Expression of A3 Adenosine Receptors in Human Lymphocytes: Up-Regulation in T Cell Activation

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ABSTRACT

The present study investigates mRNA and protein levels of A3 adenosine receptors in resting (R) and activated (A) human lymphocytes. The receptors were evaluated by the antagonist radioligand [3H]5-N-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine ([3H]MRE 3008F20), which yielded IC50 values of 125 ± 15 and 225 ± 23 fmol/mg of protein and Kd values of 1.79 ± 0.30 and 1.85 ± 0.25 nM in R and A cells, respectively. The protein seems to be induced with remarkable rapidity starting at 15 min and reaches a plateau at 30 min. Western blot assays revealed that the up-regulation of the A3 subtype after lymphocyte activation was caused by an increase in an enriched CD4+ cell fraction. Real-time reverse transcription-polymerase chain reaction experiments confirmed the rapid increase of A3 mRNA after T cell activation. Competition of radioligand binding by adenosine ligands displayed a rank order of potency typical of the A3 subtype. Thermodynamic data indicated that the binding is enthalpy- and entropy-driven in both R and A cells, suggesting that the activation process does not involve, at a molecular level, receptor alterations leading to modifications in the A3-related binding mechanisms. Functionally, the up-regulation of A3 adenosine receptors in A versus R cells corresponded to a potency increase of the A3 agonist N6-(3-iodo-benzyl)-2-chloro-adenosine-5’-N-methyluronamide in inhibiting cAMP accumulation (IC50 = 1.5 ± 0.4 and 2.7 ± 0.3 nM, respectively). This effect was antagonized by MRE 3008F20 (IC50 = 5.0 ± 0.3 nM). In conclusion, our results provide evidence, for the first time, an in-depth investigation of A3 receptors in human lymphocytes and demonstrate that, under activating conditions, they are up-regulated and may contribute to the effects triggered by adenosine.

Adenosine is an endogenous purine nucleoside that affects a number of physiological functions through its binding to specific G-protein coupled receptors named A1, A2A, A2B, and A3 (Fredholm et al., 2001). These adenosine subtypes have been classified as such because of differences at the molecular, biochemical, and pharmacological levels (Linden, 2001; Klingler et al., 2002). It is well recognized that adenosine can exert powerful effects on the immune system; this molecule has been identified as an important endogenous immunosuppressing regulator; it has been demonstrated that the lack of adenosine deaminase is responsible for severe immunodeficient disease (Apasov et al., 1995; Hirschhorn, 1995). A number of effects ascribed to adenosine on lymphocyte function, such as inhibition of interleukin-2 production, cell proliferation, and major histocompatibility complex-restricted cytotoxicity, seem to be mediated through Gs-coupled A2A receptors (Polmar, 1990). Indeed, in human lymphocytes, the A2A receptor has been thoroughly characterized based on binding (Varani et al., 1995; Murphree et al., 2002), immunohistochemistry (Koshiba et al., 1997), flow cytometry (Koshiba et al., 1999), and functional studies taking advantage of the wealth of pharmacological tools, including radio-

ABBREVIATIONS: RT-PCR, reverse transcription-polymerase chain reaction; PHA, phytohemagglutinin; NECA, 5’-N-ethylcarboxamidoadenosine; R-PtA, R(−)-N6(2-phenyl-isopropyl)-adenosine; CGS 15943, 5-amino-9-chloro-2-(furyl)1,2,4-triazolo[1,5-c] quinazoline; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; SCH 58261, 7-(2-phenylethyl)2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c] pyrimidine; MRE 31052F, 5-[4-clorophenyl]amino-carbamoylaminio-8-butyl-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine; MRE 3008F20, 5-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; MRE 3016F, 5-Amino-8-(3-methyl-2-buten-1-yl)-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine; GDPD, glyceraldehyde-3-phosphate dehydrogenase; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PBL, peripheral blood lymphocytes; CI-IB-MECA, N6-(3-iodo-benzyl)-2-chloro-adenosine-5’-N-methyluronamide; PMA, phorbol 12-myristate 13-acetate; S-PtA, S(−)-N6(2-phenylisopropyl)adenosine.
ligands, available (Gessi et al., 1999; Sullivan et al., 1999, 2001; Ohta and Sitkovsky, 2001; Thiel et al., 2003). In the case of the G1-coupled A3 receptor, the data are less extensive. It has been shown in a murine model that adenosine, through an A3 receptor, can interfere with the tumor cell recognition and the cytolytic activity of cytotoxic lymphocytes thus leading to the hypothesis that A3 antagonists might be used to relieve tumor-associated immunosuppression and facilitate adoptive immunotherapy (MacKenzie et al., 1994; Hoskin et al., 2002). As for human models, we have previously demonstrated that this adenosine subtype is highly expressed in Jurkat cells, a human tumor cell line originating from the immune system (Gessi et al., 2001), whereas a coherent picture of this subtype in human lymphocytes has not yet emerged. A considerable body of evidence has accumulated suggesting that the A3 receptor may regulate various functions in lymphoid cells by inducing both pro- and anti-inflammatory effects. Its activation results in proinflammatory activities by facilitating release of allergic mediators in mast cells (Ramkumar et al., 1993) and by initiating actin polymerization and migration in immature dendritic cells (Panther et al., 2001). In contrast, its stimulation induces anti-inflammatory effects by inhibiting pro-inflammatory cytokine release, formyl-Met-Leu-Phε-triggered respiratory burst, and tissue factor expression in monocytes cells (Sajjadi et al., 1996; Broussas et al., 1999, 2002). Moreover, anti-inflammatory effects are evoked by reduction of superoxide anion generation and degranulation in eosinophils and neutrophils (Bouma et al., 1997; Ezeamuzie and Philips, 1999; Gessi et al., 2002) and by induction of apoptosis in blood mononuclear cells (Barbieri et al., 1998). However, in the case of human lymphocytes, novel roles for the A3 receptor must be considered with caution until the ligand binding profile of this subtype has been properly defined. Prompted by these observations, we undertook an in-depth investigation using receptor-binding, western blotting studies, quantitative real-time RT-PCR and cAMP assays of A3 receptors in resting and phytohemagglutinin (PHA)-activated T cells. Our results demonstrate that activated human lymphocytes undergo a rapid induction of both transcript and protein of A3 receptors and suggest that A3 subtypes may be involved in the immunological responses mediated by adenosine in T cells.

