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Expression of A_3 Adenosine Receptors in Human Lymphocytes: Up-Regulation in T Cell Activation

Stefania Gessi, Katia Varani, Stefania Merighi, Elena Cattabriga, Arianna Avitabile, Riccardo Gavioli, Cinzia Fortini, Edward Leung, Stephen Mac Lennan, and Pier Andrea Borea

Department of Clinical and Experimental Medicine, Pharmacology Unit (S.G., K.V., S.M., E.C., A.A., P.A.B.) and Department of Biochemistry and Molecular Biology (R.G., C.F.), University of Ferrara, Ferrara, Italy; "Centro Nazionale di Eccellenza per lo Sviluppo di Metodologie Innovative per lo Studio ed il Trattamento delle Patologie Infiammatorie" Ferrara, Ferrara, Italy (S.G., K.V., S.M., E.C., A.A., P.A.B.); and King Pharmaceuticals, Cary, North Carolina (E.L., S.M).

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ABSTRACT

The present study investigates mRNA and protein levels of A_3 adenosine receptors in resting (R) and activated (A) human lymphocytes. The receptors were evaluated by the antagonist radioligand [^{3}H]5-*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo-[1,5-c]-pyrimidine ([^{3}H]MRE 3008F20), which yielded B_{max} values of 125 ± 15 and 225 ± 23 fmol/mg of protein and K_D 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 A_3 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 A_3 mRNA after T cell activation. Competition of radioligand binding by adenosine ligands displayed a rank order of potency typical of the A_3 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 A_3-related binding mechanisms. Functionally, the up-regulation of A_3 adenosine receptors in A versus R cells corresponded to a potency increase of the A_3 agonist N^6-(3-iodo-benzyl)-2-chloro-adenosine-5′-*N*-methyluronamide in inhibiting cAMP accumulation (IC_{50} = 1.5 ± 0.4 and 2.7 ± 0.3 nM, respectively); this effect was antagonized by MRE 3008F20 (IC_{50} = 5.0 ± 0.3 nM). In conclusion, our results provide, for the first time, an in-depth investigation of A_3 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 A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). These adenosine subtypes have been classified as such because of differences at the molecular, biochemical, and pharmacological levels (Linden, 2001; Klinger 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 the enzyme 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 G_s-coupled A_{2A} receptors (Polmar, 1990). Indeed, in human lymphocytes, the A_{2A} receptor has been thoroughly characterized based on binding (Varani et al., 1997; 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*-ethyl-carboxamidoadenosine; *R*-PIA, *R*(−)-N^6(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 3105F20, 5-[[(4-clorophenyl)amino]carbamoyl]amino-8-butyl-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3008F20, 5-*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo [1,5-*c*] pyrimidine; MRE 3016F20, 5-Amino-8-(3-methyl-2-buten-1-yl)-2-(2-furyl)pyrazolo-[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PBL, peripheral blood lymphocytes; Cl-IB-MECA, N^6-(3-iodo-benzyl)-2-chloro-adenosine-5′-*N*-methyluronamide; PMA, phorbol 12-myristate 13-acetate; *S*-PIA, *S*(−)-N^6(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 G_i-coupled A_3 subtype, the data are less extensive. It has been shown in a murine model that adenosine, through an A_3 receptor, can interfere with the tumor cell recognition and the cytolytic activity of cytotoxic lymphocytes thus leading to the hypothesis that A_3 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 A_3 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-Phe–triggered respiratory burst, and tissue factor expression in monocytic 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 A_3 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 A_3 receptors in resting and phytohemagglutinin (PHA)-activated T cells. Our results demonstrate that activated human lymphocytes undergo to a rapid induction of both transcript and protein of A_3 receptors and suggest that A_3 subtypes may be involved in the immunological responses mediated by adenosine in T cells.

Materials and Methods

Materials. [^{3}H]MRE 3008F20 (specific activity, 67 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, UK). NECA, *R*-PIA, *S*-PIA, Cl-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 Inalco SpA (Milano, Italy). Ficoll-Hypaque was purchased from Amersham. The A_3 antibody was purchased from Alpha Diagnostic (San Antonio, TX). TaqMan MGB probe and A_3 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 gradients. Subsequently, they were washed in 0.02 M phosphate-buffered saline at pH 7.2 containing 5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. Finally, they were decanted into a culture flask and placed in a humidified incubator (5% CO_2) 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 400*g* 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 20,000*g* for 15 min, the resulting pellet was resuspended in 50 mM Tris-HCl, 10 mM $MgCl_2$, 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 $MgCl_2$, 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 K_D 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 A_3 antagonist binding is prevalently 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 $K_A = 1/K_D$ is directly related to the standard free energy ΔG° (ΔG° = −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° = ΔH°−TΔS°. The standard free energy was calculated as ΔG° = −RTlnK_A at 298.15 K, the standard enthalpy, ΔH°, from the van't Hoff plot lnK_A versus (1/T) (the slope of which is −ΔH°/R) and the standard entropy as ΔS° = (ΔH° − ΔG°)/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 A_3 adenosine receptors in human lymphocytes performed at 4, 10, 15, 20, 25, and 30°C.

