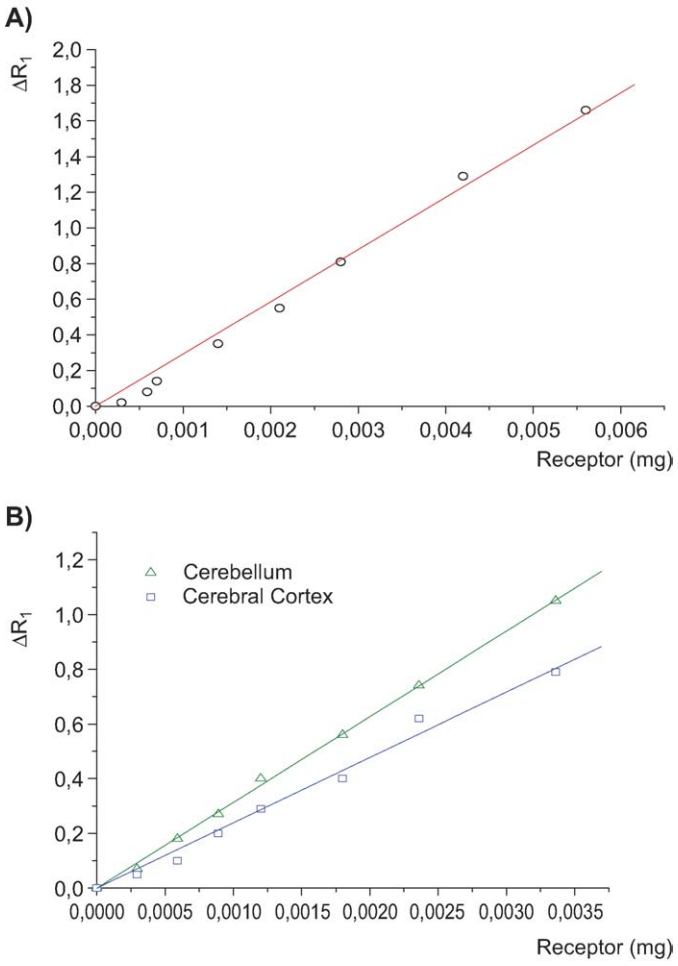

Fig. 4. Comparison of the linear regression analysis of the NH selective relaxation enhancement, ΔR_1^{SE}, as a function of receptor concentration: A) Experimental data obtained for the interaction between anandamide (1.4×10^{-2} M) solution and whole brain receptor ($[A]_L^T = 292.78 \pm 3.25$ mg^{-1} dm^3 s^{-1}). B) Experimental data obtained for the interaction between anandamide (1.4×10^{-2}M) solution and cerebral cortex ($[A]_L^T = 238.01 \pm 2.50$ mg^{-1} dm^3 s^{-1}) and cerebellum ($[A]_L^T = 313.16 \pm 2.74$ mg^{-1} dm^3 s^{-1}). The R^2 values obtained by the linear regression analysis were 0.998, 0.998 and 0.999 for the AEA–whole brain interaction, the AEA–cerebral cortex and the AEA–cerebellum, respectively.

ferentially with the receptors present in the cerebellum, since it is known from the literature that CB1 receptors are present in this brain section at very high concentration [39]. The lowest affinity index value obtained for the AEA–cerebral cortex interaction is also in perfect agreement with the cannabinoid receptor distribution found in the work by Breivogel and Childers [39].

The information which can be derived from the above analysis can be summarized as follows:

- AEA was involved in interaction processes with the receptors present in the three brain samples under study, confirming its ability to bind to cannabinoid receptors.
- The affinity index values calculated in this work suggest a preferential interaction between AEA and cerebellum receptors ($[A]_L^T = 313.16 \pm 2.74$ $mg^{-1}.dm^3.s^{-1}$) in respect to AEA–whole brain receptors ($[A]_L^T = 292.78 \pm 3.25$ $mg^{-1}dm^3.s^{-1}$) and AEA–cerebral cortex receptors ($[A]_L^T = 238.01 \pm 2.50$ $mg^{-1}.dm^3.s^{-1}$).

These results are in agreement with the distribution of CB1 receptors in different portions of the brain [6], confirming the potentiality of this experimental approach for studying recognition processes occurring in complex biological systems.

Moreover, these results underline the importance of $[A]_L^T$, as a powerful index to compare the ability of a ligand to interact with different receptors.

