Expression, Pharmacological Profile, and Functional Coupling of A_{2B} Receptors in a Recombinant System and in Peripheral Blood Cells Using a Novel Selective Antagonist Radioligand, [3H]MRE 2029-F20

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ABSTRACT

In this study, we compared the pharmacological and biochemical characteristics of A_{2B} adenosine receptors in recombinant (hA_{2B}HEK293 cells) and native cells (neutrophils, lymphocytes) by using a new potent 8-pyrazole xanthine derivative, [3H]benzyl adenosine-5'-monophosphate ([3H]MRE 2029-F20), that has high affinity and selectivity for hA_{2B} versus hA_1, hA_2A, and hA_3 subtypes. [3H]MRE 2029-F20 bound specifically to the hA_{2B} receptor stably transfected in human embryonic kidney (HEK) 293 cells with K_D of 2.8 ± 0.2 nM and B_max values of 450 ± 42 fmol/mg of protein. Saturation experiments of [3H]MRE 2029-F20 binding in human neutrophils and lymphocytes detected a second high-affinity binding site in human embryonic kidney (HEK) 293 cells with K_D values of 2.4 ± 0.5 and 2.7 ± 0.7 nM, respectively, and B_max values of 79 ± 10 and 54 ± 8 fmol/mg of protein, respectively, in agreement with real-time reverse transcription polymerase chain reaction studies showing the presence of A_{2B} mRNA. The rank order of potency of typical adenosine ligands with recombinant hA_{2B} receptors was consistent with that typically found for interactions with the A_{2B} subtype and was also similar in peripheral blood cells. 5'-N-Ethyl-carboxamidoadenosine stimulated cAMP accumulation in both hA_{2B}HEK293 and native cells, whereas phospholipase C activation was observed in recombinant receptors and endogenous subtypes expressed in neutrophils but not in lymphocytes. MRE 2029-F20 was revealed to be a potent antagonist in counteracting the agonist effect in both signal transduction pathways. In conclusion, [3H]MRE 2029-F20 is a selective and high-affinity radioligand for the hA_{2B} adenosine subtype and may be used to quantify A_{2B} endogenous receptors. In this work, we demonstrated their presence and functional coupling in neutrophils and lymphocytes that play a role in inflammatory processes in which A_{2B} receptors may be involved.

- Adenosine is a ubiquitous modulator that exerts its physiological functions through the interaction with four G protein-coupled receptors classified as A_1, A_2A, A_2B, and A_3.

ABBREVIATIONS: RT, reverse transcription; PCR, polymerase chain reaction; CGS 21680 2-[p-[2-carboxyethyl]-phenylethylamino]-5'-N-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ZM 241385, 4-(2-[7-amino-2-[2-furyl]-[1,2,4]triazolo-[2,3,4]triazinyl-amino) ethyl)-phenol; MRS 1754, 8-[4-[(4-cyano-[2,6]-phenyl)carbamoylmethyleneoxy]phenyl]-1,3-di-[p-propyl]xanthine; OSI339391, N-2-[2-phenyl]-6-[4-[(3-phenylpropyl)]-piperazine-1-carbonyl]-7-hydropyrrolo[2,3,d]pyrimidin-4-ylamino)-ethyl)-acetamide; HEK, human embryonic kidney; MRE 3008-F20, 5-N-[4-methoxyphenyl-carbamoylamino]-8-propyl-2(furyl)-pyrazolo-[4,3-e]-pyridazolo-[1,5-c]-pyrimidine; MRE 2029-F20, N-benzo[1,3]dioxol-5-yl-2-[5-[1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl]-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; CHA, 5'-N-ethylcarboxamidoadenosine; (R)-PIA, (S)-PIA, (R)-N-2-(phenyl-isopropyl)-adenosine; (S)-PIA, (S)-N-2-(phenylisopropyl)adenosine; IB-MECA, (S)-N-[3-(iodobenzyl) adenosine-5'-N-methyluronamide; CGS 15943, 5-amino-9-chloro-2-(furyl)-1,2,4-triazolo-[1,5-c]-quinazoline; AS 16, 2-(4-benzyloxy-phenyl)-N-[5-[2,6-dioxo-1,3-dipropyl-2,6,7-tetrahydro-1H-purin-8-yl]-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide]; AS 100, N-2,3,4-dichlorophenyl-2-[5-[2,6-dioxo-1,3-dipropyl-2,6,7-tetrahydro-1H-purin-8-yl]-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide; Ct, cycle threshold; KRPG, Krebs-Ringer phosphate buffer; SCH 52861, 7-(2-phenylthiole)-2-(furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine; IP_3, inositol trisphosphate; PLC, phospholipase C; hA_{2B}HEK 293 cells, human embryonic kidney-293 cells transfected with human adenosine A_{2B} receptor; IMLF, N-formyl-l-methionyl-l-leucyl-l-phenylalanine; U73122, 1-[6-[(17)-3-methoxyestra-1,3,5(10)-triien-17-yl]amino]hexyl]-1H-pyrrrole-2,5-dione.
(Fredholm et al., 2001; Fredholm, 2003). In particular, A_2B receptors are associated with stimulation of the adenylate cyclase and activation of phospholipase C through the coupling to G_s and G_{q/11} proteins, respectively. A_2B receptors have been found on practically every cell in most species. RT-PCR studies revealed their highest expression in cecum, large intestine, and urinary bladder, but lower levels in brain, spinal cord, lung, and vas deferens (Rivkees and Reppert, 1992). However, besides regulation at the level of gene expression, targeting of the receptor protein to specific cells or tissues is crucial in understanding their role in pathophysiological conditions. The lack of selective pharmacological tools has hampered research in this field; in particular, the lack of selective A_2B receptor agonists has undoubtedly contributed to the general lack of information of their physiological functions. The characterization of A_2B receptors, therefore, often relies on the lack of effectiveness of compounds that are potent and selective agonists of other receptor subtypes. The agonist CGS 21680, for example, has been useful in differentiating between A_2A and A_2B receptors (Feoktistov and Biaggioni, 1995; Fiebich et al., 1996, 1997). However, pharmacological characterization of receptors based on apparent agonist potencies is far from ideal, because it depends not only on agonist binding to the receptor but also on multiple processes involved in signal transduction (Feoktistov et al., 2001). Based on functional assays using novel A_2B selective antagonists, significant progress has been made in the understanding of the molecular pharmacology and physiology of A_2B adenosine receptors. They seem to be implicated in mast-cell secretion (Feoktistov et al., 2001), coronary flow regulation (Talukder et al., 2003), neangiogenesis (Grant et al., 2001; Feoktistov et al., 2002, 2003; Afzal et al., 2003), cytokine release by bronchial smooth muscle cells (Zhong et al., 2004), and nociception (Abo-Salem Afzal et al., 2003), but they were not commercially available at the time of this study. Our group has identified a series of 8-pyrazolo xanthine derivatives as potent and selective human A_2B adenosine receptor subtypes. The agonist CGS 21680, for example, has been shown in different studies to be useful in differentiating between A_2A and A_2B receptors (Feoktistov and Biaggioni, 1995; Fiebich et al., 1996, 1997). However, pharmacological characterization of receptors based on apparent agonist potencies is far from ideal, because it depends not only on agonist binding to the receptor but also on multiple processes involved in signal transduction (Feoktistov et al., 2001). Based on functional assays using novel A_2B selective antagonists, significant progress has been made in the understanding of the molecular pharmacology and physiology of A_2B adenosine receptors. They seem to be implicated in mast-cell secretion (Feoktistov et al., 2001), coronary flow regulation (Talukder et al., 2003), neangiogenesis (Grant et al., 2001; Feoktistov et al., 2002, 2003; Afzal et al., 2003), cytokine release by bronchial smooth muscle cells (Zhong et al., 2004), and nociception (Abo-Salem Afzal et al., 2003), suggesting a possible role of the A_2B subtype in the modulation of inflammatory processes involved in asthma, tumor growth, tissue injury, ischemia, and pain (Feoktistov et al., 2003; Livingston et al., 2004).

