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Alteration of A_3 adenosine receptors in human neutrophils and low frequency electromagnetic fields

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Abstract

The present study was designed to evaluate the binding and functional characterization of A_3 adenosine receptors in human neutrophils exposed to low frequency, low energy, pulsing electromagnetic fields (PEMFs). Great interest has grown concerning the use of PEMF in the clinical practice for therapeutic purposes strictly correlated with inflammatory conditions. Saturation experiments performed using the high affinity and selective A_3 adenosine antagonist 5*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]-MRE 3008F20) revealed a single class of binding sites with similar affinity in control and in PEMF treated human neutrophils ($K_D = 2.36 \pm 0.16$ and 2.45 ± 0.15 nM, respectively). PEMFs treatment revealed that the receptor density was statistically increased ($P < 0.01$) ($B_{max} = 451 \pm 18$ and 736 ± 25 fmol mg^{-1} protein, respectively). Thermodynamic data indicated that [^{3}H]-MRE 3008F20 binding in control and in PEMF-treated human neutrophils was entropy and enthalpy driven. Competition of radioligand binding by the high affinity A_3 receptor agonists, N^6-(3-iodo-benzyl)-2-chloro-adenosine-5′-*N*-methyluronamide (Cl-IB-MECA) and N^6-(3-iodo-benzyl)adenosine-5′-*N*-methyl-uronamide (IB-MECA), in the absence of PEMFs revealed high and low affinity values similar to those found in the presence of PEMFs. In both experimental conditions, the addition of GTP 100 μM shifted the competition binding curves of the agonists from a biphasic to a monophasic shape. In functional assays Cl-IB-MECA and IB-MECA were able to inhibit cyclic AMP accumulation and their potencies were statistically increased after exposure to PEMFs. These results indicate in human neutrophils treated with PEMFs the presence of significant alterations in the A_3 adenosine receptor density and functionality.

Keywords: Adenosine A_3 receptors; Human neutrophils; [^{3}H]-MRE 3008F20 binding; Binding thermodynamics; Cyclic AMP; Pulsing electromagnetic fields (PEMFs)

1. Introduction

Adenosine mediates a number of physiological functions through the interaction with four cell surface subtypes classified as A_1, A_{2A}, A_{2B} and A_3 receptors which are coupled to G proteins [1]. The A_1 and A_3 subtypes, via Gi and Go family, mediate inhibition of the adenylyl cyclase activity, in contrast both A_{2A} and A_{2B} subtypes, coupled to Gs, determine the stimulation of cAMP production [2,3]. In particular, the A_3 adenosine receptor subtype causes adenylyl cyclase inhibition and phospholipase C activation [4]. The A_3 receptor plays a role in modulation of cerebral ischemia [5] with dual and opposite neuroprotective and neurodegenerative effects [6]. At cardiovascular level, A_3 adenosine receptors are involved in the ischemic heart preconditioning [7], hypotension [8] and exert many cardioprotective effects through modulation of tissue factor release [9]. More recently, several papers point to the A_3 adenosine receptor as a promising therapeutic target on cell growth [10] and on apoptosis [11]. Adenosine, through the A_3 receptor, induces a differential effect on tumor and

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Abbreviations: cAMP, cyclic AMP; [^{3}H]-MRE 3008F20, 5*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo-[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine; Cl-IB-MECA, N^6-(3-iodo-benzyl)-2-chloro-adenosine-5′-*N*-methyluronamide; IB-MECA, N^6-(3-iodo-benzyl)adenosine-5′-*N*-methyluronamide; Ro 20-1724, 4-(3-butoxy-4-methoxy benzyl)-2-imidazolidinone; PEMFs, pulsing electromagnetic fields.

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normal cells [12,13]. Moreover, the presence of A_3 adenosine receptors on leukemic Jurkat T cells [4] and on human malignant melanoma A375 cell line [14] suggests a potential role for adenosine in modulating tumoral processes [15]. It has been demonstrated that anti-inflammatory actions produced by adenosine involves A_{2A} and A_3 adenosine receptor subtypes [16]. Of particular interest is the presence of a strong link between responsiveness to adenosine and mast-like cells [17] in inflammatory disease such as asthma [18,19]. In human neutrophils A_3 receptors exert their anti-inflammatory properties by inhibiting degranulation, chemotaxis and superoxide anion production [20,21]. To date binding and functional characterization of A_3 receptors has been also reported in the promyelocytic HL 60 human leukemia line and in human neutrophils [21]. There is also evidence that adenosine may play a physiological role in the regulation of chondrocytes representing a novel target for therapeutic intervention linked to inflammatory responses [22,23].

The discovery of different biological effects by low frequency electric and magnetic fields (PEMFs) from power lines, diagnostic apparatus and therapy devices has caused great interest [24]. In particular, the osteoinductive activity of PEMFs and their action on the local inflammation, on the osteogenesis and on the different phases of bone repair is well established [25,26]. It has been observed that PEMFs modulate stimulatory effect on the osteoblast early stages of culture and bone tissue-like formation [27]. Several works have investigated the dependence of the prolonged exposure to PEMFs on proliferative effects and on the cell density [28]. New hypotheses have been proposed to explain the influence of PEMFs on the cell membrane and in particular on ligand binding with membrane receptors which can also affect the membrane protein distribution [29]. Recently, it has been demonstrated that the effect of PEMFs evokes an upregulation of the A_{2A} adenosine receptors and alters the response of this receptor subtype in human neutrophils. Moreover, PEMF treatment causes an increase of adenylyl cyclase activity and a reduction of superoxide anion production as a result of upregulation of the A_{2A} receptors located on the neutrophil surface [30].