Western Blotting. T cells (5×10^6) were washed with ice-cold PBS and centrifuged. The pellets were resuspended in buffer solution (200 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 bicinchoninic acid protocol (Pierce Chemical, Rockford, IL). Equal amounts of proteins were loaded on a 12% SDS-polyacrylamide gel and electroblotted 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 A_3 mRNA transcript was carried out using gene-specific double fluorescently labeled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems). The following primer and probe sequences were used for real-time RT-PCR: A_3 forward primer, 5′-ATGCCTTTGGCCATTGTTG-3′; A_3 reverse primer, 5′-ACAATCCACTTCTACAGCTGCCT-3′; A_3 MGB probe, 5′-FAM-TCAGCCTGGGCATC-TAMRA-3′ (in which the fluorescent reporter FAM and the quencher TAMRA are 6-carboxy fluorescein and 6-carboxy-*N*,*N*,*N*′,*N*′-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 (5×10^6 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_2PO_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 solution, as phosphodiesterase inhibitor, and 2.0 IU/ml adenosine deaminase and preincubated for 10 min in a shaking bath at 37°. Then the A_3 adenosine agonist Cl-IB-MECA plus forskolin (10 μM) was added to the mixture and incubated for a further 5 min. The effect of increasing concentrations of MRE 3008F20 in the range 0.1 to 100 nM was determined by antagonism of the inhibition of cyclic AMP production induced by Cl-IB-MECA (100 nM). Analogous experiments were performed by using the nonselective agonist NECA in the range 10 nM to 10 μM. The effect of a fixed concentration of NECA (1 μM) was antagonized by increasing doses of SCH 58261 (0.1–100 nM), in the absence of forskolin. The reaction was terminated by the addition of ice-cold 6% trichloroacetic acid. The trichloroacetic acid suspension was centrifuged at 2000*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 essentially according to Varani et al. (1997). Samples of cyclic AMP standards (0–10 pmol) were added to each test tube containing 0.1 M Trizma base, 8.0 mM aminophylline, 6.0 mM 2-mercaptoethanol, pH 7.4, and [^{3}H]cyclic AMP in a total volume of 0.5 ml. The binding protein, previously prepared from bovine adrenal glands, was added to the samples and incubated at 4°C for 150 min. After the addition of charcoal, samples were centrifuged at 2000*g* for 10 min. The clear supernatant (0.2 ml) was mixed with 4 ml of Atomlight (PerkinElmer Life and Analytical Sciences) and counted in a liquid scintillation counter (LS-1800; Beckman Coulter, Fullerton, CA).

Data Analysis. All binding studies (kinetics, saturation, competition) were analyzed with the program LIGAND (Cambridge, UK) (Munson and Rodbard, 1980). EC_{50} and IC_{50} values in the cyclic AMP and Ca^{2+} assays were calculated with the nonlinear least-squares curve fitting program Prism (GraphPad Software, San Diego, CA). Statistical analyses were performed using Student's *t* test.