The characterization of A_2B receptors through radioligand binding studies has been performed, now, by using low-affinity and nonselective antagonists, such as [3H]-DPCPX, [3H]ZM 241385, and 3-(3-[125I]iodo-4-aminobenzyl)-8-(4-oxyacetate)phenyl-1-propylxanthine, that, as a consequence of their low affinity, display a rapid dissociation rate from the receptor. In addition, because these ligands are nonselective, their utility in native systems is hampered because many tissues and cell lines express several adenosine subtypes. Based on these considerations, it is evident that, to obtain more information about the physiological role of A_2B receptors, new radiolabeled compounds with high affinity and selectivity should be synthesized. High-affinity radioligands for A_2B receptors, [3H]MRS 1754, and [3H]-OSIP339391 have been introduced (Ji et al., 2001; Stewart et al., 2004), but they were not commercially available at the time of this study. Our group has identified a series of 8-pyrazolo xanthine derivatives as potent and selective human A_2B adenosine antagonists (Baraldi et al., 2004a,b), and a radiolabeled form of one compound of this series was used as a new pharmacological tool to describe the comparison between human recombinant A_2B receptors stably transfected in HEK 293 cells (hA_2B HEK 293 cells) and endogenous receptors present in neutrophils and lymphocytes, which represent inflammatory cells potentially involved in the exacerbation of asthma and other inflammatory processes in which A_2B receptors are thought to be involved. In this study, we demonstrated that A_2B receptors are strongly linked to adenylyl cyclase in both transfected and native cells, whereas for phospholipase C activity, they are well coupled in transfected cells, less in neutrophils, and not at all in lymphocytes, where the A_2B expression is quite low.

### Materials and Methods

**Materials.** [3H]MRE 2029-F20 (specific activity, 123 Ci/mmol) and [3H]MRE 2008-F20 (specific activity, 67 Ci/mmol) were synthesized at Amersham Biosciences (Little Chalfont, Buckinghamshire, UK); [3H]ZM 241385 (specific activity, 20 Ci/mmol) was purchased from Tocris (Boston, MA). [3H]DPCPX (specific activity, 120 Ci/mmol) and [3H]Ins(1,4,5)P_3 (specific activity, 21 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). CHA, NECA, (R)-PIA, (S)-PIA, CGS 21680, IB-MECA, CGS 15943, and DPCPX were obtained from Sigma-RBI (Natick, MA). MRS 1754 was obtained from Sigma-Aldrich (Milano, Italy). SCH 58261, MRE 3008-F20, MRE 2029-F20, AS 16, and AS 100 were synthesized by Prof. P. G. Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy). All other reagents were of analytical grade and obtained from commercial sources.

**Synthesis of [3H]MRE 2029-F20.** The synthesis of [3H]MRE 2029-F20 was prepared through custom synthesis at Amersham Bioscience from tritium gas by a method developed by Nycomed Amersham plc. The product was purified by reversed-phase high-performance liquid chromatography using an acetonitrile/trifluoroacetic acid gradient (Baraldi et al., 2004a).