**Materials and Methods**

**Materials.** [H]MRE 3008F20 (specific activity, 67 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, UK). NECA, R-PIA, S-PIA, CI-IB-MECA, CGS 15943, and DPCPX were obtained from Sigma/RBI (Natick, MA). SCH 58261, MRE 3105F20, MRE 3008F20, and MRE 3016F20 were synthesized by Prof. P. G. Baraldi (University of Ferrara, Italy). Fura-2 acetoxymethyl ester was from Inalo SpA (Milano, Italy). Ficoll-Hypaque was purchased from Amersham. The A3 antibody was purchased from Alpha Diagnostic (San Antonio, TX). TaqMan MGB probe and A3 primers were obtained from Applied Biosystems (Warrington, UK). All other reagents were of analytical grade and obtained from commercial sources. Stock solutions of all adenosine receptor agonists and antagonists were made in DMSO at a concentration of 1 × 10^{-2} M and then diluted directly in buffer. The final concentration of DMSO present at the highest drug concentrations did not exceed 0.001%, a dose that has no effect on lymphocytes responses.

**Lymphocyte Isolation.** Lymphocytes were isolated from buffy coats kindly provided by the Blood Bank of the University Hospital of Ferrara, according with the methods reported by Varani et al. (1997). Briefly, the blood was centrifuged on Ficoll-Hypaque density gradients. The human peripheral blood mononuclear cells were isolated and removed from the Ficoll-Hypaque density gradients. Subsequently, they were washed in 0.02 M phosphate-buffered saline at pH 7.2 containing 5 mM MgCl2 and 0.15 mM CaCl2. Finally, they were decanted into a culture flask and placed in a humidified incubator (5% CO2) for 2 h at 37°C. This procedure, aimed at removing monocytes, which adhere to the culture flask, resulted in a purified lymphocyte preparation containing at least 99% small lymphocytes identified by morphological criteria (PBL). Then, T cells were activated with 2 μg/ml PHA and, for Western blot experiments, CD8+ and CD4+ enriched T cells were purified from the PBL using dynabeads coated with an anti-CD8 mAb (Dynal ASA, Oslo, Norway).

**Membrane Preparation.** After centrifugation at 400g for 15 min at 4°C, the mononuclear cell pellets were resuspended in ice-cold, glass-distilled water and allowed to stand on ice for 60 min to ensure adequate lysis. The preparation was centrifuged at 20,000g for 15 min, the resulting pellet was resuspended in 50 mM Tris-HCl, 10 mM MgCl2, 1 mM EDTA, pH 7.4 at 25°C, at a concentration of 60 μg protein/100 μl, and this suspension was incubated with 3 IU/ml of adenosine deaminase for 30 min at 37°C before use for binding studies.