Results

Radioligand Binding Studies

Saturation Experiments. To evaluate the affinity and density of A_3 receptors on human lymphocytes expressed on plasma membranes, saturation binding experiments were performed using the potent and selective antagonist radioligand [^{3}H]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 A_3 receptors was analyzed in activated lymphocytes. One-hour treatment with PHA, a T cell mitogen, caused an increase of binding sites to A_3 receptors (B_{max} of 225 ± 23 fmol/mg of protein, $P < 0.01$), whithout 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 A_3 receptors, which reached a maximal expression at 2 μg/ml, as demonstrated by binding studies (Fig. 1B). Comparable results were obtained by activating 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 A_3 receptor was obtained at 25 ng/ml PMA plus 1 μg/ml ionomycin (data not shown). Therefore, the concentration of 2 μg/ml PHA was chosen to perform a more detailed kinetic analysis of the increase in expression of the A_3 receptor protein. The time course of A_3 receptor up-regulation was followed at 15, 30, 60, 180, 480, and 1440 min. Saturation experiments revealed a rapid increase of binding to the A_3 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} values 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). [^{3}H]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: $k_{obs} = 0.049 \pm 0.001$ min^{-1} and $k_{-1} = 0.024 \pm 0.001$ min^{-1}. The apparent equilibrium dissociation constant (K_D) was calculated from the k_{+1} ($k_{+1} = 0.0125$ min^{-1} nM^{-1}) and k_{-1} 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 [^{3}H]MRE 3008F20 binding, in human lymphocytes, in a manner consistent with the labeling of the A_3 adenosine receptor, as shown in Fig. 3A. The order of potency in [^{3}H]MRE 3008F20 inhibition assays was Cl-IB-MECA > NECA > *R*-PIA > *S*-PIA. *R*-PIA was approximately six to seven times more active ($K_H = 38$ nM; $K_L = 2100$ nM) than its stereoisomer, *S*-PIA ($K_H = 260$ nM; $K_L = 19{,}000$ 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 A_3 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 μM 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}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 μM.

Thermodynamic Experiments. Saturation experiments of [^{3}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-