**Stable Transfection of HEK 293 Cells.** cDNA encoding human A_2B receptors was a gift of Prof. Karl-Norbert Klotz (Institut für Pharmakologie und Toxikologie, Universität Würzburg, Würzburg, Germany) and subcloned into the expression plasmid pcDNA 3.1 (Invitrogen). The plasmid was amplified into a competent Escherichia coli strain and plasmid DNA isolated by using QIAGEN Maxiprep columns (plasmid purification kit; QIAGEN, Valencia, CA). cDNA was then sequenced on both strands in the University of Padova, DNA Service Sequence CRIBI, and transfected into HEK 293 cells by using the calcium phosphate precipitation method (Chen and Okayama, 1987). Colonies were selected by growth of cells on 0.8 mg/ml G-418. Stably transfected cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Dulbecco’s modified Eagle’s medium/F-12 medium) with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.3 mg/ml G-418, at 37°C in 5% CO_2/95% air.

**Membrane Preparation.** For membrane preparation, the culture medium was removed. The cells were washed with phosphate-buffered saline and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl and 2 mM EDTA, pH 7.4). The cell suspension was homogenized using a Polytron homogenizer, and the homogenate was spun for 30 min at 36,000 g. The membrane pellet was resuspended in 50 mM Tris-HCl buffer, containing 10 mM MgCl_2, 1 mM EDTA, and 0.1 mM benzamidine, pH 7.4, and incubated with 2 IU/ml adenosine deaminase for 30 min at 37°C. The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) with bovine serum albumin as reference standard.

**Peripheral Blood Cells Isolation.** Lymphocytes and neutrophils were isolated from buffy coats kindly provided by the Blood Bank of the University Hospital of Ferrara, according to Gessi et al. (2002, 2004). In brief, the blood was centrifuged on Ficoll-Hypaque density gradients. The human peripheral blood mononuclear cells were isolated and removed from the Ficoll-Hypaque gradients. Thereafter, they were washed in 0.02 M phosphate-buffered saline at pH 7.2. Further purification of lymphocytes from peripheral blood mononuclear cells was performed by adhesion of monocytes to plastic
plates for 2 h at 37°C. To obtain neutrophils, the lower phase of Ficoll-Hypaque gradients was washed and supplemented with 20 ml of 6% Dextran T500. After gentle mixing, erythrocytes were allowed to settle at 20°C for 60 min. The 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. Neutrophils were sedimented by centrifugation at 20°C for 20 min at 250g. This procedure resulted in approximately 95% neutrophils, and the cell viability was more than 95% as detected by trypan blue exclusion test. Rat neutrophils and lymphocytes were isolated from male Sprague-Dawley rats (300–350 g; Stefano Morini, Reggio Emilia, Italy). Blood samples from the inferior vena cava were collected in an EDTA-anticoagulated tube, diluted in phosphate-buffered saline, and isolated by density gradient centrifugation (Ficoll-Hypaque) as described above for human blood cells. Membrane preparations were performed essentially according to Gessi et al. (2002; 2004).

**Real-Time RT-PCR Experiments.** Total cytoplasmic RNA was extracted from HEK 293 and peripheral blood cells by the acid-guanidinium-thiocyanate-phenol method (Chomczynski and Sacchi, 1987). Quantitative real-time RT-PCR assay (Higuchi et al., 1993) of A2B mRNA transcript was carried out using gene-specific double fluorescence labeled TaqMan MGB probe (smaller groove binder) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington Cheshire, UK). For the real-time RT-PCR of the A2B gene, the assays-on-demand gene expression product (GenBank accession no. NM_0006756) was used with the fluorescent reporter 6-carboxy fluorescein and the quencher 6-carboxy-methylrhodamine. For the real-time RT-PCR of the reference gene, the endogenous control human β-actin kits was used, and the probe was fluorescence-labeled with VIC (Applied Biosystems, Monza, Italy). Moreover, a curve of A2B cDNA plasmid standards with a range spanning at least 6 orders of magnitude (10–11–10–16 g/μl) was generated. This standard curve displayed a linear relationship between Ct values and the logarithm of plasmid amount. Therefore, quantification of A2B message in blood cells was made by interpolation from standard curve of Ct values generated from the plasmid dilution series.

**Binding Assays to Human Cloned Adenosine Receptors.** Binding assays to human cloned A1, A2A, and A3 receptors were performed at 4°C using [3H]DPCPX, [3H]ZM 241385, and [3H]MRE 3008-F20, respectively, as described by Varani et al. (2000).

**[3H]MRE 2029-F20 Binding Assays.** Kinetic studies of [3H]MRE 2029-F20 (2.5 nM) were performed incubating membranes obtained by HEK 293 cells transfected with the human A2B receptors in a thermostatic bath at 4°C. A total assay volume of 250 μl was employed in which the final protein level was 70 μg per well. For the measurement of the association rate, the reaction was terminated at different times (from 5 to 180 min) by rapid filtration under vacuum, followed by washing with 5 ml of ice-cold buffer four times. For the measurement of dissociation rate, the samples were incubated at 4°C for 90 min, and then 1 μM MRE 2029-F20 was added to the mixture. The reaction was terminated at different times from 5 to 100 min. Saturation binding experiments of [3H]MRE 2029-F20 (0.3 to 30 nM) to hA2B HEK293 cell membranes were performed by incubation for 90 min at 4°C. Competition experiments of 3 nM [3H]MRE 2029-F20 were performed in duplicate in a final volume of 250 μl in test tubes containing 50 mM Tris HCl buffer, 10 mM MgCl2, 1 mM EDTA, and 0.1 mM benzamidine, pH 7.4, 100 μl of membranes, and at least 12 to 14 different concentrations of typical adenosine receptor agonists and antagonists. Nonspecific binding was defined as binding in the presence of 1 μM MRE 2029-F20 and, at the Kd value of the radioligand, was approximately 30 to 35% and 40 to 45% of total binding in HEK 293 cells and in blood cells, respectively. Similar results were obtained in the presence of 10 μM DPCPX and 100 μM NECA. 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). The filter bound radioactivity was counted with a Top Count microplate scintillation counter (efficiency, 57%) with MicroScint 20. Kd values were calculated from IC50 values according to the Cheng and Prusoff equation, Kd = IC50/(1 + [C]/Kp), where [C] is the concentration of the radioligand and Kp is its dissociation constant. A weighted nonlinear least-squares, curve-fitting program LIGAND (Munson and Rodbard, 1980) was used for computer analysis of saturation and inhibition experiments.