The present paper describes a significant alteration of A_3 adenosine receptor density and functionality in human neutrophils exposed to PEMFs. Saturation binding experiments were performed using a high affinity radioligand antagonist [^{3}H]-MRE 3008F20, and revealed a significant increase of the A_3 adenosine receptor density in PEMF-treated human neutrophils. A thermodynamic analysis of [^{3}H]-MRE 3008F20 binding was performed [31] with the aim of obtaining new insights into the effect of PEMFs on the forces driving drug–receptor coupling. Competition of radioligand binding by the high affinity A_3 receptor agonists Cl-IB-MECA and IB-MECA in the absence and in the presence of PEMFs has been performed. In functional assays the effect of Cl-IB-MECA and IB-MECA in the inhibition of adenylyl cyclase activity has been evaluated.

2. Methods

2.1. Field characteristics

The neutrophils or neutrophil membranes were exposed to a PEMF generated by a pair of rectangular horizontal coils (14 cm × 23 cm) each made of 1400 turns of copper wire; coils were powered by a pulse generator (IGEA). The general characteristics of the field were reported in previous works [30,32]. The neutrophils or neutrophil membranes were PEMF treated or untreated at different incubation times (90, 120, 150, 180 and 240 min). The temperature, continuously monitored by a thermoresistor within the incubator, was constant through the exposure time and maintained exactly during the binding and functional experiments. The intensity of the magnetic field varying from 0.2 to 3.5 mTesla (mT) were necessary to evaluate the effect of different intensities on the binding parameters.

2.2. Preparation of neutrophil suspensions and membranes

Neutrophils were isolated from buffy coats provided by the Blood Bank of the University Hospital of Ferrara. Blood was donated by healthy human volunteers after informed consent for research was obtained. Human neutrophils were prepared according to the procedure of Varani *et al.* [33]. Blood was supplemented with 20 mL of a solution consisting of 6% dextran T500. After gentle mixing, erythrocytes were allowed to settle at 20° for 60 min, and the turbid upper layer containing leukocytes was carefully removed and placed into centrifuge tube. Leukocytes were pelleted by centrifugation at 20° for 10 min at 100 *g*. Remaining erythrocytes were lysed by suspending the cell pellet in 10 mL of distilled water at 4° under gentle agitation. After 30 s, isotonicity was restored by adding 3 mL of a solution containing 0.6 M NaCl. Cells were pelletted by centrifugation at 20° for 5 min at 250 *g*, suspended in 10 mL of the Krebs Ringer phosphate buffer consisting of 136 mM NaCl, 5 mM KCl, 0.67 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 3 mM $NaHCO_3$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM HEPES, 10 mM $MgCl_2$, pH 7.45 and layered into 10 mL of Fycoll-Hypaque. Neutrophils were sedimented by centrifugation at 20° for 20 min at 250 *g*. This procedure allowed studies of cell suspensions containing 98 ± 2% neutrophils with few contaminating red blood cells or platelets. This cell suspension was used for the measurement of cyclic AMP.

Human neutrophils were suspended in 50 mM Tris–HCl pH 7.4 containing 10 mM $MgCl_2$ and centrifuged at 12,000 *g* for 15 min at 4°. The supernatant was discarded and the pellet resuspended in 10 mL of 50 mM Tris–HCl and centrifuged at 12,000 *g* for 15 min. The resulting pellet was suspended again at a concentration of 100–150 μg protein 100 μL^{-1} and this homogenate was used for the

assay of [^{3}H]-MRE 3008F20 binding. The protein concentration was determined according to a Bio Rad method [34] with bovine albumin as reference standard.

2.3. [^{3}H]-MRE 3008F20 saturation binding assay in the neutrophil membranes

Binding assays were carried out according to the method of Varani *et al.* [35]. In kinetic studies, neutrophil membranes (PEMF treated or untreated) were incubated with 2 nM [^{3}H]-MRE 3008F20 in a thermostatic bath at 4°. For the measurement of the association rate, the reaction was terminated at time points ranging from 2 to 120 min by rapid filtration under vacuum. For the measurement of the dissociation rate, membranes were pre-incubated with [^{3}H]-MRE 3008F20 at 4° for 120 min and after the addition of 1 μM MRE 3008F20 the reaction was terminated at time points ranging from 2 to 60 min. In saturation studies, neutrophil membranes (PEMF treated or untreated) were incubated with 8–10 different concentrations of [^{3}H]-MRE 3008F20 ranging from 0.2 to 20 nM in a total volume of 250 μL containing 50 mM Tris–HCl buffer, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4. Different incubation times were utilized (90, 120, 150, 180 or 240 min at 4°) according to the results of previous time course experiments. Saturation experiments were also performed under different magnetic fields (0.2, 0.5, 1, 1.5, 2.5, 3.5 mT) to evaluate the effect of intensity on binding parameters. Saturation experiments of [^{3}H]-MRE 3008F20 binding to the human PEMF treated or untreated neutrophil membranes were carried out at 0, 10, 15, 20, 25 and 30° at different incubation time from 150 min at 0° to 60 min at 25° according to the results of previous time course experiments.

2.4. Competition binding assay in the neutrophil membranes

In competition experiments, carried out to determine the K_i values, 2 nM of [^{3}H]-MRE 3008F20, 100 μL of neutrophil membranes (150 μg of protein per assay) and at least 6–8 different concentrations of Cl-IB-MECA or IB-MECA as typical A_3 adenosine receptor agonists were incubated at 4° for 120 min. Analogous experiments were performed in the presence of 100 μM GTP. Nonspecific binding was defined as binding in the presence of 1 μM MRE 3008F20 and was about 30% of total binding. Bound and free radioactivity were separated by rapid filtration through Whatman GF/B filters with a Micro-Mate 196 cell Harvester (Packard Instrument Co.). The filter bound radioactivity was counted using a microplate scintillation counter (Top Count) at an efficiency of 57% with a Micro-Scint 20.