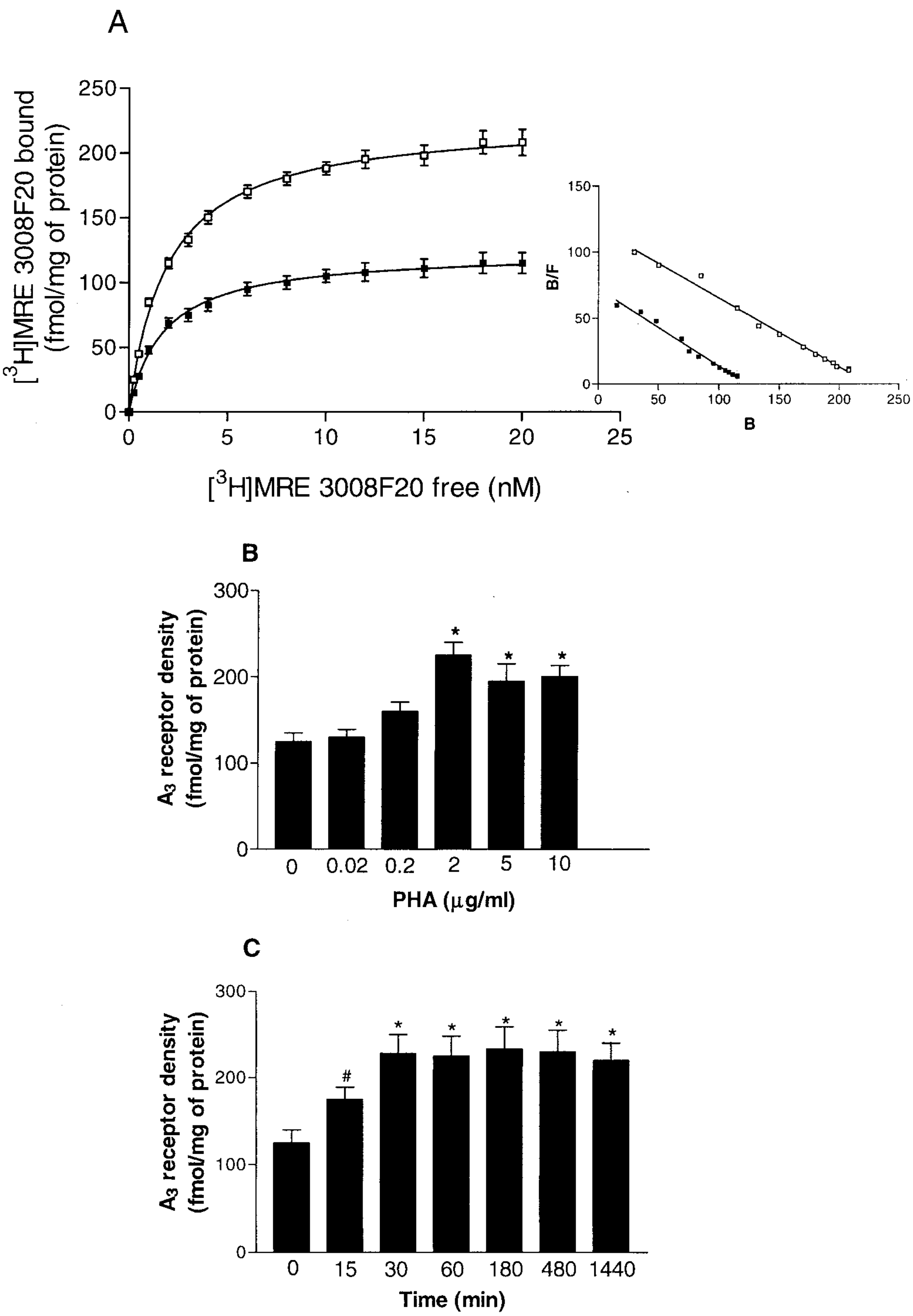

Fig. 1. A, saturation of [^{3}H]MRE 3008F20 binding to A_3 adenosine receptors in human lymphocytes under resting (■) and activated conditions (□); K_D values were 1.79 ± 0.30 and 1.85 ± 0.25 nM and B_{max} values were 125 ± 15 and 225 ± 23 fmol/mg of protein, respectively. Experiments were performed 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}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 [^{3}H]MRE 3008F20 saturation experiments seemed to be largely independent. The van't Hoff plot of lnK_A versus 1/T for [^{3}H]MRE 3008F20 binding to the A_3 adenosine receptor in human lymphocytes gives the following final equilibrium thermodynamic parameters (expressed as mean values ± S.E. of three independent determinations): $\Delta G° = -47.76 \pm 0.16$ kJ/mol; $\Delta H° = -19.78 \pm 1.50$ kJ/mol; $\Delta S° = 94 \pm 7$ 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 ($\Delta G° = -48.17 \pm 0.17$ kJ/mol; $\Delta H° = -19.37 \pm 1.75$ kJ/mol; $\Delta S° = 97 \pm 8$ J/mol/K).

Western Blotting Studies

To compare the expression of A_3 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 A_3 receptor by Western blot analysis. A significant change in the expression of the A_3 subtype within the total unseparated population was observed between resting and activated cells confirming our binding

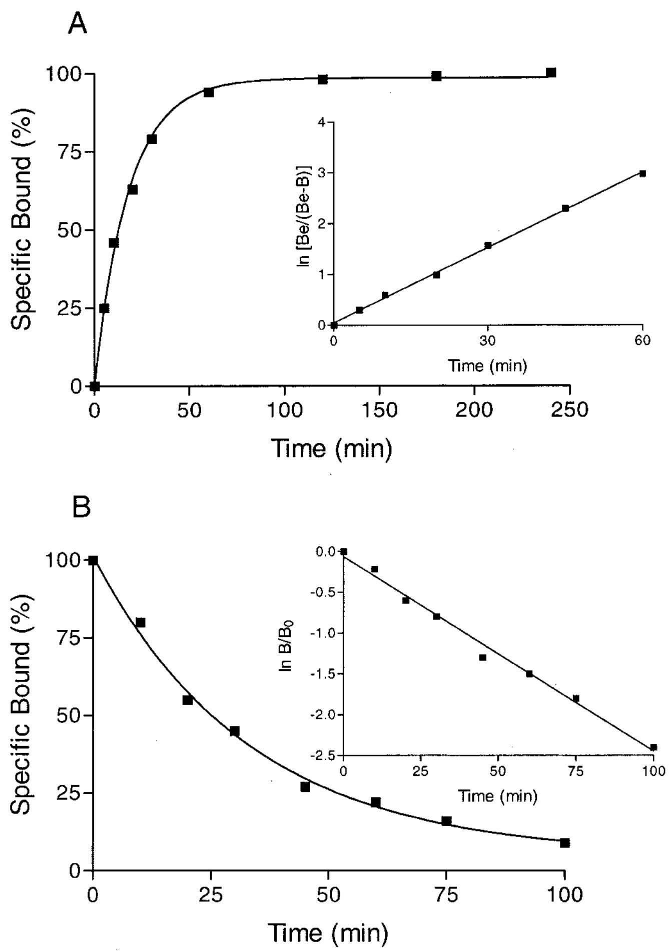

Fig. 2. A, kinetics of [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors with association curves representative of a single experiment that was replicated three times with similar results, in human lymphocytes. Inset, first-order plot of [^{3}H]MRE 3008F20 binding. Be, amount of [^{3}H]MRE 3008F20 bound at equilibrium; B, amount of [^{3}H]MRE 3008F20 bound at each time. Association rate constant was $k_{+1} = 0.0125 \pm 0.0010$ min^{-1} nM^{-1} in human lymphocytes. B, kinetic of [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors with dissociation curve representative of a single experiment. Inset, first-order plot of [^{3}H]MRE 3008F20 binding. Dissociation rate constant was $k_{-1} = 0.024 \pm 0.001$ min^{-1} in human lymphocytes.