4. Conclusions

Selective and non-selective proton spin-lattice relaxation rate analysis of AEA in the presence of different cerebral receptors allowed the identification of interaction processes occurring at different strengths. In particular AEA was found to interact preferentially with cannabinoid receptors present in the cerebellum.

This study confirms the biological role played by AEA in simulating trans-Δ-9-tetrahydrocannabinol effects on cerebral receptors.

5. Experimental

5.1. Materials

Cis-5,8,11,14 eicosatetraenoylethanolamine or anandamide (Fig. 1) was purchased from Sigma Chemical Co. and used without any further purification. The solvent used for NMR spectra was mixture DMSO-d_6/D_2O (1:1). The anandamide solution was used as 1.4×10^{-2} M.

Cannabinoid receptors were obtained from brain of rat in PBS and H_2O solution.

All the receptor suspensions have been specifically prepared in order to obtain the same amount of total protein within all the samples. This procedure was indispensable to compare the results obtained by various experiments. The affinity index is expressed as (mg^{-1} dm^3 s^{-1}).

All measurements, unless otherwise specified, were performed at 298 K.

5.2. Methods

5.2.1. Membranes preparations

Male Sprague–Dawley rats weighing 200–250 g (Harlan Italy s.r.l., Correzzana, Milan) were sacrificed by decapitation. The brains were rapidly removed and dissected on ice. Whole brain, cortex and cerebellum were used for separate membrane preparations according to Compton et al. [40] with some modifications. Each of them was homogenized with Polytron® Apparatus (Kinematica GmbH, Littau, Switzerland) in 10 volumes of phosphate-buffered saline (PBS), pH 7.4 and then centrifuged at 1600 × g for 10 min. The supernatant was saved and combined with two subsequent supernatant fractions obtained washing and centrifuging at the same speed the pellet

two more times. The combined supernatants fractions were centrifuged at 39,000 × *g* for 15 min. The resulting pellet (P_2) was suspended in 5 volumes of deuterium oxide (D_2O) (Acros Organics-Geel, Belgium), homogenized and centrifuged at 23,000 × *g* for 10 min. The obtained pellet (P_3) was resuspended in 2.5 volumes of D_2O, homogenized and centrifuged at 23,000 × *g* for 10 min. At least the pellet was suspended in 1.25 volumes of D_2O and then frozen at –80 °C before being assayed.

5.2.2. NMR measurements

The solutions for the NMR experiments were obtained by dissolving the appropriate amounts of anandamide and cannabinoid receptor in DMSO-d_6/D_2O (1:1).

^{1}H-NMR spectra were obtained with a Bruker DRX 600 AVANCE spectrometer operating at 14.7 T. The spin-lattice relaxation rates were measured using the "inversion-recovery" $(RD - 180° - \tau - 90° - t)_n$ sequence, where RD = 20 s. The 90° pulse length was 9.2 μs. The τ values used for the selective and non-selective experiments were: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.5, 2, 3, 4, 5, 7, 10 s, respectively. The selective inversion of the proton spin population was obtained by a selective soft perturbation pulse [41]. The shaped pulse of 80 ms corresponds to a field strength of 15 Hz. All the selective and non-selective spin-lattice relaxation rates refer to the NH proton of anandamide. The T_1^{SE} inversion recovery data in the presence of whole brain receptors is described in Supplementary Material (Fig. 1). In order to evaluate the possible exchange of NH proton in the presence of H_2O, the ^{1}H spectrum of anandamide in 100% DMSO was compared to the ^{1}H spectrum in a DMSO/D_2O 1:1 solution (spectra are available upon request). The chemical shift as well as the line width of NH resonance didn't change appreciably, indicating that proton-deuterium exchange can be considered negligible.

In general the recovery of proton longitudinal magnetization after a 180° pulse is not a single exponential, due to the sum of different relaxation terms, the selective spin-lattice relaxation rates were calculated using the initial slope approximation and subsequent three parameter exponential regression analysis of the longitudinal recovery curves.

The maximum experimental error in the relaxation rate measurements was estimated to be 5%. The affinity index was calculated by linear regression analysis of the experimental data.

The NMR data were processed with XWINNMR software (version 2.5) on Silicon Graphics O2 equipped with RISC R5000 processors, working under the IRIX 6.3 operating system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2006.05.017.

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