2.5. Thermodynamic analysis

For a generic binding equilibrium L + R = LR (L = ligand, R = receptor) the affinity association constant $K_A = K_D^{-1}$ is directly related to the standard free energy ΔG° ($\Delta G^\circ = -RT \ln K_A$) which can be separated in its enthalpic and entropic contributions according to the Gibbs' equation: $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. The standard free energy was calculated as $\Delta G^\circ = -RT \ln K_A$ at 298.15 K, the standard enthalpy, ΔH°, from the van't Hoff plot $\ln K_A$ vs. (T^{-1}) (the slope of which is $-\Delta H^\circ R^{-1}$) and the standard entropy as $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)T^{-1}$ with $T = 298.15$ K and $R = 8.314$ J K^{-1} mol^{-1} [31]. K_A values were obtained from saturation experiments of [^{3}H]-MRE 3008F20 binding to the human PEMF treated or untreated neutrophil membranes carried out at 0, 10, 15, 20, 25 and 30° in a thermostatic bath assuring a temperature of ±0.1°.

2.6. Measurement of cyclic AMP levels in human neutrophils

PEMF treated or untreated human neutrophils (10^6 cells mL^{-1}) were suspended in 0.5 mL incubation mixture Krebs Ringer phosphate buffer, containing 1.0 IU adenosine deaminase mL^{-1} and 0.5 mM Ro 20-1724 as phosphodiesterase inhibitor and preincubated for 10 min in a shaking bath at 37°. Then forskolin 10 μM, Cl-IB-MECA or IB-MECA at different concentrations (1 nM–10 μM) were added to the mixture and the incubation continued for a further 5 min. The effect of a selective A_3 antagonist MRE 3008F20 (1 μM) on IB-MECA or Cl-IB-MECA (100 nM)-induced reduction of cyclic AMP levels was evaluated. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). Human neutrophils were also incubated with forskolin (10 μM) and/or Ro 20-1724 (0.5 mM) to evaluate the adenylyl cyclase activity. The TCA suspension was centrifuged at 2000 *g* for 10 min at 4° and the supernatant was extracted four times with water saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay carried out according to the method of Varani *et al.* [36,37]. Samples of cyclic AMP standards (0–10 pmol) were added to each test tube containing trizma base 0.1 M, aminophylline 8.0 mM, mercaptoethanol 6.0 mM, pH 7.4 and [^{3}H]-cyclic AMP in a total volume of 0.5 mL. The binding protein, previously prepared from beef adrenals, was added to the samples and incubated at 4° for 150 min. At the end of the incubation time and after the addition of charcoal, the samples were centrifuged at 2000 *g* for 10 min. The clear supernatant was mixed with 4 mL of Atomlight and counted in a LS-1800 Beckman scintillation counter.

2.7. Materials

Dextran and Ficoll-Paque were purchased from Pharmacia. [^{3}H]-MRE 3008F20 (specific activity 67 Ci mmol^{-1}) was obtained from Amersham International. Cl-IB-MECA, IB-MECA, Ro 20-1724, cAMP, bovine serum albumin, and adenosine deaminase were obtained from Sigma-RBI.

[^{3}H]-cAMP (specific activity 21 Ci mmol^{-1}), was obtained by NEN Research Products. All other reagents were of analytical grade and obtained from commercial sources.

3. Data analysis

All binding studies (kinetic, saturation and competition experiments) were analyzed with the program LIGAND [38,39] which performs weighted, nonlinear, least squares curve fitting. The EC_{50} and IC_{50} values obtained in cyclic AMP were calculated by nonlinear regression analysis using the equation for a sigmoid concentration–response curve (GraphPAD Prism). All experimental data were expressed as the arithmetic mean ± SEM. Analysis of data was done with Student's *t*-test (unpaired analysis). Differences were considered significant at a value of $P < 0.01$.

4. Results

4.1. Kinetic studies

Kinetic studies (N = 3) showed that [^{3}H]-MRE 3008F20 binding reached equilibrium after approximately 90 min and was stable for at least 5 hr in the absence (Fig. 1A) or in the presence of PEMF treatment (Fig. 2A). [^{3}H]-MRE 3008F20 binding was rapidly reversed by the addition of 1 μM MRE 3008F20 in the absence (Fig. 1B) or in the presence of PEMF treatment (Fig. 2B). Computer analysis revealed that both association and dissociation data fit to a two site was not significantly better than to a one-component model ($P < 0.05$) in untreated human neutrophils. The following rate constants were obtained: $K_{obs} = 0.056 \pm 0.003$ min^{-1} and $K_{-1} = 0.030 \pm 0.002$ min^{-1}. From the $K_{+1} = 0.013 \pm 0.002$ min^{-1} nM^{-1} and the K_{-1} values a kinetic dissociation constant K_D of 2.31 ± 0.25 nM was calculated. In human neutrophils treated with PEMFs, the kinetic data are: $K_{obs} = 0.059 \pm 0.004$ min^{-1}, $K_{-1} = 0.034 \pm 0.002$ min^{-1} and $K_{+1} = 0.013 \pm 0.003$ min^{-1} nM^{-1}. Consequently, the apparent equilibrium dissociation constant (K_D) was calculated showing a value of 2.62 ± 0.28 nM.

4.2. Saturation binding assays to A_3 adenosine receptor

A series of experiments were carried out to determine the effect of PEMFs on the binding parameters of A_3 adenosine receptors. Table 1 reports affinity (K_D, nM) and density (B_{max}, fmol mg^{-1} protein) of A_3 adenosine receptors on human neutrophil membranes exposed to different PEMF incubation times (90, 120, 150, 180, 240 min). The PEMF incubation time was chosen according to the time course experiments. The incubation time does not modify the affinity values of both PEMF treated or untreated neutrophils. On the contrary the B_{max} values of A_3 adenosine receptors present in human neutrophils are altered after 90 min of PEMF treatment and remain at a steady value also during other incubation times. A series of experiments were performed to evaluate the relationship between intensity of PEMFs and changes in the binding parameters of [^{3}H]-MRE 3008F20 to A_3 adenosine receptors by studying the potential effect of various intensities of PEMFs ranging from 0.2 to 3.5 mT. For each intensity of PEMF, saturation curves of A_3 adenosine receptors were performed and corresponding binding parameters were determined. The incubation time of each intensity was 120 min according to the time course experiments. No effect was observed under 0.5 mT and the maximum effect appeared between 1 and 3.5 mT whereby reaching a stable plateau. Table 2 reports the affinity (K_D, nM) and density