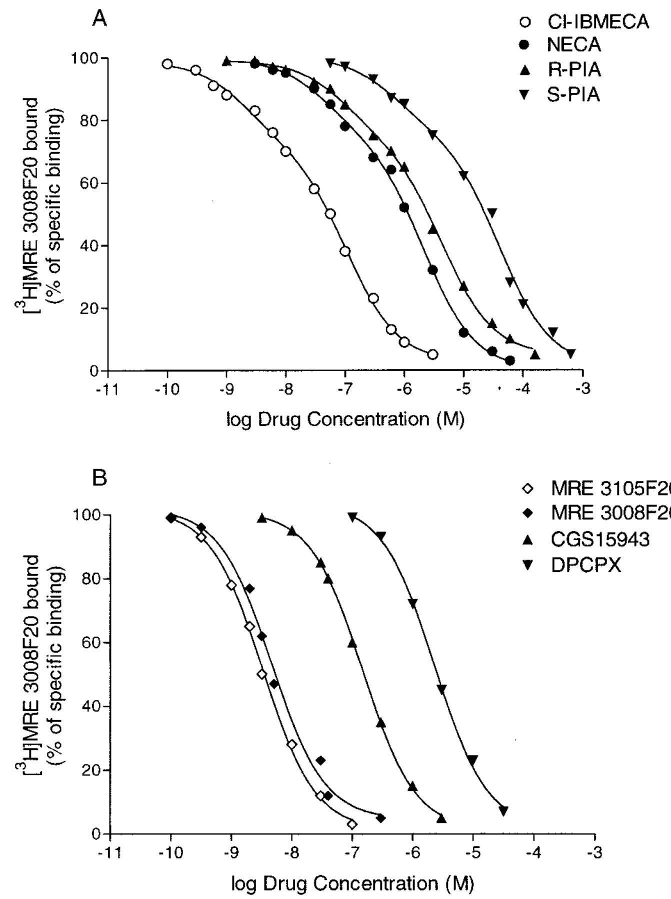

Fig. 3. Competition curves of specific [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors in human lymphocytes by adenosine agonists (A) and antagonists (B). Curves are representative of a single experiment from a series of four independent experiments. Competition experiments were perfomed as described under *Materials and Methods*.

TABLE 1

Affinities, expressed as K_H, K_L, and K_i values, of selected adenosine receptor agonists and antagonists to A_3 receptors in human lymphocytes

Displacement of [^{3}H]MRE 3008F20 binding was determined in the absence and presence of 100 μM GTP. K_H and K_L are the K_i values of the high- and low-affinity states for agonists, respectively. R_H indicates the percentage of A_3 receptors in the high affinity state ± S.E.M.

	[^{3}H]MRE 3008F20 Binding		
Compounds	K_H, K_L	K_i + GTP	R_H
	nM		
Cl-IB-MECA	0.80 ± 0.1		28 ± 3
	67 ± 6	75 ± 7	
NECA	18 ± 3		24 ± 2
	1250 ± 250	1300 ± 200	
R-PIA	38 ± 6		26 ± 3
	2100 ± 250	2200 ± 280	
S-PIA	260 ± 29		24 ± 3
	19,000 ± 2500	21,000 ± 2800	
MRE 3008F20	2.0 ± 0.3	1.8 ± 0.2	
MRE 3105F20	1.7 ± 0.2	1.6 ± 0.1	
CGS 15943	78 ± 6	85 ± 8	
DPCPX	850 ± 90	920 ± 97	
SCH 58261	>10,000	>10,000	

experiments (Fig. 5 A, lines 1 and 2). The increase in A_3 receptor expression after PHA activation was attributed to the $CD4^+$ enriched fraction (Fig. 5A, lines 3 and 4) whereas $CD8^+$ cells, which do express A_3 receptors, failed to show any significant change after T cell activation (Fig. 5A, lines 5 and 6); CHO cells transfected with human A_3 receptors were chosen as positive controls (line 7). When earlier time points were investigated in separated $CD4^+$ and $CD8^+$ cells, we observed that the increase of protein in $CD4^+$ cells started at 15 min and was maximal at 30 min (Fig. 5 B). As for $CD8^+$ cells, the density of A_3 subtype showed no change after T cell activation (data not shown).