$[^{3}H]MRE$ 3008F20 **Binding Assay.** Binding assays were carried out according to Gessi et al. (2002). Association kinetic studies were performed incubating lymphocyte membranes with 2 nM $[^{3}H]MRE$ 3008F20 in a thermostatic bath at 4°C. For the measurement of the association rate, the reaction was terminated at time points ranging from 5 to 200 min by rapid filtration under vacuum, followed by four washes with ice-cold buffer. For dissociation experiments, membranes were preincubated with $[^{3}H]MRE$ 3008F20 at 4°C for 120 min. Specific binding was then evaluated at 5 to 100 min after the addition of 1 μM MRE 3008F20. The binding of $[^{3}H]MRE$ 3008F20 to human lymphocytes was carried out in a 96-well microplate in a total volume of 250 μl containing 50 mM Tris HCl buffer, 10 mM MgCl2, and 1 mM EDTA, pH 7.4. In saturation experiments, 100 μl of membrane homogenate (60 μg protein/assay) were incubated in duplicate with 10 to 12 different concentrations of $[^{3}H]MRE$ 3008F20 in the range of 0.1 to 20 nM. In competition experiments, 2 nM $[^{3}H]MRE$ 3008F20 was incubated in duplicate with at least 12 to 14 different concentrations of typical adenosine receptor agonists and antagonists. Analogous experiments were performed in the presence of 100 μM GTP. Nonspecific binding, defined as binding in the presence of 1 μM MRE 3008F20 at the Kd value for the radioligand was ~35% of total binding. Incubation time was 120 min at 4°C to allow equilibrium to be reached. This temperature was chosen in consideration of the fact that A3 antagonist binding is prevailently enthalpy-driven (Varani et al., 2000). Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass-fiber filters using a Micro-Mate 196 cell harvester (PerkinElmer Life and Analytical Sciences, Boston, MA). The filter bound radioactivity was counted on a TopCount microplate scintillation counter (efficiency 57%) with MicroScint 20 (both from PerkinElmer). The protein concentration was determined according to a Bio Rad method (Bradford, 1976) with bovine albumin as a standard reference.

**Thermodynamic Analysis.** For a generic binding equilibrium the affinity association constant $K_a = [L][R]$ is directly related to the standard free energy $ΔG^o = -RT lnK_a$, where $R$ is the gas constant and T is the absolute temperature) that can be separated in its enthalpic and entropic contributions according to the Gibbs equation:

$$ΔG^o = ΔH^o - TΔS^o$$

The standard free energy was calculated as $ΔG^o = -RTlnK_a$ at 298.15 K, the standard enthalpy, $ΔH^o$, from the van't Hoff plot $lnK_a$ versus $1/T$ (the slope of which is $-ΔH^o/R$) and the standard entropy as $ΔS^o = (ΔH^o - ΔG^o)/T$, with $T = 298.15$ K and $r = 8.314$ J/K/mol. $K_a$ values were obtained from saturation experiments of $[^{3}H]MRE$ 3008F20 binding to the A3 adenosine receptors in human lymphocytes performed at 4, 10, 15, 20, 25, and 30°C.
Western Blotting. T cells (5 x 10^6) were washed with ice-cold PBS and centrifuged. The pellets were resuspended in buffer solution (300 μl of 2% SDS, 10% glycerol, and 60 mM Tris-HCl, pH 6.8) and lysed for 7 min at 100°C. Protein concentration was determined using a biocinchoninic acid protocol (Pierce Chemical, Rockford, IL). Equal amounts of proteins were loaded on a 12% SDS-polyacrylamide gel and electrophoresed to Protran nitrocellulose membranes (Schleicher and Schuell, Keene, NH). The blots were probed with the appropriate dilution of primary antibody in fresh blocking buffer (5% skim milk powder in phosphate-buffered saline containing 0.1% Tween 20) overnight at 4°C. Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin G (1:5000). Specific reactions were revealed with the enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences).

Real-Time RT-PCR Experiments. Total cytoplasmic RNA was extracted by the acid guanidinium-thiocyanate-phenol method (Chomczynski and Sacchi, 1987). Quantitative real-time RT-PCR assay (Higuchi et al., 1993) of A3 mRNA transcript was carried out using gene-specific double fluorescently labeled TaqMan MGB probe (minor groove binder) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The following primer and probe sequences were used for real-time RT-PCR: A3 forward primer, 5'-ATGCCCTTTGGCCATTGTTG-3'; A3 reverse primer, 5'-ACAATCTCATTCTACAGCTGCCT-3'; A3 MGB probe, 5'-FAM-TCAGGCTGGGATC-TAMRA-3' (in which the fluorescent reporter FAM and the quencher TAMRA are 6-carboxy fluorescein and 6-carboxy-N_2,N_4,N_6,N_8-tetramethylrhodamine, respectively). For the real-time RT-PCR of the reference gene, the endogenous control human GAPDH kit was used, and the probe was fluorescence-labeled with VIC™ (Applied Biosystems, Monza, Italy).