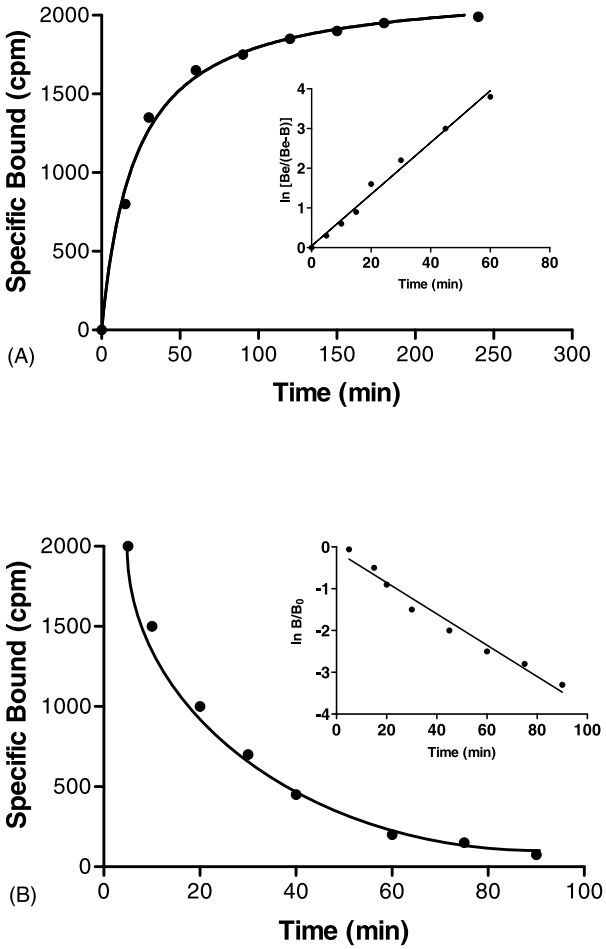

Fig. 1. (A) Kinetics of 1 nM [^{3}H]-MRE 3008F20 binding to A_3 adenosine receptors in untreated human neutrophil membranes with association curves representative of a single experiment (N = 3). Inset, first-order plots of [^{3}H]-MRE 3008F20 binding. B_{eq} represents the amount of [^{3}H]-MRE 3008F20 bound at equilibrium and B represents the amount of [^{3}H]-MRE 3008F20 bound at each time. Association rate constant was $K_{+1} = 0.013 \pm 0.002$ min^{-1} nM^{-1}. (B) Kinetics of 1 nM [^{3}H]-MRE 3008F20 binding to human A_3 adenosine receptors with dissociation curves representative of a single experiment. Inset, first-order plots of [^{3}H]-MRE 3008F20 binding. Dissociation rate constant was: $K_{-1} = 0.030 \pm 0.002$ min^{-1}.

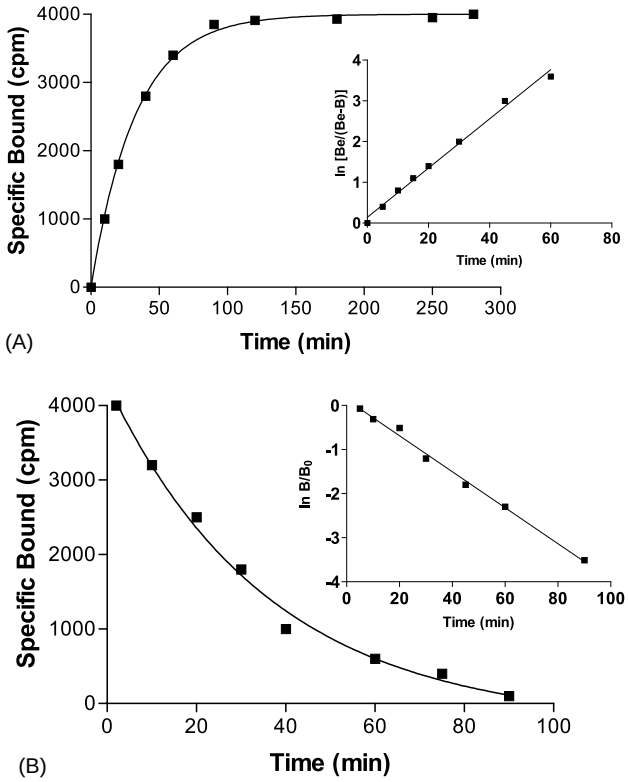

Fig. 2. (A) Kinetics of 1 nM [^{3}H]-MRE 3008F20 binding to A_3 adenosine receptors in PEMF-treated human neutrophil membranes with association curves representative of a single experiment (N = 3). Inset, first-order plots of [^{3}H]-MRE 3008F20 binding. B_{eq} represents the amount of [^{3}H]-MRE 3008F20 bound at equilibrium and B represents the amount of [^{3}H]-MRE 3008F20 bound at each time. Association rate constant was $K_{+1} = 0.013 \pm 0.003\ min^{-1}\ nM^{-1}$. (B) Kinetics of 1 nM [^{3}H]-MRE 3008F20 binding to human A_3 adenosine receptors with dissociation curves representative of a single experiment. Inset, first-order plots of [^{3}H]-MRE 3008F20 binding. Dissociation rate constant was: $K_{-1} = 0.034 \pm 0.002\ min^{-1}$.