Real-Time RT-PCR Experiments

To determine whether the increase in A_3 density was caused by an increase in mRNA encoding the A_3 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_3 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_3 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_3 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 $CD4^+$ and $CD8^+$ cells, a rapid increase was observed starting within 15 min (3.5 ± 0.4 fold, $P < 0.01$) in $CD4^+$ 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_3 receptor mRNA in $CD8^+$ cells (data not shown).

Cyclic AMP Assays

To demonstrate the existence of functional A_3 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_3 agonist Cl-IB-MECA in the inhibition of cAMP levels. Cl-IB-MECA was able to inhibit forskolin-stimulated cAMP levels with an IC_{50} 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_{50} value (1.5 ± 0.4 nM). In resting lymphocytes, the selective A_3 ligand MRE 3008F20 antagonized 100 nM Cl-IB-MECA–induced inhibition of cAMP levels with an IC_{50} of 5.0 ± 0.3 nM (Fig. 7B), suggesting the involvement of A_3 receptors. A low-affinity analog of MRE 3008F20, MRE 3016F20 (hA_3CHO cells; K_i = 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_3 receptors. As shown in Fig. 7B, the failure of this ligand to antagonize Cl-IB-MECA effects on cAMP levels confirms the involve-

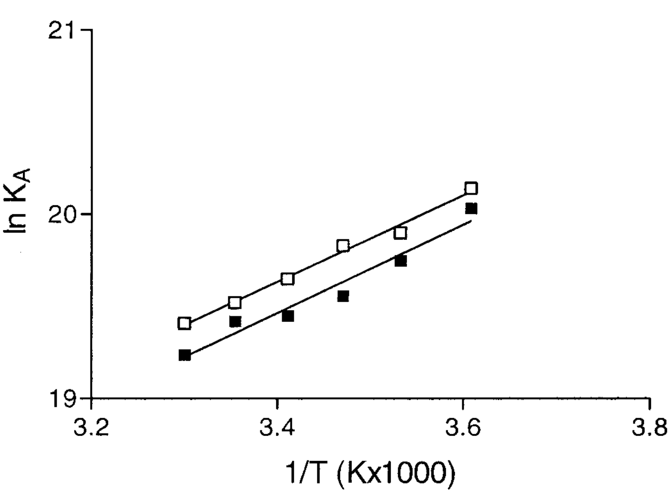

Fig. 4. Van't Hoff plot showing the effect of temperature on the equilibrium binding association constant, $K_A = 1/K_D$ of [^{3}H]MRE 3008F20 in human lymphocytes under resting (■) and activated (□) conditions. The plot is essentially linear in the temperature range investigated 0 to 30°C.

