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Effect of low frequency electromagnetic fields on A_{2A} adenosine receptors in human neutrophils

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1 The present study describes the effect of low frequency, low energy, pulsing electromagnetic fields (PEMFs) on A_{2A} adenosine receptors in human neutrophils.

2 Saturation experiments performed using a high affinity adenosine antagonist [^{3}H]-ZM 241385 revealed a single class of binding sites in control and in PEMF-treated human neutrophils with similar affinity ($K_D = 1.05 \pm 0.10$ and 1.08 ± 0.12 nM, respectively). Furthermore, after 1 h of exposure to PEMFs the receptor density was statistically increased ($P < 0.01$) ($B_{max} = 126 \pm 10$ and 215 ± 15 fmol mg^{-1} protein, respectively).

3 The effect of PEMFs was specific to the A_{2A} adenosine receptors. This effect was also intensity, time and temperature dependent.

4 In the adenylyl cyclase assays the A_{2A} receptor agonists, HE-NECA and NECA, increased cyclic AMP accumulation in untreated human neutrophils with an EC_{50} value of 43 (40–47) and 255 (228–284) nM, respectively. The capability of HE-NECA and NECA to stimulate cyclic AMP levels in human neutrophils was increased ($P < 0.01$) after exposure to PEMFs with an EC_{50} value of 10(8–13) and 61(52–71) nM, respectively.

5 In the superoxide anion (O_2^-) production assays HE-NECA and NECA inhibited the generation of O_2^- in untreated human neutrophils, with an EC_{50} value of 3.6(3.1–4.2) and of 23(20–27) nM, respectively. Moreover, in PEMF-treated human neutrophils, the same compounds show an EC_{50} value of 1.6(1.2–2.1) and of 6.0(4.7–7.5) nM respectively.

6 These results indicate the presence of significant alterations in the expression and in the functionality of adenosine A_{2A} receptors in human neutrophils treated with PEMFs.
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Keywords: Adenosine A_{2A} receptors; human neutrophils; [^{3}H]-ZM241385 binding; binding thermodynamics; cyclic AMP; superoxide anion production; pulsing electromagnetic fields (PEMFs)

Abbreviations: fMLF, N-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine; HE-NECA, 2hexynyl-5′N-ethylcarboxamidoadenosine; [^{3}H]-ZM241385, 4-(2-[7-amino-2 (2-furyl)-[1,2,4] triazolo [2,3-a]-[1,3,5]triazin-5-y-lamino]ethyl)phenol; NECA, 5′N-ethylcarboxamidoadenosine; O_2^-, superoxide anion; PEMFs, pulsing electromagnetic fields; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; SCH 58261, 7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine

Introduction

Adenosine, an endogenous modulator of a wide range of biological functions, interacts with at least four cell surface subtypes classified as A_1, A_{2A}, A_{2B} and A_3 receptors which are coupled to G proteins (Fredholm *et al*., 2000; Klotz, 2000). In the peripheral system, A_{2A} receptors are present on numerous tissues including neutrophils, monocytes, macrophages, lymphocytes, platelets, mast cells and show simi-larities to those obtained in CHO cells transfected with the human A_{2A} receptor (Gessi *et al*., 2000; Fredholm *et al*., 2001). Activation of A_{2A} receptors seems to be associated with inhibition of tumour necrosis factor (TNF)-α, IL-6, IL-8 and elastase release by activated mononuclear phagocytes (Elenkov *et al*., 2000; Tsuruta *et al*., 2000). Recently, it has been revealed that an A_{2A} adenosine receptor-dependent mechanism exists by which aspirin retains its anti-inflammatory activity (Cronstein *et al*., 1999). It has also been demonstrated that anti-inflammatory effects of methotrexate are mediated *via* adenosine receptors (Montesinos *et al*., 2000). Moreover, it is generally accepted that adenosine stimulates accumulation of intracellular cyclic AMP, which acts as a second messenger to alter cellular function (Sullivan & Linden, 1998). A good correlation was found between cyclic AMP accumulation data and inhibition of superoxide anion generation by adenosine receptor agonists studied suggesting that the cyclic AMP could be involved in the action of A_{2A} receptors to inhibit superoxide anion formation (Varani *et al*., 1998).

Interest has grown concerning the exposure to extremely low frequency electric and magnetic fields (PEMFs) originating from power lines and from diagnostic apparatus (Bersani *et al*., 1997). The osteo-inductive activity of PEMFs and their action on the local inflammation (Canè *et al*., 1993; De

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Mattei *et al*., 1999), on the osteogenesis and on the different phases of bone repair (Matsumoto *et al*., 2000; Satter *et al*., 1999) is well established. A careful investigation of possible genotoxic effects of PEMFs has suggested that the exposure does not induce any chromosomal alteration such as breakages, translocations or inversion (Cadossi *et al*., 1992). Recently, it has been observed that PEMFs modulate the neurite outgrowth *in vitro* and nerve regeneration *in vivo* (Shah *et al*., 2001). Several hypotheses have been proposed to explain the influence of PEMFs on living systems, and most of them share the common assumption that the cell membrane is most likely the target for field interaction (Bonhomme-Faivre *et al*., 1998). It has been proposed that electromagnetic exposure may affect ligand binding with membrane receptors and can also affect the membrane protein distribution (Chiabrera *et al*., 2000).

The present paper describes, for the first time, the effect of PEMFs on A_{2A} adenosine receptors in human neutrophils. Saturation binding experiments performed using a high affinity radioligand antagonist, [^{3}H]-ZM 241385, revealed a significant increase of the A_{2A} adenosine receptor density in neutrophils treated with PEMFs. The effect of PEMFs was specific to the A_{2A} adenosine receptors, intensity, time and temperature dependent. Finally, a significant increase of the A_{2A} adenosine receptor signalling in the adenylyl cyclase assay and in the capability by adenosine agonists to inhibit the generation of superoxide anion production has been reported.

Methods

Field characteristics

The neutrophils or neutrophil membranes were exposed to a PEMFs generated by a pair of rectangular horizontal coils (14×23 cm) each made of 1400 turns of copper wire; coils were powered by a pulse generator (IGEA, Italy). The general characteristics of the field were reported in previous work (Cadossi *et al*., 1992). Briefly, the exposure system has three components: (1) the signal generator, which produces the input voltage of pulses at 75 Hz; (2) the amplifier, which produces the electrical voltage output (200 V) supplying coils; (3) the coils which produce the magnetic field varying from 0.2 to 3.5 mTesla (mT) to evaluate the effect of intensity on binding parameters. The induced electric field in air is 0.04 $mVcm^{-1}$.