**Cyclic AMP Assay.** hA2B HEK-293 and peripheral blood cells were suspended in 0.5 ml of Krebs-Ringer phosphate buffer (KRPB) (136 mM NaCl, 5 mM KCl, 0.67 mM Na2HPO4, 0.2 mM KH2PO4, 3 mM NaHCO3, 1 mM CaCl2, 5 mM glucose, 5 mM HEPES, 10 mM MgCl2, and 2.0 IU/ml adenosine deaminase, pH 7.4) containing 0.5 mM 4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor, and preincubated for 10 min in a shaking bath at 37°C. Then the nonselective adenosine agonist NECA was added to the mixture, and the incubation continued for a further 10 min. The reaction was terminated by the addition of ice-cold 6% trichloroacetic acid. The trichloroacetic acid suspension was centrifuged at 2000g 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 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 the incubation buffer (0.1 M Trizma base, 8.0 mM aminophylline, and 6.0 mM 2-mercaptoethanol, pH 7.4) and [3H]cyclic AMP in a total volume of 0.5 ml. The binding protein, previously prepared from beef adrenal glands, was added to the samples and incubated at 4°C for 150 min. After the addition of chesual, samples were centrifuged at 2000g for 10 min. The clear supernatant (0.2 ml) was mixed with 4 ml of Atomilight and counted in a LS-1800 Beckman scintillation counter.

**Ins(1,4,5)P3 Binding Assay.** IP3 generation in hA2B HEK 293 and peripheral blood cells was measured by [3H]Ins(1,4,5)P3 competition assay to IP3 binding protein according to the method described by Challiss et al. (1990). hA2B HEK 293 cells (2 × 106 cells in each tube) and peripheral blood cells (8 × 106 cells in each tube) were suspended in KRPB buffer and stimulated with agonists at 37°C. The reaction was terminated by addition of 0.5 M trichloroacetic acid. Acidified samples were left on ice for 15 min, centrifuged at 3000g for 15 min at 4°C and trichloroacetic acid was extracted with five 2-ml washes with water-saturated diethyl ether. Finally, 125 μl of 30 mM EDTA and 125 μl of 60 mM NaHCO3 were added to 500 μl of cell extract, and samples were taken for analysis. Buffer samples were also taken through the acidification/extraction protocol to provide diluent for the Ins(1,4,5)P3 assay standard curve. A 30-μl portion of sample or of trichloroacetic acid-extracted buffer containing standard amounts of Ins(1,4,5)P3 (0.12–12 pmol) or Ins(1,4,5)P3 (0.3 mmol to define nonspecific binding) was added to 30 μl of assay buffer (25 mM Tris HCl and 1 mM EDTA, pH 8) and 30 μl of [3H]Ins(1,4,5)P3 (7000 dpm/assay). Then, 30 μl (0.4 μg of protein) of the adrenal-cortical binding protein preparation was added and incubation continued for 30 min. Bound and free [3H]Ins(1,4,5)P3 were separated by rapid filtration through Whatman GF/B glass fiber filters with four 3-ml washes of ice-cold 25 mM Tris HCl, 1 mM EDTA, and 5 mM NaHCO3, pH 8. Scintillator was added to the filter discs, and radioactivity was determined after a 12-h extraction period by scintillation counting.

**Measurement of Cytosolic Ca2+ Concentration.** (Ca2+), was evaluated by incubating hA2B HEK 293 cells with the Ca2+-sensitive fluorescent dye Fura 2-acetoxymethyl ester (5 μM) in KRPB buffer for 45 min at 37°C. Alternate excitation at 340 nM and 380 nM was supplied, and the F340/F380 emission ratio was recorded with a dynamic image analysis system (Laboratory Automation 2.0, RCS, Florence, Italy). For calcium measurements in human lymphocytes and neutrophils, cells were loaded with fura 2-acetoxymethyl ester (2 μM) in KRPB buffer, for 30 min at 37°C, according to Gessi et al. (2002). The cells were centrifuged at 1000g for 10 min to remove
extracellular dye and were resuspended in KRPG solution at 4 x 10^6 cells/ml. Fluorescence was monitored with a LS50 spectrofluorometer (PerkinElmer Life and Analytical Sciences), at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm, in cuvettes thermostatically controlled at 37°C and continuously stirred.