Table 1
Binding parameters of the A_3 adenosine receptor antagonist [^{3}H]-MRE 3008F20 in human neutrophil membranes at different PEMF incubation times

PEMF incubation time (min)	Treatment	K_D (nM)	B_{max} (fmol mg^{-1} protein)
90	Control	2.25 ± 0.06	448 ± 18
	PEMF	2.30 ± 0.08	702 ± 10*
120	Control	2.36 ± 0.16	451 ± 16
	PEMF	2.45 ± 0.15	736 ± 25*
150	Control	2.30 ± 0.09	447 ± 21
	PEMF	2.40 ± 0.08	715 ± 27*
180	Control	2.20 ± 0.08	443 ± 20
	PEMF	2.29 ± 0.07	710 ± 27*
240	Control	2.31 ± 0.21	454 ± 11
	PEMF	2.35 ± 0.25	741 ± 28*

Data are mean ± SEM, where N = 3. Intensity of PEMFs was 2.5 for 120 min. Analysis was by Student's *t*-test.
* $P < 0.01$ vs. control.

Table 2
Relationship between PEMF intensity and binding parameters of the A_3 adenosine receptor antagonist [^{3}H]-MRE 3008F20 in human neutrophil membranes

PEMF intensity (mT)	K_D (nM)	B_{max} (fmol mg^{-1} protein)
0.2	2.34 ± 0.23	445 ± 42
0.5	2.25 ± 0.27	482 ± 44
1	2.40 ± 0.28	638 ± 36*
1.5	2.32 ± 0.24	645 ± 33*
2.5	2.45 ± 0.15	736 ± 25*
3.5	2.50 ± 0.26	755 ± 56*

Data are mean ± SEM, where N = 3. PEMF exposure time was 120 min according to the time course experiments. Analysis was by Student's *t*-test.
* $P < 0.01$ vs. control.

(B_{max}, fmol mg^{-1} protein) of A_3 adenosine receptors linked to different intensity of PEMFs. From the analysis of these data binding and functional experiments were performed to 2.5 mT PEMF intensity for the incubation time of 120 min. Figs. 3 and 4 report a saturation curve of [^{3}H]-MRE 3008F20 binding to A_3 adenosine receptors in human neutrophil untreated or treated for 120 min with PEMFs, respectively. The Scatchard plot analysis (inset) indicated the presence of a single class of binding sites with a K_D value of 2.36 ± 0.16 nM and B_{max} value of 451 ± 18 fmol mg^{-1} protein in untreated human neutrophils (N = 4). In human neutrophils treated with PEMFs the K_D value was 2.45 ± 0.15 nM and B_{max} value was 736 ± 25 fmol mg^{-1} protein (N = 4).

4.3. Thermodynamic studies

Saturation experiments were performed at 0, 10, 15, 20, 25 and 30° and the Scatchard plots resulted to be linear within the concentration range investigated (N = 3). In untreated neutrophils K_D values of [^{3}H]-MRE 3008F20 ranged from 2.28 ± 0.32 nM (0°) to 5.07 ± 0.52 nM (30°). However, B_{max} values are independent of temperature (mean value being 445 ± 48 fmol mg^{-1} protein). Also in PEMF-treated neutrophils K_D values of [^{3}H]-MRE 3008F20 ranged from 2.37 ± 0.35 nM (0°) to 5.28 ± 0.55 nM (30°), whereas B_{max} values appear independent of temperature (mean value being 745 ± 65 fmol mg^{-1} protein). Fig. 5 shows the van't Hoff of ln K_A vs. T^{-1} for [^{3}H]-MRE 3008F20 binding to the A_3 adenosine receptors in untreated or PEMF-treated human neutrophils. The final equilibrium thermodynamic parameters (expressed as mean values ± standard error of three independent experiments) in untreated human neutrophils were: $\Delta G^\circ = -47.41 \pm 0.15$ kJ mol^{-1}; $\Delta H^\circ = -21.27 \pm 2.52$ kJ mol^{-1} and $\Delta S^\circ = 87.73 \pm 8.85$ J mol^{-1} K^{-1}. The thermodynamics parameters in human neutrophils treated with PEMFs were: $\Delta G^\circ = -47.13 \pm 0.14$ kJ mol^{-1}; $\Delta H^\circ = -20.69 \pm 2.25$ kJ mol^{-1} and $\Delta S^\circ = 88.72 \pm 8.28$ J mol^{-1} K^{-1}.