The neutrophils or neutrophil membranes were PEMF-treated or untreated for different incubation times (30, 45, 60, 90 and 120 min). The temperature, continuously monitored by a thermoresistor within the incubator, was constant through the exposure time and exactly maintained during the binding and functional experiments.

Preparation of cell suspensions

The cell preparation were isolated from heparin-treated peripheral blood (100–200 ml) provided by the Blood Bank of the University Hospital of Ferrara. Blood was donated by healthy human volunteers after written informed consent for research. Human neutrophils were prepared according to the procedure of Varani *et al*. (1998). Blood was supplemented with 20 ml of a solution consisting of 6% by weight dextran T500. After gentle mixing, erythrocytes were allowed to settle down at 20°C 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°C for 10 min at $100 \times g$. Remaining erythrocytes were lysed by suspending the cell pellet in 10 ml of distilled water at 4°C 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°C for 5 min at $250 \times g$, suspended in 10 ml of the Krebs–Ringer phosphate buffer and layered into 10 ml of Fycoll-Hypaque. Neutrophils were sedimented by centrifugation at 20°C for 20 min at $250 \times g$. 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 measurement of cyclic AMP and superoxide anion production experiments.

Preparation of neutrophil membranes

Human neutrophils were suspended in 50 mM tris HCl pH 7.4 containing 10 mM $MgCl_2$ and centrifuged at $12{,}000 \times g$ for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in 10 ml of 50 mM Tris HCl and centrifuged at $12{,}000 \times g$ 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]-ZM 241385 binding. The protein concentration was determined according to a Bio Rad method (Bradford, 1976) with bovine albumin as reference standard.

[^{3}H]-ZM 241385 binding assay in the neutrophil membranes

Binding assays were carried out according to Varani *et al*. (1997). In kinetic studies, neutrophil membranes (PEMF-treated or untreated) were incubated with 1 nM [^{3}H]-ZM 241385 in a thermostatic bath at 4°C. For the measurement of the association rate, the reaction was terminated at time points ranging from 2–120 min by rapid filtration under vacuum, followed by washing with 5 ml ice-cold buffer four times. For the measurement of the dissociation rate, membranes were pre-incubated with [^{3}H]-ZM 241385 at 4°C for 120 min and after the addition of 1 μM ZM 241385 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]-ZM 241385 ranging from 0.05 to 10 nM in a total volume of 250 μl containing 50 mM Tris HCl buffer, 10 mM $MgCl_2$, pH 7.4. Saturation experiments of [^{3}H]-ZM 241385 binding to the human PEMF-treated or untreated neutrophil membranes were performed using different conditions to evaluate their effect on binding parameters: (i) at different incubation times as 30, 45, 60, 90 or 120 min at 4°C; (ii) at different intensity of the magnetic fields as 0.2, 0.5, 1, 1.5, 2.5, 3.5 mT; (iii) at different temperatures as 0, 10, 15, 20, 25 and 30°C carried out at various incubation time from 60 min at 0°C to 20 min at 25°C according to the results of previous time course experiments. In competition experiments, carried out to determine the IC_{50} values, 1 nM of [^{3}H]-ZM 241385, 100 μl

of neutrophil membranes (150 μg of protein assay^{-1}) and at least 6–8 different concentrations of HE-NECA or NECA as typical adenosine receptor agonists were incubated at 4°C for 60 min. Analogous experiments were performed in the presence of 100 μM GTP. Non specific binding was defined as binding in the presence of 1 μM ZM 241385 or 1 μM SCH 58261 and was about 30% (35%) 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, Meriden, CT, U.S.A.) at an efficiency of 57% with a Micro-Scint 20.

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°C. Then forskolin 1 μM, HE-NECA or NECA at different concentrations (1 nM–10 μM) were added to the mixture and the incubation continued for a further 5 min. The effect of typical selective A_{2A} antagonist, SCH 58261 (1 μM) was determined by antagonism of the HE-NECA or NECA (100 nM)-induced stimulation of cyclic AMP levels. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at $2000 \times g$ for 10 min at 4°C 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*. (1996). 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°C for 150 min. At the end of the incubation time and after the addition of charcoal the samples were centrifuged at $2000 \times g$ for 10 min. The clear supernatant was mixed with 4 ml of Atomlight and counted in a LS-1800 Beckman scintillation counter.

Superoxide anion production in human neutrophils

O_2^- production was measured by the superoxide dismutase (0.5 mg ml^{-1})-inhibitable reduction of ferricytochrome *c* modified for microplate-based assays (Varani *et al*., 1998). The tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nM of cytochrome *c* and Krebs Ringer phosphate buffer. At zero time 1 μM fMLF was added, and the plates were incubated in a microplate reader (Ceres 900, Bio-TeK Instruments, VT, U.S.A.) with the compartment T set at 37°C. Absorbance was recorded at wavelengths of 550 and 468 nm and the differences in absorbance at the two wavelengths were used to calculate nmoles of O_2^- produced, using a molar extinction coefficient for cytochrome *c* of 18.5 mM^{-1} cm^{-1}. Neutrophils were preincubated in the presence of 5 μg ml^{-1} cytochalasin B for 5 min prior to activation by fMLF. The net nmoles of O_2^- released were calculated from the formula: nmoles released by stimulated neutrophils minus nmoles released by resting neutrophils alone. Inhibitory activity was determined by measuring the A_{2A} agonist's ability to inhibit O_2^- production as activated by fMLF. The percentage of activity was obtained by comparing the nmoles of O_2^- in the absence (100%) and in the presence of HE-NECA or NECA at different concentrations (1 nM–1 μM). The effect of typical selective A_{2A} antagonist, SCH 58261 (1 μM) was determined by antagonism of the HE-NECA or NECA at the concentration of 100 nM.

PEMF response specificity

Binding to α_2 adrenergic receptors was carried out with [^{3}H]-UK 14304 (0.2–10 nM) on human neutrophils in a 50 mM Tris HCl buffer pH 7.4, containing $MgCl_2$ 10 mM for 60 min at 25°C. Non-specific binding was determined with 1 μM of UK 14304 (Zaccaria *et al*., 1997). Binding to α_2 adrenergic receptors was carried out with [^{3}H]-CGP 12177 (0.1–10 nM) on human neutrophils in a 50 mM Tris HCl buffer pH 7.4, containing $MgCl_2$ 10 mM for 60 min at 25°C. Non-specific binding was determined with 10 μM of CGP 12177 (Borea *et al*., 1992). Binding to α- and κ-opioid receptors were performed on human neutrophils with [^{3}H]-DAMGO and [^{3}H]-U69593 in a 50 mM Tris HCl buffer pH 7.4 for 60 min at 25°C. Non-specific binding was determined with 100 μM of bremazocine (Varani *et al*., 1999). The effects of isoproterenol (1 nM–10 μM) on stimulation of adenylate cyclase activity and on superoxide anion production were evaluated to verify if PEMFs involve the functionality of another G protein coupled receptor.