**Results**

**Real-Time RT-PCR Experiments.** HEK 293 cells are recognized as cells expressing native A2B receptors. Therefore, the mRNA presence of this adenosine subtype was investigated in both wild-type and hA2B HEK 293 cells. Transfection of A2B receptors in HEK 293 cells produced a 350 ± 30-fold increase of A2B mRNA accumulation with respect to the corresponding wild-type cells; the expression level of A2B receptors was normalized to the expression level of the endogenous receptor (β-actin) in each sample (Fig. 1, A and B). In addition, the mRNA level of A2B receptors was investigated in primary cells in which this adenosine subtype is supposed to play an important regulatory role, such as peripheral blood cells. The amount of product was expressed as the ratio of mRNA (determined by interpolation from standard curve of Ct values generated from the plasmid dilution series) and total RNA and indicated the following rank order: monocytes < lymphocytes ≈ neutrophils, with monocytes expressing approximately 4-fold less mRNA compared with the other blood cells (Student's t test; *, P < 0.01 versus monocytes) (Fig. 1C).

**Radioligand Binding Studies in Recombinant and Native Cells.** Fig. 2A shows that [3H]MRE 2029-F20 binding to hA2B HEK 293 cells reached equilibrium after approximately 45 min and was stable for at least 3 h. [3H]MRE 2029-F20 binding was rapidly reversed by the addition of 1 μM MRE 2029-F20 as shown in Fig. 2B. A one-component model fit association and dissociation curves significantly better than a two-component model (P < 0.05). The rate constants were: $k_{on} = 0.046 ± 0.002$ min⁻¹ and $k_{off} = 0.025 ± 0.003$ min⁻¹. From these values, a kinetic dissociation constant ($K_D$) of 2.98 nM was calculated. Figure 3 shows a saturation curve of [3H]MRE 2029-F20 to adenosine A2B receptor, and the linearity of the Scatchard plot in the inset is indicative, in our experimental conditions, of the presence of a single class of binding sites with a $K_D$ value of 2.8 ± 0.2 nM and a $B_{max}$ value of 450 ± 42 fmol/mg of protein. In contrast to the presence of mRNA message, no specific binding of [3H]MRE 2029-F20 was detectable in HEK 293 wild-type cells (data not shown). Table 1 shows the affinities, expressed as $K_i$ values, of selected adenosine receptor agonists and antagonists to hA2B receptors expressed in HEK 293 cells using [3H]MRE 2029-F20. The order of potency in [3H]MRE 2029-F20 displacement assays for adenosine receptor agonists was NECA > (R)-PIA > CHA > IB-MECA > (S)-PIA > CGS 21680 (Fig. 4A). The selectivity of MRE 2029-F20 for the human A2B over A1, A2A, and A3 receptors was evaluated in radioligand binding assays by using [3H]DPCPX, [3H]ZM 241385, and [3H]MRE 3008-F20, respectively. MRE 2029-F20 displays low affinity for the human A1 receptor ($K_i = 245 ± 31$ nM) and no significant affinity for the human A2A and A3 subtypes ($K_i > 1000$ nM). This indicates that MRE 2029-F20 is 88-fold selective for A2B over A1 receptors and more than 300-fold selective for A2B over A2A and A3 subtypes, which means a range of selectivity similar to [3H]MRS 1754 (selectivity of 210-, 260-, and 290-fold for A2B over A1, A2A, and A3 subtypes, respectively) and

**Fig. 1.** Representative amplification plots for the A2B receptors mRNA in transfected (●) and untransfected (○) HEK 293 cells (A) compared with the respective β-actin mRNA (B). C, expression of A2B receptors mRNA in monocytes, lymphocytes, and neutrophils. The amount of product in blood cells was expressed as the ratio of mRNA (micrograms) determined by interpolation from standard curves of Ct values generated from the plasmid dilution series and total RNA (micrograms) used in retrotranscription reaction. Results are presented as the mean ± S.E.M. of four independent experiments (Student's t test; *, P < 0.01 versus monocytes).
better than \[^{3}H\]OSIP339391 (selectivity of 70-fold for A\(_{2B}\) over A\(_{1A}\), A\(_{2A}\), and A\(_{3}\) subtypes) (Ji et al., 2001; Stewart et al., 2004). Saturation experiments of \[^{3}H\]MRE 2029-F20 binding performed at 21 and 37°C revealed \(K_D\) values of 3.5 ± 0.4 and 6.5 ± 0.7 nM and \(B_{\text{max}}\) values of 460 ± 50 and 430 ± 48 fmol/mg of protein, respectively, thus suggesting that dissociation constants increased with temperature, whereas \(B_{\text{max}}\) data were largely independent of it. This binding behavior has previously been found to be typical of adenosine A\(_{1A}\), A\(_{2A}\), and A\(_{3}\) subtypes (Borea et al., 1996; Varani et al., 2000). To investigate whether MRE 2029-F20 would be a useful tool to detect A\(_{2B}\) receptors in primary cells, we isolated monocytes, lymphocytes, and neutrophils from peripheral blood. As for human monocytes, it was not possible to detect A\(_{2B}\) receptors, even though the mRNA was present, suggesting that possibly in these cells their level was extremely low. On the other hand, in agreement with real-time RT-PCR experiments, neutrophils and lymphocytes both express A\(_{2B}\) subtype with the following binding parameters: \(K_D\) of 2.4 ± 0.5 and 2.7 ± 0.7 nM and \(B_{\text{max}}\) of 79 ± 10 and 54 ± 8 fmol/mg of protein, respectively (Fig. 5, A and B). The pharmacological profile of selected adenosine agonists and antagonists obtained from inhibition binding experiments in peripheral blood cells revealed a rank order of potency strictly similar to that obtained in transfected cells: NECA > (R)-PIA > CHA > IB-MECA > (S)-PIA > CGS 21680 and MRE 2029-F20 > MRS 1754 > AS100 > ZM 241385 > AS16 > DPCPX > enprofyline > theophylline (Table 1). Specific binding of \[^{3}H\]MRS 2029-F20 (55–60%) was also detected in rat neutrophils and lymphocytes.