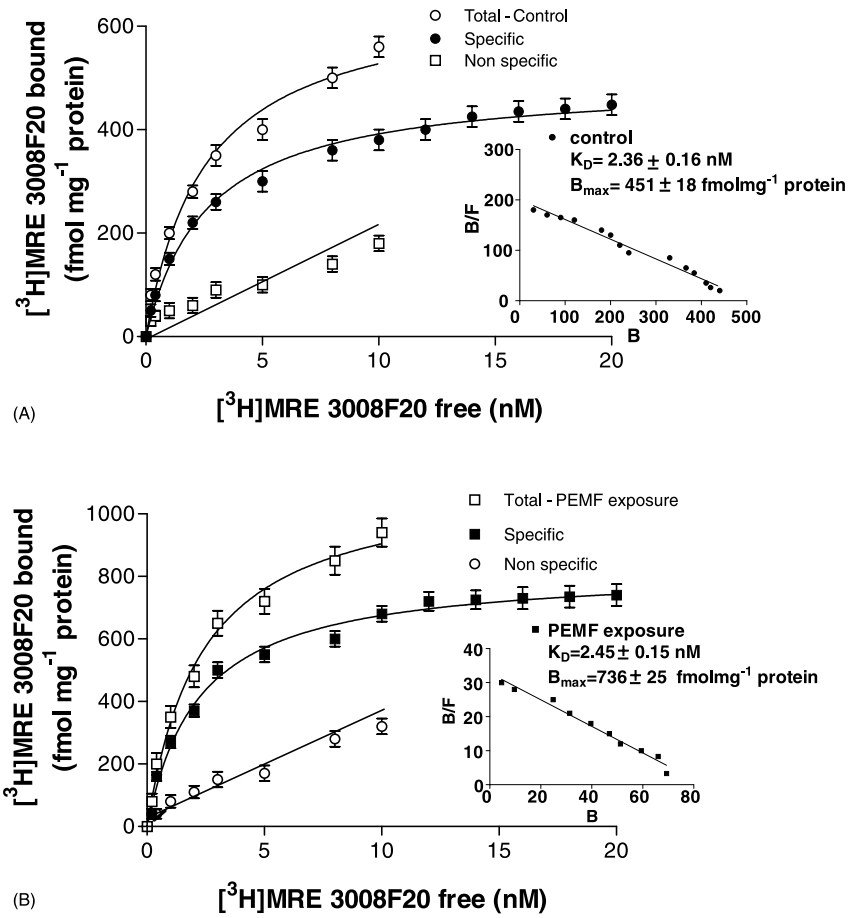

Fig. 3. (A) Saturation of [^{3}H]-MRE 3008F20 binding to A_3 adenosine receptors on untreated neutrophil membranes. The K_D value was 2.36 ± 0.16 nM and the B_{max} value was 451 ± 18 fmol mg^{-1} protein. (B) Saturation of [^{3}H]-MRE 3008F20 binding to A_3 adenosine receptors on PEMF-treated human neutrophil membranes. The K_D value was 2.45 ± 0.15 nM and the B_{max} value was 736 ± 25 fmol mg^{-1} protein. Experiments were performed as described in Section 2. Values are the means and vertical lines SEM of four separate experiments performed in triplicate. In the inset, the Scatchard plot of the same data is shown.

4.4. Competition studies

Fig. 6 shows the competition curves of Cl-IB-MECA (A) and IB-MECA (B) in untreated or PEMF-treated human neutrophils, respectively. In untreated human neutrophils Cl-IB-MECA shows a K_H value of 0.68 ± 0.20 nM and a K_L value of 89 ± 12 nM (N = 4). In parallel studies, Cl-IB-MECA in human neutrophils treated with PEMFs, has a K_H value of 0.65 ± 0.14 nM and a K_L value of 72 ± 10 nM (N = 4). In untreated human neutrophils IB-MECA shows a K_H value of 1.83 ± 0.32 nM and a K_L value of 102 ± 15 nM (N = 4) whereas in PEMF-treated human neutrophils IB-MECA reveals a K_H value of 1.64 ± 0.43 nM and a K_L value of 98 ± 11 nM (N = 4). The competition curves of both the agonists examined performed in the absence and in the presence of PEMFs exhibited Hill coefficients less than unity (0.54 and 0.62 for Cl-IB-MECA; 0.52 and 0.64 for IB-MECA, respectively) and were best described by the existence of one high-affinity (K_H) and one low-affinity (K_L) agonist-receptor binding state. Coupling of the A_3 receptor to G protein was investigated in the presence of 100 μM GTP that is able to shift the competition binding curves of the agonist from a biphasic to a monophasic shape with a K_i value near to that of the low affinity sites. The K_i values for Cl-IB-MECA were 93 ± 11 and 78 ± 9 nM and for IB-MECA were 122 ± 16 and 115 ± 13 nM in the absence and in the presence of PEMF treatment, respectively.

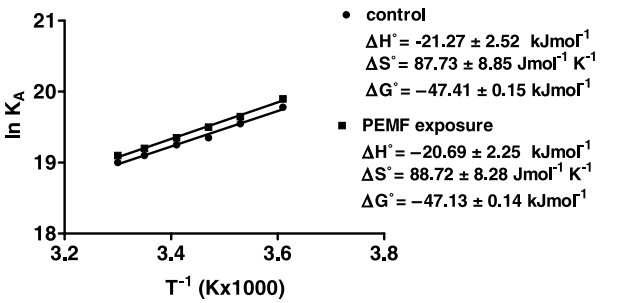

Fig. 4. van't Hoff plot showing the effect of temperature on the equilibrium binding association constant, $K_A = 1/K_D$ of [^{3}H]-MRE 3008F20. The plot is essentially linear in the temperature range investigated (4–30°). Binding experiments were performed as described in Section 2 (N = 3).

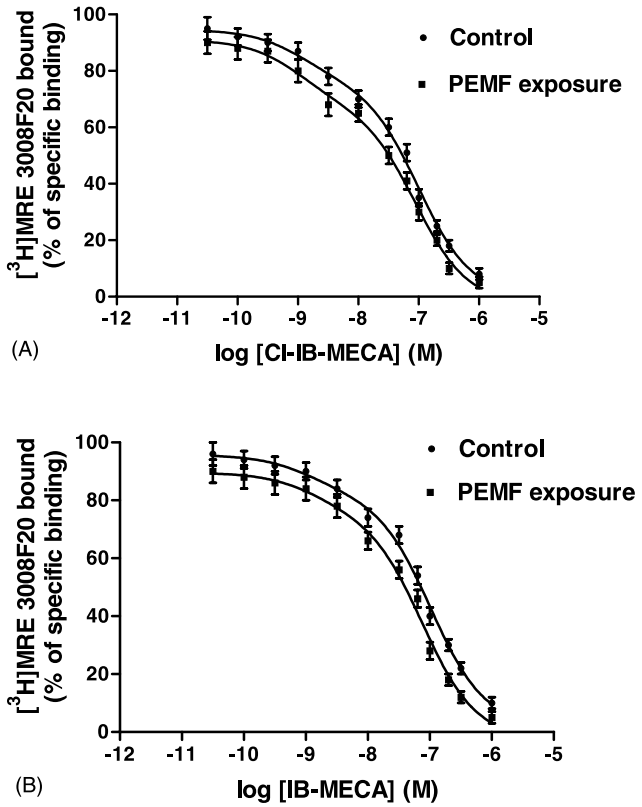

Fig. 5. Competition experiments of specific [^{3}H]-MRE 3008F20 binding to A_3 adenosine receptors of Cl-IB-MECA (A) and IB-MECA (B) in untreated and PEMF-treated human neutrophil membranes. Competition experiments were performed as described in Section 2 (N = 4).