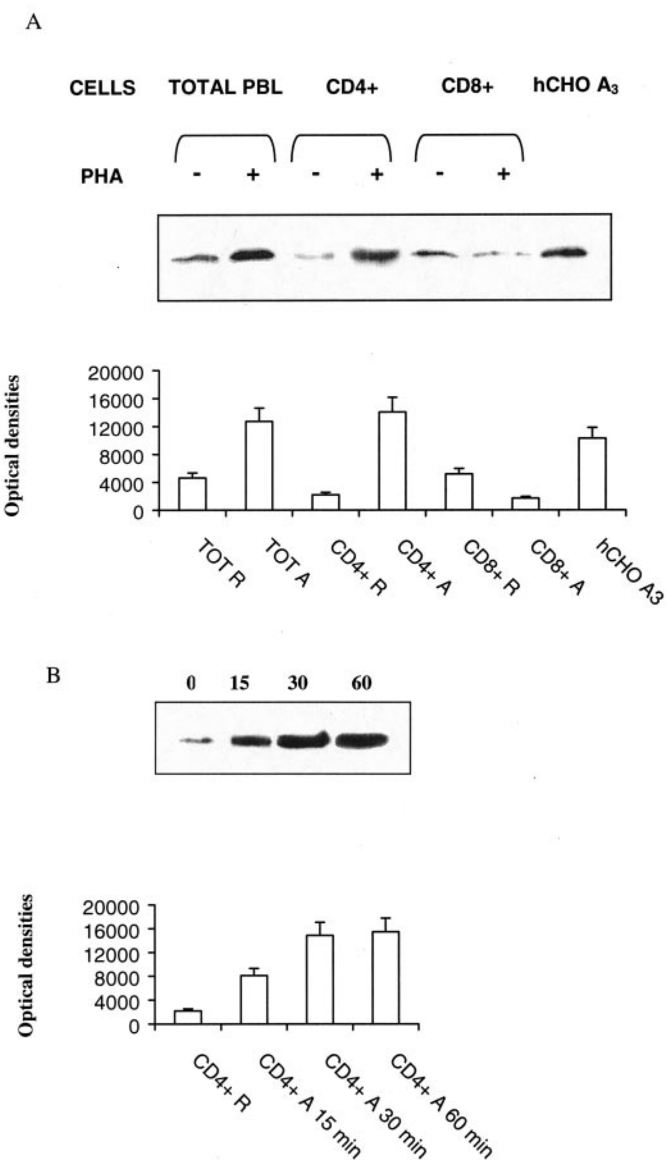

Fig. 5. A, Western blotting experiments showing the A_3 receptor expression, using an A_3 specific antibody, in total, $CD4^+$ enriched, and $CD8^+$ human lymphocytes under resting (R) and activated (A) conditions; hCHO A_3 cells were used as positive controls. B, time course of A_3 receptor protein in separated $CD4^+$ 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 A_3 receptors in this response. DPCPX (100 nM), a selective blocker of A_1 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 A_1 receptors. The nonselective agonist NECA was able to induce a stimulation of cyclic AMP levels, with an EC_{50} 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 A_{2A} 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 IC_{50} value of 15 ± 3 nM, suggesting the involvement of A_{2A} 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 A_{2A} receptors. However, it has been suggested that the A_3 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 A_3 subtype in the regulation of human immune system, it is important to identify the leukocytes that express functional A_3 receptors. Prompted by these considerations and after the pharmacological characterization by means of receptor-binding of the A_3 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 A_3 adenosine receptor in human lymphocytes.

Different experimental approaches attest to the existence of adenosine A_3 receptors on these peripheral blood cells and

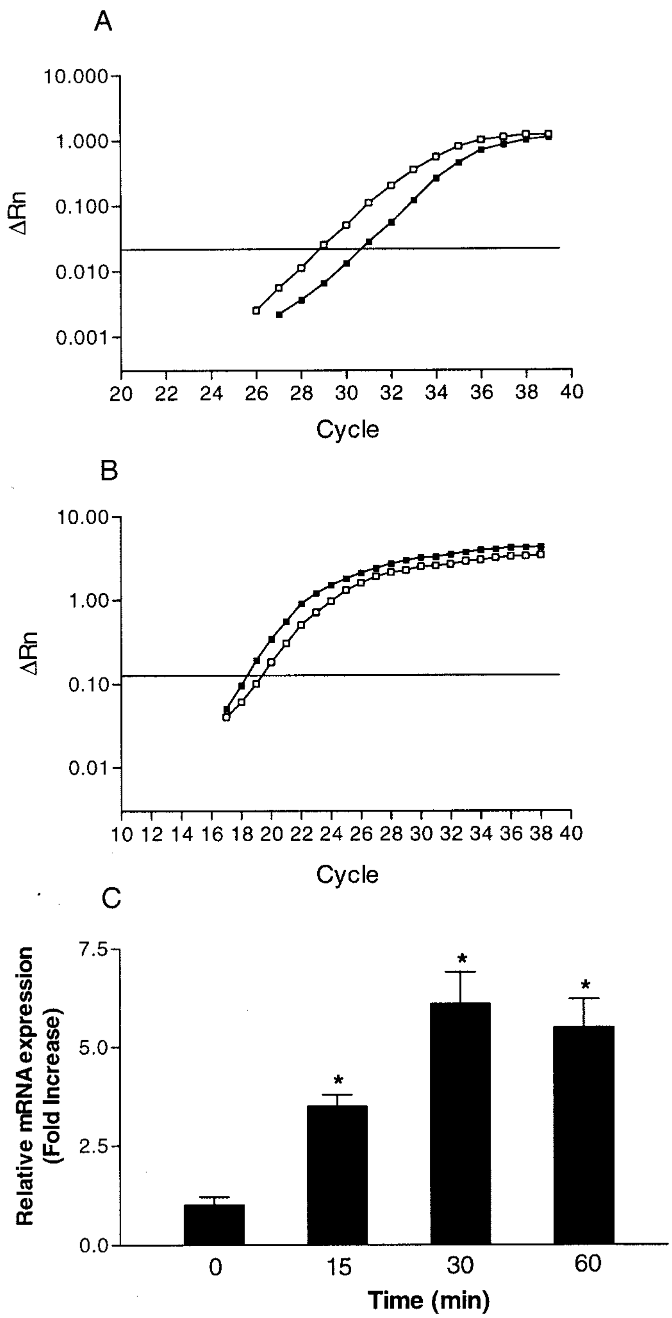

Fig. 6. Representative amplification plots for the A_3 receptors mRNA in resting (■) and one hour-activated (□) human lymphocytes (A) compared with the respective GAPDH mRNA (B). C, time course of A_3 receptor mRNA in separated $CD4^+$ subsets stimulated with 2 μg/ml PHA. The values represent the means ± 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).