Data analysis

All binding studies (kinetic, saturation and competition experiments) were analysed with the program LIGAND (Munson & Rodbard, 1980) which performs weighted, non linear, least squares curve fitting program. The EC_{50} and IC_{50} values obtained in cyclic AMP and superoxide anion production assays were calculated by non linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPAD Prism, San Diego, CA, U.S.A.). EC_{50} values were expressed as geometric means with 95% confidence intervals and all other data were expressed as the arithmetic mean $\pm$ s.e.mean. Analysis of data was done with Student's *t*-test (unpaired analysis). Differences were considered significant at a value of $P < 0.01$.

Materials

Dextran and Ficoll-Paque were purchased from Pharmacia (Uppsala, Sweden). [^{3}H]-ZM 241385 (specific activity 17 Ci mmol^{-1}) was obtained by Tocris Cookson Ltd (Northpoint Fourth Way, Bristol, U.K.). HE-NECA, NECA, cytochrome *c*, fMLF, Ro 20-1724, cyclic AMP, bovine serum albumin, cytochalasin B and adenosine deaminase were obtained from Sigma-RBI (St. Louis, MO, U.S.A.). SCH 58261 was a kind gift of Dr Ennio Ongini, Schering Plough Research Institute, Milan, Italy. [^{3}H]-cyclic AMP (specific activity 21 Ci mmol^{-1}), [^{3}H]DAMGO (specific activity 65 Ci mmol^{-1}), [^{3}H]U69593 (specific activity 44 Ci mmol^{-1}),

[^{3}H]-UK 14304 (specific activity 62 Ci mmol^{-1}), [^{3}H]-CGP12177 (specific activity 43 Ci mmol^{-1}) were obtained by NEN Research Products (Boston, MA, U.S.A.). All other reagents were of analytical grade and obtained from commercial sources.

Results

Kinetic studies

Kinetic studies ($n=3$) showed that [^{3}H]-ZM 241385 binding reached equilibrium after approximately 20 min and was stable for at least 3 h in the absence (Figure 1A) or in the presence of PEMF treatment (Figure 2A). [^{3}H]-ZM 241385 binding was rapidly reversed by the addition of 1 μM ZM 241385 in the absence (Figure 1B) or in the presence of PEMF treatment (Figure 2B). Computer analysis revealed that both association and dissociation data fit a two site was not significantly better than a one-component model ($P<0.05$) in untreated human neutrophils with the following rate constants: $K_{obs}=0.100\pm0.005$ min^{-1} and $K_{-1}=0.055\pm0.003$ min^{-1}. From the $K_{+1}=0.045\pm0.002$ min^{-1} nM^{-1} and the K_{-1} values gave a kinetic dissociation constant K_D of 1.22 ± 0.12 nM. In human neutrophils treated with PEMFs the kinetic data are: $K_{obs}=0.103\pm0.005$ min^{-1}, $K_{-1}=0.059\pm0.002$ min^{-1} and $K_{+1}=0.044\pm0.003$ min^{-1} nM^{-1}. Consequently, the apparent equilibrium dissociation constant (K_D) was calculated showing a value of 1.33 ± 0.15 nM.

Saturation binding assays to A_{2A} adenosine receptor

A series of experiments were carried out to determine the effect of PEMFs on the binding parameters of A_{2A} adenosine receptors. Table 1 reports affinity (K_d, nM) and density (B_{max}, fmol mg^{-1} protein) of A_{2A} adenosine receptors on human neutrophil membranes exposed to different PEMF incubation times (30, 45, 60, 90, 120 min). 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_{2A} adenosine receptors present in human neutrophils are altered after 30 min

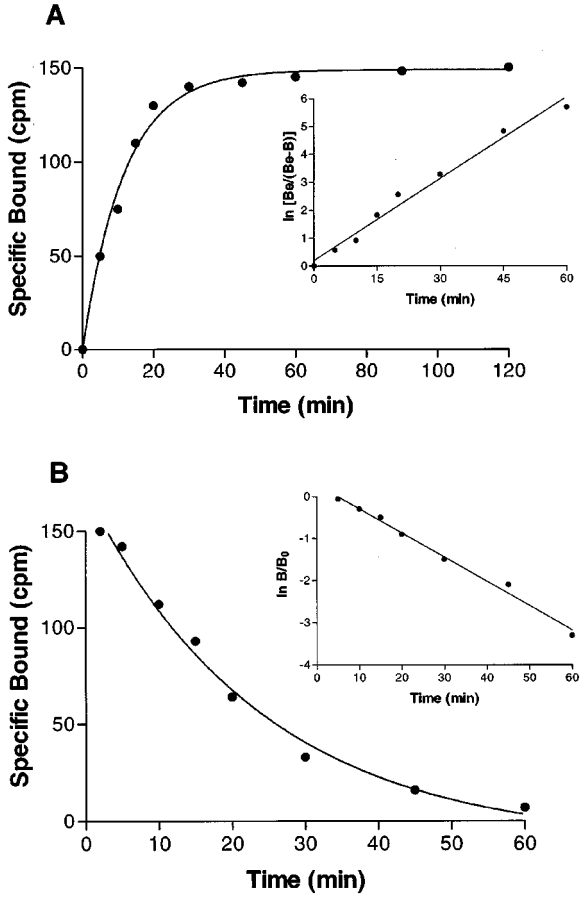

Figure 1 (A) Kinetics of 1 nM [^{3}H]-ZM 241385 binding to A_{2A} adenosine receptors in untreated human neutrophil membranes with association curves representative of a single experiment. Inset, first-order plots of [^{3}H]-ZM 241385 binding. Be represents the amount of [^{3}H]-ZM 241385 bound at equilibrium and B represents the amount of [^{3}H]-ZM 241385 bound at each time. Association rate constant was $K_{+1}=0.045\pm0.002$ min^{-1} nM^{-1}. (B) Kinetics of 1 nM [^{3}H]-ZM 241385 binding to human A_{2A} adenosine receptors with dissociation curves representative of a single experiment. Inset, first-order plots of [^{3}H]-ZM 241385 binding. Dissociation rate constant was: $K_{-1}=0.055\pm0.003$ min^{-1}.