Measurement of Cyclic AMP Levels. Human lymphocytes (5 x 10^6 cells assay) were suspended in 0.5 ml of Krebs Ringer phosphate buffer (136 mM NaCl, 5 mM KCl, 0.67 mM Na_2HPO_4, 0.2 mM KH.PO_4, 3 mM NaHCO_3, 1 mM CaCl_2, 5 mM glucose, 5 mM HEPES, and 10 mM MgCl_2, at pH 7.45), containing 0.5 mM Ro 20-1724, water (Chomczynski and Sacchi, 1987). Quantitative real-time RT-PCR (Amersham Biosciences).

Radioiogand Binding Studies

Saturation Experiments. To evaluate the affinity and density of A3 receptors on human lymphocytes expressed on plasma membranes, saturation binding experiments were performed using the potent and selective antagonist radioligand [3H]MRE 3008F20 (range 0.1 to 20 nM). The linearity of the Scatchard plots in the inset failed to show a significantly better fit to a two-site than to a one-site binding model, indicating that only one class of high-affinity binding sites was present under our experimental conditions, with an equilibrium dissociation constant of 1.79 ± 0.30 nM and a receptor density of 125 ± 15 fmol/mg of protein (Munson and Rodbard, 1980). The expression of A3 receptors was analyzed in activated lymphocytes. One-hour treatment with PHA, a T cell mitogen, caused an increase of binding sites to A3 receptors (B_max of 225 ± 23 fmol/mg of protein, P < 0.01), with changes in K_d value (1.85 ± 0.25 nM) (Fig. 1A). Incubation for 1 h of the cells with PHA at different concentrations caused an increase of A3 receptors, which reached a maximal expression at 2 μg/ml, as demonstrated by binding studies (Fig. 1B). Comparable results were obtained by activatin T cells with PMA (range, 0.1–100 ng/ml) plus 1 μg/ml ionomycin for 1 h; in this case, the maximal expression of A3 receptor protein was chosen to perform a more detailed kinetic analysis of the increase in expression of the A3 receptor protein. The time course of A3 receptor up-regulation was followed at 15, 30, 60, 180, 480, and 1440 min. Saturation experiments revealed a rapid increase of binding to the A3 subtype starting within 15 min, with a B_max value of 175 ± 14 fmol/mg of protein (P < 0.05), and reaching a plateau at 30 min (B_max value of 228 ± 22, 225 ± 23, 233 ± 26, 230 ± 25, and 220 ± 20 fmol/mg of proteins at 30, 60, 180, 480, and 1440 min, respectively, P < 0.01) (Fig. 1C); the respective equilibrium dissociation constants were not significantly different.

Kinetic Experiments. Association studies showed that binding equilibrium was reached after approximately 60 min and was stable for at least 5 h (Fig. 2A). [3H]MRE 3008F20 binding was rapidly reversed by the addition of 1 μM MRE 3008F20 (Fig. 2B). A two-site fit of association and dissociation curves was not significantly better than a one-site fit (P < 0.05). The rate constants were: k_on = 0.049 ± 0.001 min^-1 and k_off = 0.024 ± 0.001 min^-1. The apparent equilibrium dissociation constant (K_d) was calculated from the k_on/k_off value of 0.0125 min^-1 nM^-1 and k_off values to be 1.92 nM. Similar results were obtained in activated T cells (data not shown).

Competition Experiments. Adenosine agonists were found to inhibit [3H]MRE 3008F20 binding, in human lymphocytes, in a manner consistent with the labeling of the A3 adenosine receptor, as shown in Fig. 3A. The order of potency in [3H]MRE 3008F20 inhibition assays was CI-IB-MECA > NECA > R-PIA > S-PIA. R-PIA was approximately six to seven times more active (K_H = 38 nM; K_I = 2100 nM) than its stereoisomer, S-PIA (K_H = 260 nM; K_I = 190,000 nM) showing the stereoselectivity of agonist binding. The competition curves of all agonists were best fit assuming a two-state model corresponding to high- and low-affinity states of the A3 adenosine receptor (Fig. 3A), a phenomenon expected
for a G-protein-coupled receptor. Affinities are reported in Table 1 as $K_i$, $K_H$, and $K_L$ values, and the percentage of receptors in the high-affinity state ($R_H$) is also shown. To test whether the high-affinity state of the $A_3$ receptors was linked to a guanine nucleotide regulatory protein, the effect of GTP on the affinity states was examined. The addition of 100 $\mu$M GTP shifted the competition curves with agonists from a biphasic to a monophasic shape (LIGAND, $P < 0.01$), with a $K_i$ value near that of the low affinity sites, as shown in Table 1. In contrast, the above treatment did not change the shape of the competition curves with antagonists. Antagonist competition curves for $[^{3}\text{H}]$MRE 3008F20 binding showed a rank order of potency of MRE3105F20 > MRE3008F20 > CGS 15943 > DPCPX, each of which exhibited Hill slopes near unity (Fig. 3B; Table 1). SCH 58261 showed a $K_i$ value $> 10$ $\mu$M.