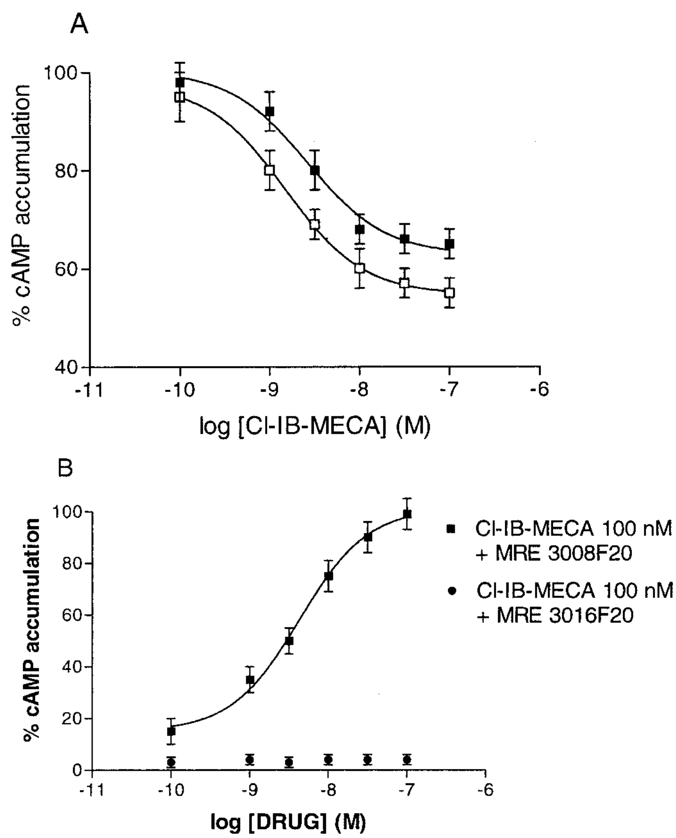

Fig. 7. A, inhibition curves of forskolin (10 μM) stimulated cAMP levels by Cl-IB-MECA under resting (■) and activated (□) conditions (IC_{50} = 2.7 ± 0.3 and 1.5 ± 0.4 nM, respectively). Results are presented as the mean ± S.E.M. of five independent experiments (Student's *t* test; *, $P <$ 0.01 versus resting cells). B, antagonism by MRE 3008F20 (■) and lack of effect by MRE 3016F20 (●), an analog of MRE 3008F20, with low affinity to A_3 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 [^{3}H]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_H) and one low-affinity (K_L) 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 [^{3}H]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; Gessi et al., 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_{2B} 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 Cl-IB-MECA to modulate adenylyl cyclase activity. Forskolin-stimulated cAMP levels were inhibited by Cl-IB-MECA, and this effect was potently antagonized by MRE 3008F20. The agonist IC_{50} 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 [^{3}H]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_i 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 (Sitkovsky, 2003).

At variance with adenylyl cyclase activity, only high micromolar doses of Cl-IB-MECA induced Ca^{2+} increase in both resting and activated conditions. The low potency of the agonist in the stimulation of Ca^{2+} increase indicates that Cl-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 laboratory are aimed at evaluating the A_3 receptor involvement in cytokine release and its role in the expression of T cells activation markers.

In conclusion, all the data reported in this study indicate that human lymphocytes present A_3 adenosine receptors with a pharmacological, biochemical, and thermodynamic profile typical of the human A_3 subtype. The rapid up-regulation of A_3 receptors functionally coupled with adenylyl cyclase in activated T cells may indicate another potential candidate of biological significance for adenosine-mediated responses in the immune system.

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Address correspondence to: Prof. Dr. Pier Andrea Borea, Chair of Pharmacology, Faculty of Medicine, University of Ferrara, Department of Clinical and Experimental Medicine, Pharmacology Unit, Via Fossato di Mortara 17-19, 44100 Ferrara, Italy. E-mail: bpa@dns.unife.it