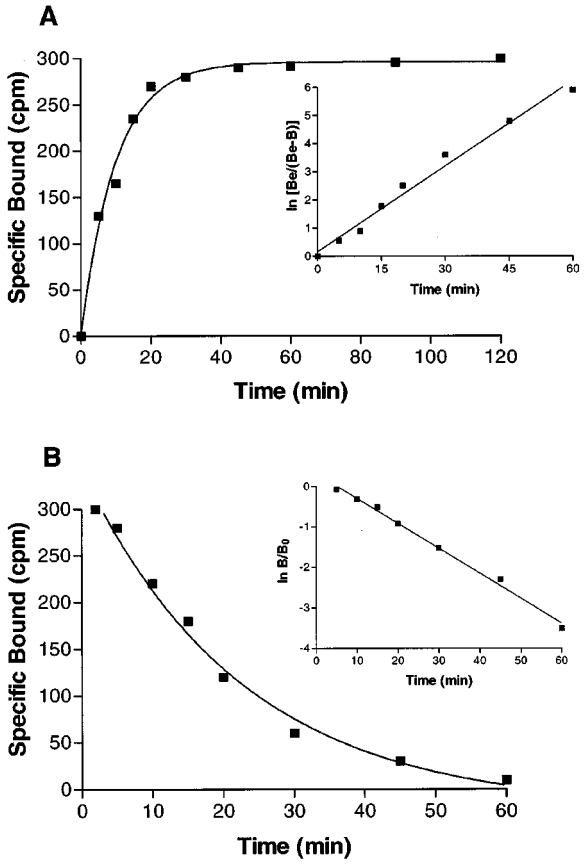

Figure 2 (A) Kinetics of 1 nM [^{3}H]-ZM 241385 binding to A_{2A} adenosine receptors in PEMF-treated human neutrophil membranes with association curves representative of a single experiment. Inset, first-order plots of [^{3}H]-ZM 241385 binding. Be represents the amount of [^{3}H]-ZM 241385 bound at equilibrium and B represents the amount of [^{3}H]-ZM 241385 bound at each time. Association rate constant was $K_{+1}=0.044\pm0.003$ min^{-1} nM^{-1}. (B) Kinetics of 1 nM [^{3}H]-ZM 241385 binding to human A_{2A} adenosine receptors with dissociation curves representative of a single experiment. Inset, first-order plots of [^{3}H]-ZM 241385 binding. Dissociation rate constant was: $K_{-1}=0.059\pm0.002$ min^{-1}.

Table 1 Binding parameters of the A_{2A} adenosine receptor antagonist [^{3}H]-ZM 241385 in human neutrophil membranes at different incubation times

Incubation time (min)	*Treatment*	K_D (nM)	B_{max} (fmol mg^{-1} protein)
30	Control	0.96±0.09	118±11
	PEMF	0.98±0.10	205±13*
45	Control	1.00±0.14	121±12
	PEMF	1.03±0.11	218±16*
60	Control	1.05±0.10	126±10
	PEMF	1.08±0.12	215±15*
90	Control	0.99±0.13	115±14
	PEMF	1.02±0.15	225±18*
120	Control	1.08±0.16	112±15
	PEMF	1.15±0.19	220±13*

Each value represented the mean±s.e.m. of three independent experiments performed in duplicate. Analysis was by Student's *t*-test. *$P<0.01$ vs control.

Table 2 Relationship between PEMF intensity and binding parameters of the A_{2A} adenosine receptor antagonist [^{3}H]-ZM 241385 in human neutrophil membranes

PEMF intensity (mTesla)	K_D (nM)	B_{max} (fmol mg^{-1} protein)
0.2	1.05±0.10	126±10
0.5	1.06±0.09	138±15
1	1.03±0.11	185±16*
1.5	1.11±0.15	210±14**
2.5	1.08±0.12	215±15**
3.5	1.15±0.19	218±13**

Each value represented the mean±s.e.m. of three independent experiments performed in duplicate. Analysis was by Student's *t*-test, *$P<0.05$ vs control; **$P<0.01$ vs control.

of PEMF treatment and remain to a steady value also for the 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]-ZM 241385 to A_{2A} 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_{2A} adenosine receptors were performed and corresponding binding parameters were determined. No effect was observed under 0.5 mT and the maximum effect appeared from 1 to 3.5 mT reaching a stable plateau. Table 2 reports the affinity (K_D, nM) and density (B_{max}, fmol mg^{-1} protein) of A_{2A} adenosine receptors linked to different intensity of PEMFs. Figures 3 and 4 report a saturation curve of [^{3}H]-ZM 241385 binding to A_{2A} adenosine receptors in human neutrophil untreated or treated for 1 h with PEMFs, respectively. The linearity of the Scatchard plot in the inset is indicative of the presence of a single class of binding sites with a K_D value of 1.05±0.10 nM and B_{max} value of 126±10 fmol mg^{-1} protein in human neutrophils untreated ($n=4$). Moreover, in human neutrophils treated with PEMFs the K_D value was 1.08±0.12 nM and B_{max} value was 215±15 fmol mg^{-1} protein ($n=4$). Temperature dependence were evaluated through saturation experiments performed at 0, 10, 15, 25 and 30°C and the Scatchard plots are linear in the concentration range investigated. In untreated neutrophils K_D values of [^{3}H]-ZM 241385 range from 1.04±0.09 nM (0°C) to 4.52±0.42 nM (30°C). However, B_{max} values are independent

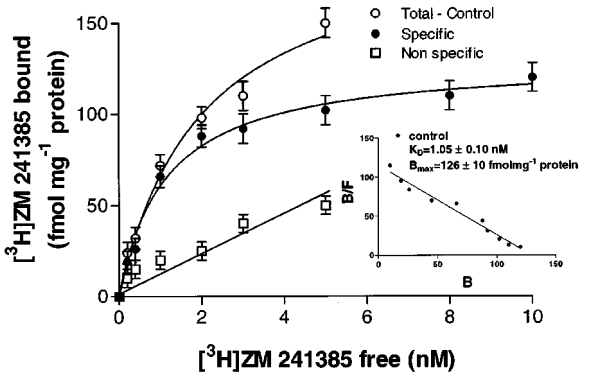

Figure 3 Saturation of [^{3}H]-ZM 241385 binding to A_{2A} adenosine receptors on untreated neutrophil membranes. The K_D value was 1.05±0.10 nM and the B_{max} value was 126±10 fmol mg^{-1} protein. Experiments were performed as described in Methods. Values are the means and vertical lines s.e.mean of four separate experiments performed in triplicate. In the inset the Scatchard plot of the same data is shown.

of temperature (mean value being 112±14 fmol mg^{-1} protein). Also in PEMF-treated neutrophils K_D values of [^{3}H]-ZM 241385 range from 1.06±0.10 nM (0°C) to 4.84±0.50 nM (30°C), whereas B_{max} values appear independent of temperature (mean value being 211±16 fmol mg^{-1} protein).