**Thermodynamic Experiments.** Saturation experiments of $[^{3}\text{H}]$MRE 3008F20 binding, performed at the six temperatures selected revealed that, in resting cells, $K_D$ and $B_{max}$ values were in the range 1.8 to 4.3 nM and 110 to 130 fmol/mg of proteins, respectively. Whereas the dissociation constant ($K_D$) changed with temperature, $B_{max}$ values ob-

![Graph A](image1.png)

**Fig. 1.** A, saturation of $[^{3}\text{H}]$MRE 3008F20 binding to $A_3$ adenosine receptors in human lymphocytes under resting (■) and activated conditions (□); $K_D$ values were 1.78 ± 0.30 and 1.85 ± 0.25 nM and $B_{max}$ values were 125 ± 15 and 225 ± 23 fmol/mg of protein, respectively. Experiments were performed as described under Materials and Methods. Insets, Scatchard plots of the same data. B, effect of increasing concentrations of PHA on $A_3$ receptor density in human lymphocytes. C, time course of $A_3$ receptor density in human lymphocytes stimulated with PHA, assayed by means of $[^{3}\text{H}]$MRE 3008F20 saturation binding experiments. Analysis was by Student’s $t$ test. #, $P < 0.05$; *, $P < 0.01$ versus resting cells (0 h). Values are the means and vertical lines are S.E.M. of five separate experiments performed in triplicate.
tained from [3H]MRE 3008F20 saturation experiments seemed to be largely independent. The van’t Hoff plot of lnK_A versus 1/T for [3H]MRE 3008F20 binding to the A₃ adenosine receptor in human lymphocytes gives the following final equilibrium thermodynamic parameters (expressed as mean values ± S.E. of three independent determinations): ΔG° = -47.76 ± 0.16 kJ/mol; ΔH° = -19.78 ± 1.50 kJ/mol; ΔS° = 94 ± 7 J/mol/K and indicates that the binding was both enthalpy- and entropy-driven. Similar results were obtained after PHA-activation as shown in Fig. 4 (ΔG° = -48.17 ± 0.17 kJ/mol; ΔH° = -19.37 ± 1.75 kJ/mol; ΔS° = 97 ± 8 J/mol/K).

**Western Blotting Studies**

To compare the expression of A₃ receptors in the most representative subset of human T lymphocytes, resting and PHA-activated PBL from different human volunteers were separated into CD8⁺ and enriched CD4⁺ fractions. The resting and activated cells of these two fractions and an aliquot of the total unseparated PBL were then evaluated for the expression of the specific A₃ receptor by Western blot analysis. A significant change in the expression of the A₃ subtype within the total unseparated population was observed between resting and activated cells confirming our binding

![Fig. 2](image)

**Fig. 2.** A, kinetics of [3H]MRE 3008F20 binding to human A₃ adenosine receptors with association curves representative of a single experiment that was replicated three times with similar results, in human lymphocytes. Inset, first-order plot of [3H]MRE 3008F20 binding. B, amount of [3H]MRE 3008F20 bound at equilibrium; C, amount of [3H]MRE 3008F20 bound at each time. Association rate constant was k₁ = 0.0125 ± 0.0010 min⁻¹ nM⁻¹ in human lymphocytes. B, kinetic of [3H]MRE 3008F20 binding to human A₃ adenosine receptors with dissociation curve representative of a single experiment. Inset, first-order plot of [3H]MRE 3008F20 binding. Dissociation rate constant was k₋₁ = 0.024 ± 0.001 min⁻¹ in human lymphocytes.

![Fig. 3](image)

**Fig. 3.** Competition curves of specific [3H]MRE 3008F20 binding to human A₃ adenosine receptors in human lymphocytes by adenosine agonists (A) and antagonists (B). Curves are representative of a single experiment from a series of four independent experiments. Competition experiments were performed as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>K_H, nM</th>
<th>K_L, nM</th>
<th>Kᵢ + GTP, pM</th>
<th>RH ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI-IB-MECA</td>
<td>0.80 ± 0.1</td>
<td>67 ± 6</td>
<td>75 ± 7</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>NECA</td>
<td>18 ± 3</td>
<td>1250 ± 250</td>
<td>1300 ± 200</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>R-PIA</td>
<td>38 ± 6</td>
<td>2100 ± 250</td>
<td>2200 ± 280</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>S-PIA</td>
<td>280 ± 29</td>
<td>19,000 ± 2500</td>
<td>21,000 ± 2800</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>MRE 3008F20</td>
<td>2.0 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>85 ± 8</td>
</tr>
<tr>
<td>MRE 3105F20</td>
<td>76 ± 8</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>85 ± 8</td>
</tr>
<tr>
<td>CGS 15943</td>
<td>850 ± 90</td>
<td>920 ± 97</td>
<td>920 ± 97</td>
<td>920 ± 97</td>
</tr>
<tr>
<td>SCH 58261</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
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</table>
experiments (Fig. 5 A, lines 1 and 2). The increase in A<sub>3</sub> receptor expression after PHA activation was attributed to the CD4<sup>+</sup> enriched fraction (Fig. 5A, lines 3 and 4) whereas CD8<sup>+</sup> cells, which do express A<sub>3</sub> receptors, failed to show any significant change after T cell activation (Fig. 5A, lines 5 and 6); CHO cells transfected with human A<sub>3</sub> receptors were chosen as positive controls (line 7). When earlier time points were investigated in separated CD4<sup>+</sup> and CD8<sup>+</sup> cells, we observed that the increase of protein in CD4<sup>+</sup> cells started at 15 min and was maximal at 30 min (Fig. 5 B). As for CD8<sup>+</sup> cells, the density of A<sub>3</sub> subtype showed no change after T cell activation (data not shown).