**Evaluation of cAMP Levels.** To investigate the functional coupling of native and recombinant A\(_{2B}\) receptors to adenylyl cyclase, we evaluated the stimulation of cAMP production in hA\(_{2B}\)HEK293 cells and in peripheral blood cells. Our results show that in hA\(_{2B}\)HEK293 cells, NECA showed a potency greater in cAMP (EC\(_{50}\) value of 4.5 ± 0.4 nM) than in radioligand binding studies (\(K_I\) value of 262 ± 30 nM) (Fig. 6A). We were surprised to find that this effect was not potently antagonized by MRE 2029-F20 that showed an IC\(_{50}\) value of 150 ± 18 nM. Because of the low potency of MRE 2029-F20 in inhibiting cAMP accumulation induced by NECA, we suspected the presence of basal levels of A\(_{2A}\) receptors in addition to A\(_{2B}\) in wild-type HEK 293 cells. This was assessed by real-time RT-PCR experiments revealing a similar amount of A\(_{2A}\) and A\(_{2B}\) mRNA expression (data not shown).

![Figure 2](image2.png)

**Fig. 2.** A, kinetics of \[^{3}H\]MRE 2029-F20 binding to human A\(_{2B}\) adenosine receptors in HEK 293 cells, with association curves representative of a single experiment that was replicated three times with similar results. Inset, first-order plot of \[^{3}H\]MRE 2029-F20 binding. Be, amount of \[^{3}H\]MRE 2029-F20 bound at equilibrium; B, amount of \[^{3}H\]MRE 2029-F20 bound at each time. Association rate constant was: \(k_{\text{on}} = 0.0084 ± 0.0009 \text{ min}^{-1} \text{ nM}^{-1}\). B, kinetic of \[^{3}H\]MRE 2029-F20 binding to human A\(_{2B}\) adenosine receptors; dissociation curve represents a single experiment. Inset, first-order plot of \[^{3}H\]MRE 2029-F20 binding. Dissociation rate constant was: \(k_{\text{off}} = 0.025 ± 0.003 \text{ min}^{-1}\).

![Figure 3](image3.png)

**Fig. 3.** A, saturation of \[^{3}H\]MRE 2029-F20 binding to A\(_{2B}\) adenosine receptors in HEK 293 cells. Data points are the means and vertical lines are the S.E.M. of four separate experiments performed in triplicate. B, Scatchard plot of the same data are shown. \(K_D\) value was 2.8 ± 0.2 nM and \(B_{\text{max}}\) value was 450 ± 42 fmol/mg of protein. Experiments were performed as described under Materials and Methods.
shown) and confirmed by saturation binding experiments of [\textsuperscript{3}H]ZM 241385 binding (0.1–10 nM) showing an affinity of 1.04 ± 0.2 nM and a density of 35 ± 4 fmol/mg of protein. This value was possibly not caused by a contaminating presence of endogenous A2B receptors, as demonstrated by the lack of [\textsuperscript{3}H]MRE 2029-F20 specific binding in untransfected HEK 293 cells. CGS 21680, an A2A-selective agonist, stimulated human A2B adenosine receptors in HEK 293 cells. CGS 21680, an A2A-selective agonist, stimulated cAMP levels with an EC\textsubscript{50} of 410 ± 52 nM, but it was less efficacious than NECA. The unstimulated level of cAMP was 15 ± 3 pmol/10\textsuperscript{6} cells; at concentrations producing maximal effects (10 μM), CGS 21680 induced a 4-fold increase, whereas NECA caused an 8-fold increase in cAMP (to 60 ± 8 and 120 ± 15 pmol/10\textsuperscript{6} cells, respectively), suggesting the activation, in the first case, of only A2A receptors and, in the second, of both A2A and A2B subtypes (Fig. 6A). Therefore to evaluate the potency of MRE 2029-F20 at A2B receptors, a Schild analysis of this compound was performed in the presence of the A2A blocker, SCH 58261 (100 nM). The pA\textsubscript{2} value of MRE 2029-F20 was 7.81 ± 0.10 nM, in agreement with its affinity in binding experiments performed at 37°C (K\textsubscript{D} = 6.5 ± 0.7 nM) (Fig. 6B). In human neutrophils and lymphocytes expressing different adenosine subtypes, the nonselective agonist NECA, through the activation of both A2A and A2B receptors, showed EC\textsubscript{50} values of 147 ± 16 and 220 ± 20 nM, respectively. The unstimulated levels of cAMP were 20 ± 5 and 16 ± 4 pmol/10\textsuperscript{6} cells and were increased by NECA stimulation to 80 ± 15 and 60 ± 9 in neutrophils and lymphocytes, respectively. To distinguish the role played by the two stimulatory adenosine subtypes, we repeated the concentration-response curve of NECA in the presence of 100 nM