Competition studies to A_{2A} adenosine receptor

Figure 5A,B shows the competition curves of HE-NECA and NECA in untreated or PEMF-treated human neutrophils, respectively. In untreated human neutrophils HE-NECA and NECA show a K_i value of 9.6 (6.6–14.2) nM (Figure 5A) and 25 (20–31) nM (Figure 5B), respectively ($n=4$). In parallel studies, the affinity of HE-NECA and NECA in human neutrophils treated with PEMFs, present a K_i values of 8.8 (7.6–10.3) nM (Figure 5A) and 23 (19–27) nM (Figure 5B), respectively ($n=4$). To assess adenosine A_{2A} receptor-G protein interactions, inhibition experiments were performed also in the presence of 100 μM GTP. The presence of GTP did not modify the affinity of HE-NECA and NECA that show in untreated and PEMF-treated human neutrophils similar K_i values ($K_i=9.2$ (8.1–10.9) nM and 25 (20–29) nM, respectively).

Cyclic AMP assays

The A_{2A} adenosine receptor is coupled to stimulation of adenylyl cyclase *via* Gs stimulatory proteins, which leads to increases of cyclic AMP formation. We evaluated the sensitivity of adenylyl cyclase to pharmacological agents that are known to specifically activate this effector system. 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 used in the absence or in the presence of the cyclic AMP-dependent phosphodiesterase inhibitor, Ro 20-1724 (Table 3). Finally, we evaluated the stimulatory effect of typical A_{2A} adenosine agonists on the adenylyl cyclase activity. When the adenosine agonists NECA or HE-NECA were incubated with PEMF-treated or untreated neutrophils, an amplification of adenylyl cyclase response was detected revealing a significant increase

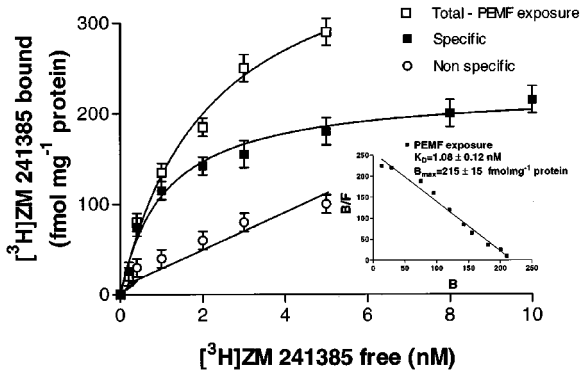

Figure 4 Saturation of [^{3}H]-ZM 241385 binding to A_{2A} adenosine receptors on PEMF-treated human neutrophil membranes. The K_D value was 1.08 ± 0.12 nM and the B_{max} value was 215 ± 15 fmol mg^{-1} protein. Experiments were performed as described in Methods. Values are the means and vertical lines s.e.mean of four separate experiments performed in triplicate. In the inset the Scatchard plot of the same data is shown.

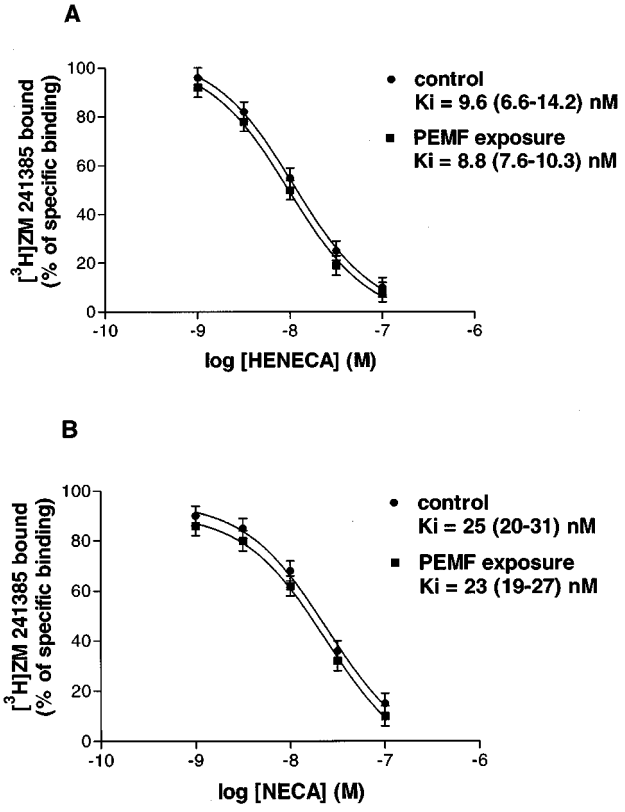

Figure 5 Competition experiments of specific [^{3}H]-ZM 241385 binding to A_{2A} adenosine receptors of HE-NECA (A) and NECA (B) in untreated and PEMF-treated human neutrophil membranes. Curves are representative of a single experiment from a series of four experiments. Competition experiments were performed as described in Methods.

of cyclic AMP production in a concentration-dependent manner. As shown in Figure 6 the log dose-response curve for HE-NECA and NECA, typical adenosine receptor agonists, in control and PEMF-exposed human neutrophils revealed an increase in cyclic AMP levels. HE-NECA determines an increase of stimulation of cyclic AMP levels