**Real-Time RT-PCR Experiments**

To determine whether the increase in A<sub>3</sub> density was caused by an increase in mRNA encoding the A<sub>3</sub> receptor, mRNA content was investigated from resting and PHA-stimulated human lymphocytes by use of quantitative real-time RT-PCR. The expression level of A<sub>3</sub> receptors was normalized to the expression level of the endogenous reference (GAPDH) in each sample. Activation of T cells by the addition of PHA for 1 h produced a 5.2 ± 0.7-fold increase of A<sub>3</sub> receptor mRNA accumulation in activated T cells with respect to the corresponding resting ones, as obtained from different human volunteers. A representative example of the real-time RT-PCR quantitation of A<sub>3</sub> receptors and GAPDH mRNAs in one individual case is shown in Fig. 6, A and B. When investigating the time-dependence of mRNA accumulation in separated CD4<sup>+</sup> and CD8<sup>+</sup> cells, a rapid increase was observed starting within 15 min (3.5 ± 0.4 fold, P < 0.01) in CD4<sup>+</sup> subset; this increase was highly reproducible and reached a plateau at 30 min (5.5 ± 0.8 fold, P < 0.01) (Fig. 6 C). Conversely, PHA treatment did not change A<sub>3</sub> receptor mRNA in CD8<sup>+</sup> cells (data not shown).

**Cyclic AMP Assays**

To demonstrate the existence of functional A<sub>3</sub> receptors in human lymphocytes and to evaluate whether changes of receptor density under activating conditions were reflected at a functional level, we determined the potency of the most selective A<sub>3</sub> agonist CI-IB-MECA in the inhibition of cAMP levels. CI-IB-MECA was able to inhibit forskolin-stimulated cAMP levels with an IC<sub>50</sub> value of 2.7 ± 0.3 nM, according to the affinity determined in radioligand binding assays (Fig. 7A); interestingly, after PHA treatment, there was a shift to the left of the agonist IC<sub>50</sub> value (1.5 ± 0.4 nM). In resting lymphocytes, the selective A<sub>3</sub> ligand MRE 3008F20 antagonized 100 nM CI-IB-MECA–induced inhibition of cAMP levels with an IC<sub>50</sub> of 5.0 ± 0.3 nM (Fig. 7B), suggesting the involvement of A<sub>3</sub> receptors. A low-affinity analog of MRE 3008F20, MRE 3016F20 (hA<sub>3</sub>CHO cells; K<sub>i</sub> = 800 nM) (Baraldi et al., 2000), was chosen to ascertain that the block of cAMP levels was caused not by the structure of this family of compounds but by the activity toward A<sub>3</sub> receptors. As shown in Fig. 7B, the failure of this ligand to antagonize CI-IB-MECA effects on cAMP levels confirms the involve-

**Fig. 4.** Van’t Hoff plot showing the effect of temperature on the equilibrium binding association constant, K<sub>A</sub> = 1/K<sub>D</sub> of [3H]MRE 3008F20 in human lymphocytes under resting (■) and activated (□) conditions. The plot is essentially linear in the temperature range investigated 0 to 30°C.