4.5. Cyclic AMP assays

The A_3 adenosine receptor is coupled to adenylyl cyclase via Gi proteins, which leads to decreases of cAMP formation. Untreated or PEMF-treated neutrophils did not reveal changes of basal enzyme activity and of the response of adenylyl cyclase to the direct activator forskolin (10 μM) used in the absence or in the presence of the cAMP-dependent phosphodiesterase inhibitor, Ro 20-1724 (0.5 mM) (Table 3). We also evaluated the inhibitory effect of typical A_3 adenosine agonists like Cl-IB-MECA or IB-MECA on the adenylyl cyclase activity. When the agonists were incubated with PEMF treated or untreated neutrophils, a reduction of adenylyl cyclase response was detected revealing a significant decrease of cAMP production in a concentration-dependent manner (N = 3) (Fig. 6). Cl-IB-MECA determines a decrease of cAMP levels in untreated or PEMF-treated human neutrophils with IC_{50} values of 2.89 ± 0.14 and 1.18 ± 0.09 nM, respectively (Fig. 6A). Moreover, IB-MECA determines a decrease of cAMP levels in untreated or PEMF-treated human neutrophils with IC_{50} values of 5.84 ± 0.62 and 2.36 ± 0.38 nM, respectively (Fig. 6B). The selective A_3 antagonist MRE 3008F20 (1 μM) antagonized Cl-IB-MECA or IB-MECA (100 nM) mediated cAMP inhibition suggesting that the inhibitory effect was essentially A_3 mediated (Table 3).

5. Discussion

The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the presence of adenosine receptor subtypes [39,40]. Adenosine, interacting with specific receptors on the surface of neutrophils, has been recognized as an endogenous anti-inflammatory agent [16]. The activation of A_3 adenosine receptors in human neutrophils determines their anti-inflammatory properties by inhibiting specific cell functions as degranulation, chemotaxis and superoxide anion production [21]. In the past, it has been verified that electric or magnetic fields can affect membrane functions [24] and specialised molecules, such as receptors, enzymes, ion channels, integrins that are essential for many fundamental cell functions. Different exposure to PEMFs induces an increase in the proliferation of human articular chondrocytes suggesting an important role also in cartilagine repair [41]. In earlier studies, it has been verified that PEMFs induce programmed cell death in cultured T cells and determine a decreased T-cell proliferative capacity [42]. Recently, it has been demonstrated that PEMF treatment evokes an upregulation of the A_{2A} adenosine receptors and

Table 3
Basal and forskolin stimulated cAMP formation in human neutrophils

	Control (pmol cAMP × 10^6 cells^{-1})	PEMF treatment (pmol cAMP × 10^6 cells^{-1})
Basal levels	20 ± 2	22 ± 4
Forskolin (10 μM)	82 ± 8	87 ± 9
Forskolin (10 μM) + Ro 20-1724 (0.5 Mm)	95 ± 9	98 ± 8
Forskolin (10 μM) + Cl-IB-MECA (100 nM)	66 ± 4	42 ± 3*
Forskolin (10 μM) + IB-MECA (100 nM)	74 ± 5	45 ± 4*
Cl-IB-MECA (100 nM) + MRE 3008F20 (1 μM)	90 ± 8	96 ± 10
IB-MECA (100 nM) + MRE 3008F20 (1 μM)	87 ± 9	92 ± 7

Inhibition of forskolin stimulated cyclic AMP levels by Cl-IB-MECA and IB-MECA and antagonism by MRE 3008F20. Formation of cAMP was detected in the absence of a stimuli (basal levels) and upon stimulation with forskolin and forskolin + Ro 20-1724. In this last experimental condition, cAMP levels were also evaluated using 100 nM Cl-IB-MECA in the presence of the A_3 selective antagonist MRE 3008F20 (1 μM). Data are mean ± SEM, where N = 3. Analysis was by Student's *t*-test.

* $P < 0.01$ vs. Cl-IB-MECA or IB-MECA inhibited cAMP formation in control human neutrophils.

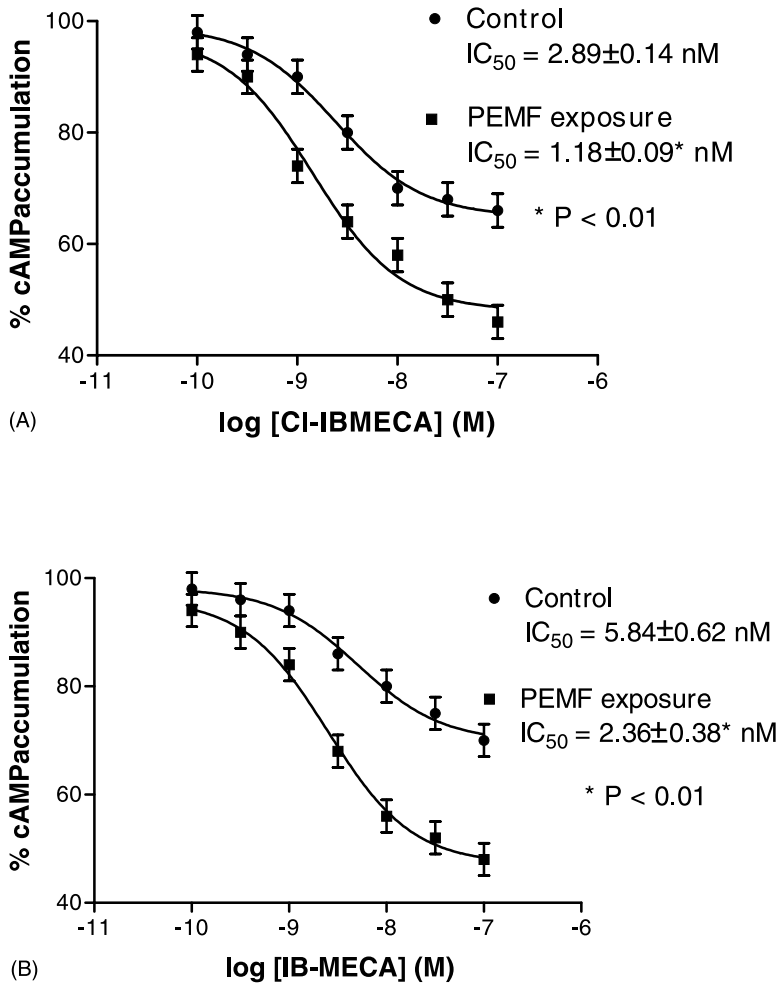

Fig. 6. Inhibition of cyclic AMP levels in untreated and PEMF-treated human neutrophils by Cl-IB-MECA (A) and IB-MECA (B). Cyclic AMP experiments were performed as described in Section 2 (N = 3).