<table>
<thead>
<tr>
<th>Agonists</th>
<th>hA\textsubscript{2B} HEK 293</th>
<th>Human Neutrophils</th>
<th>Human Lymphocytes</th>
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<tr>
<td>NECA</td>
<td>262 ± 30</td>
<td>315 ± 37</td>
<td>340 ± 42</td>
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<td>(R)-PIA</td>
<td>3500 ± 440</td>
<td>4800 ± 550</td>
<td>5300 ± 570</td>
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<tr>
<td>(S)-PIA</td>
<td>8500 ± 920</td>
<td>9200 ± 950</td>
<td>9700 ± 990</td>
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<tr>
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<td>9.8 ± 0.9</td>
<td>5.2 ± 0.4</td>
<td>4.0 ± 0.7</td>
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<tr>
<td>CGS 21680</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
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<table>
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<tr>
<th>Antagonists</th>
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<th>Human Lymphocytes</th>
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<tr>
<td>MRE 2029-F20</td>
<td>3.0 ± 0.15</td>
<td>3.5 ± 0.6</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>MRS 1754</td>
<td>3.5 ± 0.20</td>
<td>4.6 ± 0.4</td>
<td>5.2 ± 0.5</td>
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<tr>
<td>AS 16</td>
<td>22 ± 1.5</td>
<td>35 ± 5</td>
<td>40 ± 6</td>
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<tr>
<td>AS 100</td>
<td>3.8 ± 0.31</td>
<td>5.0 ± 0.4</td>
<td>5.5 ± 0.8</td>
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<td>CGS 15943</td>
<td>9.8 ± 0.9</td>
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</tr>
<tr>
<td>ZM 241385</td>
<td>9.0 ± 0.8</td>
<td>22 ± 4</td>
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<tr>
<td>DPCPX</td>
<td>35 ± 5</td>
<td>41 ± 5</td>
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<td>Theophylline</td>
<td>6700 ± 800</td>
<td>7500 ± 800</td>
<td>7900 ± 900</td>
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</table>

N.T., not tested.

**Fig. 4.** Competition curves of specific [\textsuperscript{3}H]MRE 2029-F20 binding to human A\textsubscript{2B} adenosine receptors in HEK 293 cells 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.

**Fig. 5.** A, saturation of [\textsuperscript{3}H]MRE 2029-F20 binding to A\textsubscript{2B} adenosine receptors in human neutrophils (●) and lymphocytes (▲). B, Scatchard plots of the same data are shown. K\textsubscript{D} values were 2.4 ± 0.5 and 2.7 ± 0.7 nM and B\textsubscript{max} values were 79 ± 10 and 54 ± 8 fmol/mg of protein in neutrophils and lymphocytes, respectively. Data points are the means and vertical lines are the S.E.M. of four separate experiments performed in triplicate. Experiments were performed as described under Materials and Methods.
MRE 2029-F20 or 100 nM SCH 58261. In the first case, the EC_{50} values related to the A_{2A} component were 39 ± 6 and 70 ± 10 nM in neutrophils and lymphocytes, respectively. In the second condition, the EC_{50} values of NECA related to the A_{2B} receptor stimulation were 2100 ± 220 and 2640 ± 240 nM in neutrophils and lymphocytes, respectively (Fig. 7, A and B).

**Phosphoinositide Turnover.** It is well known that A_{2B} receptors, in addition to adenylyl cyclase, are also coupled to phospholipase C and stimulate phosphoinositide production. In hA_{2B}HEK 293 cells, 1 μM NECA induced a significant stimulation of IP_{3} generation raising from basal values of 1.0 ± 0.3 to 15 ± 2 pmol of IP_{3} from 1 × 10^6 cells (P < 0.01, n = 3). Pretreatment of the cells with 100 nM MRE 2029-F20 and the aminosteroid PLC inhibitor U73122 (5 μM) antagonized the NECA-mediated IP_{3} generation, suggesting the involvement of A_{2B} receptors and PLC_{β}, respectively (Fig. 8A). When IP_{3} generation was evaluated in human lymphocytes, we could not observe any coupling to PLC_{β}. In contrast, in human neutrophils, it was possible to detect IP_{3} turnover induced by classic stimuli linked to PLC activation, such as 10 μM formyl-methionyl-leucyl phenylalanine and 100 μM NECA. This dose of NECA induced an increase from 0.45 ± 0.15 to 5.94 ± 0.5 pmol of IP_{3} from 1 × 10^6 cells that was significantly reduced in the presence of 100 nM MRE 2029-F20 and 5 μM U73122 (2.5 ± 0.5 and 0.7 ± 0.2 pmol/10^6 cells, respectively) (P < 0.01, n = 3) (Fig. 8B).

**Intracellular Calcium Measurements.** To better characterize A_{2B} coupling to PLC activation, intracellular calcium levels were determined. NECA revealed a marked concentration-dependent increase of intracellular Ca^{2+} in hA_{2B}HEK 293 cells with an EC_{50} value of 312 ± 30 nM (Fig. 9A). This effect was potently reversed by increasing concentrations of MRE 2029-F20, showing an IC_{50} value of 12 ± 2 nM (Fig. 9B). Treatment with 5 μM U73122 completely abrogated NECA-induced calcium increase, whereas no changes in calcium levels were observed in the presence of pertussis toxin that inactivate Gi and G_{i} family G proteins (Fig. 9C). These data suggest that A_{2B} receptors in hA_{2B}HEK 293 cells signal through the activation of G_{q/11} protein and PLC_{β} enzyme. To investigate the coupling of A_{2B} receptors with this pathway also in native cells, we performed the same experiments in human lymphocytes and neutrophils. Consistent with IP_{3} assay, in human lymphocytes, A_{2B} receptors did not seem to stimulate intracellular calcium levels. As for human neutrophils, we observed that 100 μM NECA increases intracellular calcium levels (Fig. 10A). This effect was antagonized by MRE 2029-F20 with an IC_{50} value of 125 ± 23 nM and completely abrogated in the presence of the PLC inhibitor.
U73122 indicating the involvement of A2B receptors and PLCβ enzyme (Fig. 10, A and B).