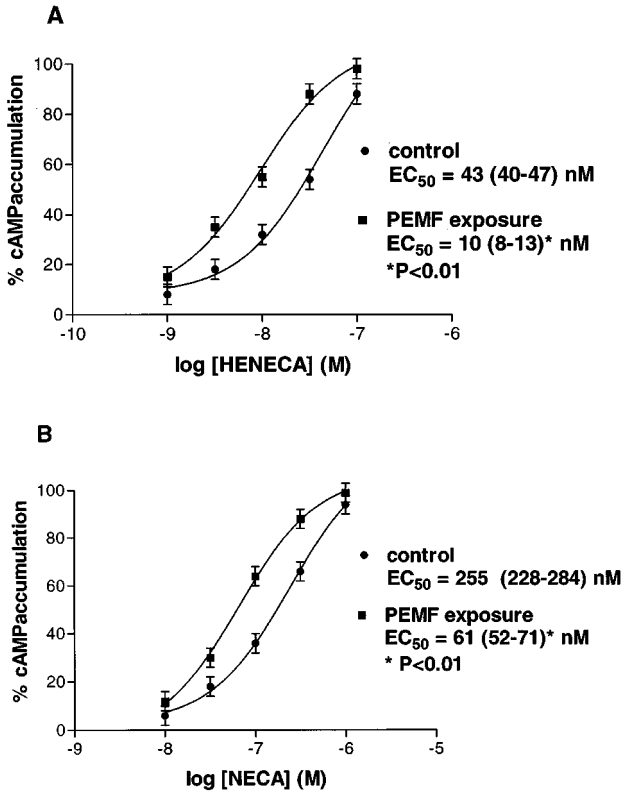

Figure 6 Stimulation of cyclic AMP levels in untreated and PEMF-treated human neutrophils by HE-NECA (A) and NECA (B). Curves are representative of a single experiment from a series of four independent experiments.

in untreated or PEMF-treated human neutrophils with EC_{50} values of 43 (40–47) nM and 10 (8–13) nM, respectively (Figure 6A). NECA elicited a stimulation of cyclic AMP levels in untreated or PEMF-treated human neutrophils with EC_{50} values of 255 (228–284) nM and 61 (52–71) nM, respectively (Figure 6B). The selective A_{2A} SCH 58261 (1 μM) totally inhibited the rise in cyclic AMP levels induced by NECA (100 nM) or by HE-NECA (10 nM) suggesting that the stimulatory effect was essentially A_{2A} mediated (Table 3).

Superoxide anion production

The next step addressed in our study was to investigate the effect of the A_{2A} adenosine receptors to inhibit the superoxide anion generation. In particular, the effect of typical A_{2A} adenosine agonists HE-NECA or NECA on O_2^- generation by fMLF-stimulated neutrophils was evaluated. In untreated neutrophils these compounds were able to inhibit the generation of O_2^- stimulated by fMLF (1 μM), in a dose-dependent manner, showing an EC_{50} value of 3.6 (3.1–4.2) nM (Figure 7A) and 23 (20–27) nM (Figure 7B), respectively. Moreover, in PEMF-treated human neutrophils HE-NECA and NECA showed an EC_{50} value of 1.6 (1.2–2.1) nM (Figure 7A) and 6.0 (4.7–7.5) nM (Figure 7B), respectively. HE-NECA and NECA (100 nM) mediated inhibitory effects which were completely blocked by using of the selective A_{2A} adenosine antagonist, SCH 58261, at the final concentration of 1 μM. Since the A_{2A} adenosine receptor is coupled to stimulation of adenylyl cyclase *via* Gs

Table 3 Basal and forskolin stimulated cyclic AMP formation in human neutrophils. Stimulation of cyclic AMP levels by NECA and HE-NECA and antagonism by SCH 58261

	Control (pmoles cyclic AMP 10^5 cells^{-1})	*PEMF treatment* (pmoles cyclic AMP 10^5 cells^{-1})
Basal levels	18±2	20±4
Forskolin (1 μM)	72±8	75±9
Forskolin (1 μM)+ Ro 201724 (0.5 mM)	78±8	82±8
NECA (100 nM)	35±3	68±6*
HE-NECA (10 nM)	32±3	62±5*
NECA (100 nM)+ SCH 58261 (1 μM)	20±2	19±2
HE-NECA (10 nM)+ SCH 58261 (1 μM)	18±2	20±3

Formation of cyclic AMP was detected in the absence of stimuli (basal levels) and upon stimulation with forskolin and forskolin+Ro 201724. Cyclic AMP levels were also evaluated using 100 nM NECA and 10 nM HE-NECA in the presence of a typical selective antagonist SCH 58261 (1 μM). Analysis was by Student's *t*-test, $*P<0.01$ vs NECA and HE-NECA stimulated cyclic AMP formation in control human neutrophils.

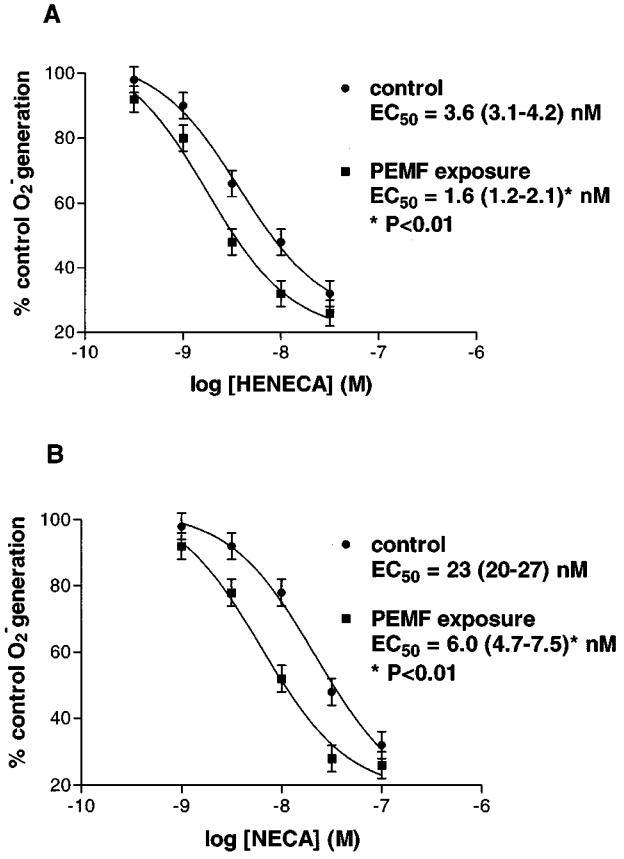

Figure 7 Effect of HE-NECA (A) and NECA (B) on O_2^- production induced by FMLF in untreated and PEMF-treated human neutrophils. Curves are representative of a single experiment from a series of four independent experiments.

stimulatory proteins leading to cyclic AMP increases, we analysed the sensitivity of adenylyl cyclase to agents that are known to specifically activate this effector system.