**Fig. 5.** A, Western blotting experiments showing the A<sub>3</sub> receptor expression, using an A<sub>3</sub> specific antibody, in total, CD4<sup>+</sup> enriched, and CD8<sup>+</sup> human lymphocytes under resting (R) and activated (A) conditions; hCHO A<sub>3</sub> cells were used as positive controls. B, time course of A<sub>3</sub> receptor protein in separated CD4<sup>+</sup> subset stimulated with 2 μg/ml PHA. The density of each band was quantified by densitometry (GS700; Bio-Rad, Hercules, CA). The values represent the means ± S.E.M. of four experiments.
ment of A3 receptors in this response. DPCPX (100 nM), a selective blocker of A1 subtypes, did not significantly modify 100 nM Cl-IB-MECA-mediated response (65 ± 5 and 62 ± 4% in the absence and in the presence of DPCPX, respectively), thus ruling out the involvement of A1 receptors. The nonselective agonist NECA was able to induce a stimulation of cyclic AMP levels, with an EC50 value of 215 ± 30 nM. Because of the stimulatory effect observed in the presence of NECA, we antagonized a fixed concentration of this agonist (1 μM) by increasing doses of the selective A2A blocker SCH 58261 (0.1–100 nM) in the absence of forskolin. Under these experimental conditions, SCH 58261 antagonized the rise in cyclic AMP levels with an IC50 value of 15 ± 3 nM, suggesting the involvement of A2A subtype.

Discussion

Adenosine has been implicated in the regulation of a number of effects on lymphocyte function, most of which are attributed to the activation of the well characterized A2A receptors. However, it has been suggested that the A3 receptor may play a more selective role in immune and inflammatory responses (MacKenzie et al., 1994; Hoskin et al., 2002). Therefore, even though many pharmacological works rely on the selectivity of adenosine agonists and antagonists, we are convinced that before establishing a role for the A3 subtype in the regulation of human immune system, it is important to identify the leukocytes that express functional A3 receptors. Prompted by these considerations and after the pharmacological characterization by means of receptor-binding of the A3 subtype in human eosinophils (Kohno et al., 1996), neutrophils (Gessi et al., 2002), and recently dendritic cells (Fossetta et al., 2003), in the present study, we provide an analysis of the A3 adenosine receptor in human lymphocytes.

Different experimental approaches attest to the existence of adenosine A3 receptors on these peripheral blood cells and

**Fig. 6.** Representative amplification plots for the A3 receptors mRNA in resting (■) and one hour-activated (□) human lymphocytes (A) compared with the respective GAPDH mRNA (B). C, time course of A3 receptor mRNA in separated CD4+ subsets stimulated with 2 μg/ml PHA. The values represent the means ± S.E.M. of four experiments. Results are presented as the mean ± S.E.M. of four independent experiments (Student’s t test; *, P < 0.01 versus resting cells).

**Fig. 7.** A, inhibition curves of forskolin (10 μM) stimulated cAMP levels by Cl-IB-MECA under resting (■) and activated (□) conditions (IC50 = 2.7 ± 0.3 and 1.5 ± 0.4 nM, respectively). Results are presented as the mean ± S.E.M. of five independent experiments (Student’s t test; *, P < 0.01 versus resting cells). B, antagonism by MRE 3008F20 (■) and lack of effect by MRE 3016F20 (△), an analog of MRE 3008F20, with low affinity to A3 receptors on the Cl-IB-MECA (100 nM) inhibition of forskolin-stimulated cAMP levels in human resting lymphocytes.
indicate an up-regulation after T cell activation. First, their presence on plasma membrane was detected through saturation experiments of [3H]MRE 3008F20 binding, revealing a single class of binding sites with a $K_d$ value of 1.79 ± 0.30 nM, comparable with that determined by kinetic experiments (1.92 nM). The binding was rapid, reversible, and saturable, with a capacity of 125 ± 15 fmol/mg of protein, indicating that the number of $A_3$ receptors in resting human lymphocytes was similar to the number of $A_{2A}$ subtypes (Varani et al., 1997) but lower than the number of $A_3$ receptors found in Jurkat cells, a human tumor leukemia line (Gessi et al., 2001).

Interestingly, after PHA activation, we observed a rapid increase of $A_3$ receptor density, starting at 15 min, that was maximal within 30 min of treatment and was stable when evaluated at 3, 8, or 24 h of PHA treatment. Analog results were obtained by stimulating lymphocytes with other activators such as PMA plus ionomycin, suggesting that $A_3$ receptors may play a role in activated T cells.

Competition experiments of various adenosine receptor ligands, performed to characterize the $A_3$ subtype in T cells, showed a rank order of potency typical of the $A_3$ subtype (Gessi et al., 2002; Fossetta et al., 2003). All agonist competition curves were best described by the existence of one high-affinity ($K_{iH}$) and one low-affinity ($K_{iL}$) agonist receptor binding state. Approximately 25 to 28% of the $A_3$ receptor existed in the high-affinity state under the present assay conditions. The addition of GTP, and subsequent uncoupling of receptors from G-proteins, converted the agonist displacement curves from biphasic to monophasic, suggesting a guanine nucleotide-mediated shift of the high-affinity binding sites to a low-affinity form, in agreement with that reported for human $A_3$ receptors (Varani et al., 2000). On the contrary, competition binding curves with antagonists, including substituted pyrazolo triazolo pyrimidine compounds (Baraldi and Borea, 2000) were monophasic and did not change upon addition of GTP. Thermodynamic parameters obtained from the van’t Hoff plot indicate that [3H]MRE 3008F20 binding to $A_3$ adenosine receptors is enthalpy- and entropy-driven in agreement with data obtained in all systems in which human $A_3$ receptors have been studied from this point of view (Varani et al., 2000, 2001, 2002; Merighi et al., 2001). A similar thermodynamic behavior was found in activated T cells, suggesting that the activation process does not involve, at a molecular level, receptor alterations, leading to modifications in the $A_3$-related binding mechanisms.