alters the response of this receptor subtype in human neutrophils. Moreover, PEMF treatment causes an increase of adenylyl cyclase activity and a reduction of superoxide anion production as a result of upregulation of the A_{2A} receptors located on the neutrophil surface [30].

On this basis, in the present study we have evaluated the effect of PEMFs on A_3 adenosine receptors in human neutrophils using a typical pharmacological approach based on binding and functional characterization of this important receptor subtype. A set of experiments were designed to studied the change in the density and affinity of A_3 adenosine receptors treated at different incubation times (90, 120, 150, 180 and 240 min) according to the previous time course experiments. These data have shown that 90 min of treatment is able to induce an upregulation of A_3 adenosine receptors. In addition, the treatment with different intensities of PEMFs reveals that lower intensities do not determine alteration in binding parameters. On the contrary, when the magnetic intensity is used in the range 1–3.5 mT a significant increase of adenosine A_3 density was demonstrated. These experimental conditions are similar to those used in other works showing the "*in vitro*" effect of PEMFs on human chondrocytes [28,41]. Moreover, the signal characteristics of PEMFs are comparable with those utilized in orthopaedic and traumatologic treatments even if the time of exposure is much more longer (30–90 days) than that used in "*in vitro* experiments" [43,44]. In saturation binding experiments [^{3}H]-MRE 3008F20 labeled a single class of recognition sites with a similar affinity in both different experimental conditions. Moreover, the number of binding sites in control or PEMF-treated human neutrophils was increased significantly ($P < 0.01$) showing a B_{max} value of 451 ± 18 fmol mg^{-1} 736 ± 25 fmol mg^{-1} protein, respectively. These data revealed that the predominant effect of PEMFs mediated a significant increase of A_3 adenosine receptor density, suggesting that the upregulation cannot be ascribed to the synthesis of new receptors during the short time of PEMF treatment. On the contrary the upregulation of A_3 adenosine receptors can probably be due to a translocation of this receptor subtype to the membrane surface. Recently, it has been demonstrated that PEMFs treatment also modifies the binding parameters of the A_{2A} adenosine receptors and not of α_2, β_2 adrenergic and μ, k opioid receptors suggesting a

specificity of PEMF treatment to adenosine receptors [30]. Thermodynamic parameters obtained from the van't Hoff plot indicate that [^{3}H]-MRE 3008F20 binding to A_3 adenosine receptors is enthalpy- and entropy-driven, with a major contribution of the enthalpic component in agreement with data obtained in other cells expressing human A_3 receptors [4,35] and using typical adenosine antagonists [31]. To determine whether the high affinity state of the A_3 receptor, that is linked to a guanine nucleotide regulatory protein, was altered by PEMFs, we performed competition experiments of Cl-IB-MECA or IB-MECA in the presence of GTP which converted the curves of the agonists from biphasic to monophasic. The similarity between K_i values determined in the presence of GTP and K_L values obtained in the absence of GTP indicated a guanine nucleotide-mediated shift of the high affinity binding sites to a low affinity form, in agreement with that reported for human A_3 adenosine receptors transfected in CHO cells [35] and on human neutrophils [21]. These results show that the affinity of Cl-IB-MECA or IB-MECA in the PEMF-treated neutrophils were strictly similar to those obtained in untreated neutrophils suggesting that the treatment did not modify the affinity values of these agonists.

Another aim of the present study was to investigate if the PEMF treatment determines a different modulation of adenylyl cyclase activity. Our results do not show any change of basal enzyme activity and of the response of adenylyl cyclase to the direct activator forskolin used in the absence or in the presence of cAMP-dependent phosphodiesterase inhibitor (Ro 20-1724) suggesting that PEMF treatment does not modify the adenylyl cyclase activity. Moreover, we have evaluated the capability of typical A_3-adenosine agonists such as Cl-IB-MECA or IB-MECA to inhibit cAMP levels. These compounds show an IC_{50} values in the nanomolar range, in agreement with their affinity in binding experiments. Interestingly, the potency of Cl-IB-MECA or IB-MECA in the PEMF-treated neutrophils is significantly increased if compared with the untreated neutrophils. To further confirm that the effect induced by the agonists examined on cAMP formation is due to stimulation of the A_3 receptor subtype, we performed experiments in the presence of MRE 3008F20, a typical selective A_3 adenosine antagonist. This antagonist, used at the final concentration of 1 μM, is able to prevent the decrease of cAMP induced by Cl-IB-MECA (100 nM) through selective inhibition of the adenylyl cyclase via the A_3 receptor.

In summary, all these data provide evidence that PEMF treatment evokes an upregulation of the A_3 adenosine receptors and alters the response of this receptor subtype in human neutrophils. It can be of interest that PEMF treatment causes an alteration of a functional response such as adenylyl cyclase activity, probably as a result of upregulation of the A_3 adenosine receptors located on the neutrophil surface. In conclusion, these results suggest that the binding and functional parameters of A_3 adenosine receptors are altered in the presence of PEMF treatment. Additional clarifications are needed to elucidate the mechanisms underlying the relationship between adenosine-mediated anti-inflammatory effects and PEMF exposure.

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