PEMF response specificity

To verify that the effect of PEMF is strictly correlated to the presence of the adenosine receptors, we studied other types of membrane receptors coupled to G proteins. The expression of α_2, β_2 adrenergic and μ and κ opioid receptors in PEMF-treated and untreated human neutrophils was determined performing saturation binding experiments using [^{3}H]-UK14304, [^{3}H]-CGP12177, [^{3}H]-DAMGO and [^{3}H]-U69593, respectively. A single saturable binding site was detected for all type of receptors. Saturation of [^{3}H]-UK14304 binding shows a K_D value of 2.12±0.04 nM and a B_{max} value of 34±2 fmol mg^{-1} protein. [^{3}H]-CGP12177 exhibits high affinity for α_2 receptors with a K_D value of 0.16±0.02 nM and B_{max} value of 9.0±0.5 fmol mg^{-1} protein. [^{3}H]-DAMGO and [^{3}H]-U69593 label α and κ opioid receptors and reveal a K_D value of 2.29±0.11 and 0.80±0.08 nM, and B_{max} value of 4.7±0.2 and 4.6±0.2 fmol mg^{-1} protein, respectively. Affinity of the different G protein coupled receptors for their ligand as measured by the dissociation constant (K_D) are comprised in the lower nM range (from 0.16 to 2.29 nM). The receptor binding capacity, as measured by B_{max}, is not abundant and varies from 4 to 34 fmol mg^{-1} protein. None of these bindings were significantly affected by PEMF-treatment at 2.5 mT, an intensity that promotes an up regulation of A_{2A} adenosine receptors. In addiction, we analysed the specificity of PEMF in functional studies as cyclic AMP and superoxide anion production assays. Isoproterenol (1 nM–10 μM) acting *via* β adrenoceptor, is able to stimulate adenylate cyclase activity and to determine an increase of cyclic AMP levels showing an EC_{50} value of 212 (194–246) nM and was not influenced by the PEMFs treatment (EC_{50}=224 (202–255) nM). Moreover, isoproterenol inhibits fMLP-induced superoxide anion generation showing an IC_{50} value of 82 (74–96) nM strictly similar to that obtained in the presence of PEMFs treatment (IC_{50}=76 (68–85) nM).

Discussion

It is well known that a biological system exposed to a physical stimulus is able to detect its presence and to modify its own biological activity depending on the characteristics of the applied stimulus such as mechanic, electric or magnetic. The cell structure, able to receive the applied energy, has been identified to be cell membrane (Cadossi *et al*., 1992). In the past, it has been verified that electric or magnetic fields can affect membrane functions not only by a local effect on ion fluxes or ligand binding, but also by altering the distribution and/or the aggregation of the intramembrane protein (Bersani *et al*., 1997). It is known that such proteins include a variety of different specialised molecules, such as receptors, enzymes, ion channels, integrins that are essential for many fundamental functions mainly related to signal transduction and cell adhesion. In particular, the influence of radio-frequency electromagnetic exposure on ligand binding to hydrophobic receptor proteins is a plausible early event of the interaction mechanism (Chiabrera *et al*., 2000). Collateral studies of magnetic or electric fields reveal a modulation in

the activity of adenylyl cyclase by coupling with specific receptor sites in the membrane surface (Massot *et al*., 2000). Static and time varying magnetic fields have been shown to alter animal and human behaviour, such as directional orientation learning, pain perception (nociception or analgesia) and anxiety-related behaviours (Thomas *et al*., 2001). Recently, it has also been demonstrated that PEMFs mediate the modulation of gene transcription suggesting new perspectives in the use of electromagnetic energy (Ventura *et al*., 2000). Nevertheless, evidence is now accumulating that PEMFs may alter human cardiac rhythm and that chronic exposure to these fields may enhance during surgery, transplantation or heart attack in humans (Di Carlo *et al*., 1999; 2001). The predominant effect of PEMF is also shown on the different phases of bone repair and have a positive effect on the repair process (Canè *et al*., 1993; Satter *et al*., 1999; Matsumoto *et al*., 2000). The stimulation of repair processes in clinical practice using the effectiveness of PEMF stimulation for enhancement of bone healing has been reported and accepted (Yanemori *et al*., 1996; Satter *et al*., 1999; Saxena *et al*., 2000). It is worth noting that the whole of these early effects of physical stimuli is able to trigger a more complex biologic response such as cell proliferation that represents the basic effect to explain some relevant clinical results (Sollazzo *et al*., 1997; De Mattei *et al*., 1999; Shah *et al*., 2001). Different exposure of PEMF induces an increase in the proliferation of human articular chondrocytes suggesting an important role also in cartilagine repair (Pezzetti *et al*., 1999). 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 (Jasti *et al*., 2001). Generally, it has been established that inflammation is characterized by massive infiltration of T lymphocytes, neutrophils and macrophages into the damaged tissue (Gessi *et al*., 2000). The available studies demonstrating the presence of A_{2A} adenosine receptors in human neutrophils, strongly suggest that adenosine could play an important role in modulating immune and inflammatory processes and the activation of A_{2A} receptors may have a relevant therapeutic potential (Cronstein *et al*., 1999; Montesinos *et al*., 2000). It is known that neutrophils are the most abundant white cells in the peripheral blood and are usually the first cells to arrive at an injured or infected site. Adenosine, interacting with specific receptors on the surface of neutrophils, has been recognized as an endogenous anti-inflammatory agent (Cronstein, 1994). The activation of A_{2A} receptors in human neutrophils affects the immune response in cancer, autoimmune and neurodegenerative diseases and decreases inflammatory reactions (Huang *et al*., 1997). Experimental evidence suggests that PEMFs are able to suppress the extravascular oedema during early inflammation (Lee *et al*., 1997). It has also been demonstrated that the complete healing of wounds depends on the presence of A_{2A} adenosine receptor agonists (Montesinos *et al*., 1997). In earlier studies, it has been reported that PEMFs mediate positive effects on a wound healing, controlling the proliferation of inflammatory lymphocytes and therefore demonstrating beneficial affects on inflammatory disease (Jasti *et al*., 2001).