In addition to the $A_3$ receptors, PHA has been shown to alter the expression of other adenosine subtypes. For example, it has been reported that $A_{2A}$ receptors are also up-regulated by PHA in both CD4+ and CD8+ cells (Mirabet et al., 1999); as for $A_{2A}$ subtypes, it has been observed that the activation process increases their expression predominantly in CD8+ T cells (Koshiba et al., 1999). Therefore, we evaluated whether activation-dependent changes in $A_3$ expression were attributable to CD8+ or CD4+ subsets of human lymphocytes, the two major T cell subsets involved in the recognition of peptide antigens presented by class I and II major histocompatibility complex, respectively. Our results allow us to conclude that CD8+ fraction does express $A_3$ receptors but is not responsible for their increase under activating conditions, which is instead caused by an enriched CD4+ cell fraction. When we investigated the kinetic of this up-regulation, we found that even at earlier time points, the increase was present only in CD4+ cell fractions, whereas it was not changed in CD8+ cells. Therefore, it is possible that in humans, as in the murine model (Hoskin et al., 2002), $A_3$ receptors play an immunosuppressive role in CD8+ T cells, but their up-regulation in CD4+ fraction strongly suggests that they might also be implicated in T helper cell activities. One method for increasing the amount of $A_3$ receptors on the cell membrane is to increase the accumulation of mRNA encoding the $A_3$ subtypes. As evaluated by means of real-time RT-PCR experiments, activation of T cells with PHA rapidly increased the level of $A_3$ message in the CD4+ subset, but not in the CD8+ cells. This increase in $A_3$ receptor mRNA, which could occur as a result of an increase in transcription and/or an increase in mRNA stability, is likely to be responsible for the increased synthesis of receptor proteins as detected by means of binding and Western blotting studies. Interestingly, it has been reported that $A_{2A}$ mRNA is also regulated after activating conditions, suggesting that adenosine receptor expression is of greater significance in the modulation of the functions of these cells (Thiel et al., 2003).

Numerous data attest to the importance of $A_{2A}$ subtype in the modulation of immune response mediated by adenosine, whereas less impressive information is actually available on the effects exerted through $A_3$ receptor. To verify whether the increase in receptor density was reflected in the regulation of second messengers activated by $A_3$ adenosine receptors in human lymphocytes, we investigated the ability of CI-IB-MECA to modulate adenylyl cyclase activity. Forskolin-stimulated cAMP levels were inhibited by CI-IB-MECA, and this effect was potently antagonized by MRE 3008F20. The agonist $I_{Ca}$ value was shifted to the left under stimulating conditions, suggesting that $A_3$ receptors may activate this pathway during T cell activation and may affect the T cell-mediated immune response. As for NECA, this compound, even if able to compete for [3H]MRE 3008F20 binding, did not inhibit CAMP levels, as already observed in Jurkat cells (Gessi et al., 2001). Most probably, its stimulatory effect on CAMP levels, raised through the activation of $A_{2A}$ receptors, prevailed over the $A_3$-mediated inhibitory action, suggesting a stronger potency of $A_{2A}$ receptors in signal transduction over the $A_3$ receptors. This is not surprising, because a similar behavior has already been reported in doubly Gs/Gi-coupled chimeric $A_1/A_{2A}$ adenosine receptors expressed in human embryonic kidney 293 cells (Tucker et al., 2000). Therefore, future studies will help to reconcile the complicated effects of adenosine through G protein-coupled $A_3$ adenosine receptors in the modulation of immune and inflammatory processes and to clarify the relevance of the differential activation by adenosine of Gs/Gi-coupled receptor subtypes to reach a balance between destruction of pathogen agents and tissue protection from excessive damage (Sikkovsky, 2003).

At variance with adenylyl cyclase activity, only high micromolar doses of CI-IB-MECA induced Ca2+ increase in both resting and activated conditions. The low potency of the agonist in the stimulation of Ca2+ increase indicates that CI-IB-MECA elevated calcium by an unknown mechanism that cannot clearly be related to $A_3$ receptor activation (data not shown). However, there are other effector systems that may be activated by $A_3$ subtypes to modulate immune functions (Butler et al., 2003), and ongoing studies in our labora-
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References


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