On this basis, in the present study we have evaluated the effect of PEMFs on A_{2A} adenosine receptors in human neutrophils using a typical pharmacological approach based on binding and functional characterization of this important receptor subtype. Analysis of association and dissociation kinetic parameters of [^{3}H]-ZM 241385 binding in untreated or PEMF-treated human neutrophils revealed a K_D value of 1.22 and 1.33 nM, respectively (Figures 1 and 2) of the same order of magnitude as that determined by saturation experiments that show a K_D value of 1.05 nM and 1.08 nM, respectively (Figures 3 and 4). We have also studied the change in the density and affinity of adenosine A_{2A} receptors treated with different incubation times (30, 45, 60, 90 and 120 min) revealing also that 30 min of treatment is able to induce an up regulation of A_{2A} adenosine receptors. The treatment with different intensity of PEMFs reveals that lower intensity does 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_{2A} density is demonstrated. The experimental results revealed a dose-response relationship between the binding parameters of A_{2A} adenosine receptors and the intensity of PEMFs reaching a stable plateau. Moreover, while K_D values are widely temperature dependent, B_{max} values are quite independent of temperature suggesting a substantial stability of the effective receptor population at all temperatures studied. (Borea *et al*., 1996; Varani *et al*., 1996; 1997; 1998; 2000). Consequently the statistical difference of B_{max} values is strictly linked to PEMF treatment. In saturation binding experiments [^{3}H]-ZM 241385 labelled a single class of recognition sites with a similar affinity in both different experimental conditions. On the contrary, the number of binding sites in control or PEMF-treated human neutrophils was increased significantly ($P<0.01$) showing a B_{max} value of 126 ± 10 fmol mg^{-1} protein and 215 ± 15 fmol mg^{-1} protein, respectively. These data revealed that PEMF treatment determines a significant increase of A_{2A} adenosine receptor density, suggesting that the upregulation can probably be due to a translocation of this receptor subtype to the membrane surface. The upregulation of A_{2A} adenosine receptors cannot probably be ascribed to the synthesis of new receptors during the short time of PEMF treatment. In competition experiments the affinity of HE-NECA and NECA in the absence of guanine nucleotides is strictly similar to those obtained in the presence of 100 μM GTP. This result excludes a guanine nucleotides modulation in the A_{2A} antagonist binding such as [^{3}H]-ZM 241385 even if it is known that the presence of GTP determines dissociation of receptor G protein complexes converting receptors from high to a low affinity state. Similar results were obtained also in other substrates as rat striatal or human blood circulating cells using [^{3}H]-SCH 58261 as radioligand (Zocchi *et al*., 1996; Dionisotti *et al*., 1997; Varani *et al*., 1997; 1998). The lack of guanine nucleotide modulation in the antagonist binding has led to the suggestion that a tight association between the A_{2A} adenosine receptor and the stimulatory G protein exists (Nanoff *et al*., 1991; Nanoff & Stiles, 1993). Another aim of the present study was to investigate if the PEMF treatment determines a different modulation of adenylyl cyclase activity. Our results showed no changes of basal enzyme activity and of the response of adenylyl cyclase to the direct activator forskolin (1 μM) used in the absence or in the presence of cyclic AMP-dependent phosphodiesterase inhibitor (Ro 20-1724) suggesting that PEMF treatment does not modify the adenylyl cyclase activity. Moreover, we have evaluated the capability

of two typical A_{2A}-adenosine agonists such as HE-NECA and NECA to stimulate cyclic AMP levels. These compounds showed an EC_{50} value in the nanomolar range, in agreement with their affinity in binding experiments (Figures 5 and 6). In particular, HE-NECA appeared to be the most potent compound in both different experimental conditions showing an EC_{50} value of 43 (40–47) nM in untreated neutrophils and 10 (8–13) nM in PEMF-treated neutrophils. Similarly, NECA showed an EC_{50} value of 255 (228–284) nM in untreated neutrophils and an EC_{50} value of 61 (52–71) nM in PEMF-treated neutrophils. Interestingly, the potency of HE-NECA and NECA in the PEMF-treated neutrophils was significantly increased if compared with the untreated neutrophils. These results suggest a selective increase of responsiveness of the adenosine A_{2A} receptor/adenylyl cyclase system in the presence of PEMF treatment. To further confirm that the effect induced by HE-NECA or NECA on cyclic AMP formation is due to stimulation of A_{2A} receptor subtype, we performed experiments in the presence of a typical selective adenosine antagonist as SCH 58261. This antagonist, used at the final concentration of 1 μM, is able to prevent the increase of cyclic AMP induced by the agonists examined (100 nM) through selective stimulation of the adenylyl cyclase *via* the A_{2A} receptor. Our study of inhibition of superoxide anion production by HE-NECA and NECA and the block with a selective A_{2A} antagonist (SCH 58261) indicates that the receptor subtype involved is a typical A_{2A} adenosine receptor. HE-NECA and NECA show EC_{50} values of 3.6 (3.1–4.2) and 23 (20–27) nM in untreated neutrophils, respectively. In PEMF-treated human neutrophils HE-NECA and NECA show EC_{50} values of 1.6 (1.2–2.1) and 6.0 (4.7–7.5) nM (Figure 7). Binding and functional results indicate that a 2 fold increase in B_{max} is sufficient to determine 4 fold shift in EC_{50}. We have no explanation for these differences at present, but it may be relevant that the functional assay was carried out in intact cells at 37°C whereas the binding assay is performed on cell membranes incubated at lower temperature. Moreover several papers are present in literature revealing that a strictly direct correlation between binding and functional parameters does not always exist (Belardinelli *et al*., 1996; Gessi *et al*., 2001). Most relevantly we observe that the rank order of potencies of agonists and antagonists in the binding assays is similar to that obtained in functional assays (Varani *et al*., 1998; 1999; 2000). HE-NECA, an A_{2A} adenosine receptor agonist known to have the highest affinity for human neutrophils, appears also to be the most potent compound in the cyclic AMP and O_2^- generation assays. A good correlation was found in PEMF-treated human neutrophils between cyclic AMP accumulation and inhibition of O_2^- generation data. The evidence from this study that HE-NECA was the most potent compound in both assays would suggest that the mechanism involves the upregulation of A_{2A} adenosine receptors. It is worth noting that the response of PEMF is receptor specific as shown by saturation studies to other G protein coupled receptors. PEMFs treatment does not modify the binding parameters of the α_2, β_2 adrenergic and μ, κ opioid receptors. As for the β adrenergic, moreover, the capability of isoproterenol to stimulate adenylate cyclase and to inhibit the FMLP-superoxide anion generation is not modified by PEMF treatment.

In summary, all these data provide evidence that PEMF treatment 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. The results reported here should serve as an impetus for further investigation of the changes in the adenosine receptors and functional alterations produced by PEMF treatment. From a clinical point of view, a clarification of the potential biological effects of electromagnetic exposure could facilitate the development of alternative treatments or the elaboration of novel promising therapeutic tools.

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