**Discussion**

In this work, we have compared the pharmacological behavior and the functional coupling of recombinant A2B adenosine receptors expressed in transfected HEK 293 cells with endogenous receptors present in peripheral blood cells by using the novel radiolabeled receptor antagonist [3H]MRE 2029-F20. In saturation assays, this radioligand shows a $K_D$ value of 2.8 nM, in agreement with data obtained from kinetic experiments, and a receptor density of 450 fmol/mg of protein. The pharmacology of A2B receptors shows that reference adenosine ligands bound to human A2B receptors with a rank order of potency typical of the A2B subtype. Adenosine antagonists show affinity values similar to those reported in literature. In particular, MRE 2029-F20 has affinity and selectivity values comparable with those of MRS 1754 and OSIP339391, ZM 241385, and DPCPX. As for agonists, they all confirm a low affinity for the A2B site. In contrast to mRNA presence of A2B receptors, in human monocytes it was not possible to detect any specific binding of [3H]MRE 2029-F20, and this is in agreement to findings reported by Thiele et al. (2004) showing an increase of A2B message in cultured human monocytes in comparison with freshly isolated cells but not A2B receptor functionality.

In 1999, Linden et al. reported that in transfected HEK 293 cells, A2B receptors seem to be coupled to adenyl cyclase and phospholipase C activity; in the same work, coupling to Gq proteins was also observed in HMC-1 cells. However, it is well known that recombinant receptors that are expressed in very high concentrations may produce different coupling in comparison with natural systems in which they are present at a lower level. Therefore, we

![Fig. 7](image-url)

**Fig. 7.** A, effect of increasing concentrations of NECA alone (■) and in the presence of a fixed concentration (100 nM) of SCH 58261 (○) or MRE 2029-F20 (▲) on cAMP accumulation in human neutrophils (EC$_{50}$ = 147 ± 16, 2100 ± 220, and 29 ± 6 nM, respectively). B, effect of increasing concentrations of NECA alone (■) and in the presence of a fixed concentration of SCH 58261 (○) or MRE 2029-F20 (▲) on cAMP accumulation in human lymphocytes (EC$_{50}$ = 220 ± 20, 2640 ± 240, and 70 ± 10 nM, respectively). 100% represents the percentage maximal response for each curve. Results are presented as the mean ± S.E.M. of five independent experiments.

![Fig. 8](image-url)

**Fig. 8.** A, effect of 1 μM NECA on IP$_3$ production in hA2BHEK 293 cells and antagonism by 100 nM MRE 2029-F20 and 5 μM U73122. B, effect of 10 μM fMLF (added for 30 s) and 100 μM NECA (added for 30 min) on IP$_3$ production in human neutrophils and antagonism by 100 nM MRE 2029-F20 and 5 μM U73122. Results are presented as the mean ± S.E.M. of three independent experiments. (Student's t test; *, P < 0.01 versus basal; #, P < 0.01 versus NECA).
compared the intracellular signaling pathways regulated by A_{2B} adenosine receptors in both recombinant systems and native cells. To achieve this, we investigated the regulation of adenylyl cyclase and phospholipase C activity by A_{2B} receptors, using NECA as the most potent nonselective A_{2B} agonist available and MRE 2029-F20 as a highly selective A_{2B} antagonist in HEK 293 cells, neutrophils, and lymphocytes. It is interesting that in the cAMP accumulation assay, NECA exhibited a potency higher than that observed in binding experiments, according to Linden et al. (1999). Because this effect was not potently reversed by MRE 2029-F20 (IC_{50} 150 nM), we investigated the possible presence of A_{2A} receptors in these cells. Even though previous studies reported the absence of A_{2A} transcript and protein in HEK 293 cells (Cooper et al., 1997; Gao et al., 1999), in our conditions, real-time RT-PCR and binding studies revealed the presence of A_{2A} receptors (B_{max} of 35 fmol/mg of protein). Therefore, new experiments to determine the potency of MRE 2029-F20 to counteract the NECA-induced cAMP levels were performed in the presence of a fixed concentration of the A_{2A} blocker SCH 58261. With this experimental approach, the A_{2B} antagonist showed a pA_{2} of 7.8, which was consistent with its binding affinity. The discrepancy between our data and previous data might be related either to a different method used to reveal A_{2A} receptors or to a different strain.

Fig. 9. A, effect of increasing concentrations of NECA on intracellular Ca^{2+} levels in hA_{2B}HEK 293 cells (IC_{50} = 312 ± 30 nM). [Ca^{2+}]_{i} was monitored at 37°C with Fura-2 in hA_{2B}HEK 293 cells. Responses were measured relative to the peak response to 10 μM NECA. Forty-five cells were analyzed in each experiment. The results are typical of four experiments. B, antagonism by MRE 2029-F20, added 10 min before agonist, on the 500 nM NECA-induced stimulation of calcium release in hA_{2B}HEK 293 cells (IC_{50} = 12 ± 2 nM). C, typical traces that visualize stimulation of intracellular Ca^{2+} levels in hA_{2B}HEK 293 cells by 1 μM NECA in the absence and in the presence of PTX (200 ng/ml for 18 h) (top traces, bold and light, respectively) and effect of U73122 5 μM (bottom trace). The results are typical of four experiments.

Fig. 10. A, effect of 100 μM NECA, on intracellular Ca^{2+} levels in human neutrophils in the absence (top trace) and in the presence (bottom trace) of U73122 5 μM. B, antagonism by MRE 2029-F20 on the 100 μM NECA-stimulated intracellular calcium levels in human neutrophils. The IC_{50} value was calculated to be 125 ± 23 nM. The results are typical of four experiments.
of HEK 293 cells. In native cells, the coexpression of all adenosine subtypes coupled to the same effector system makes it impossible to study the contribution of each receptor subtype without the availability of selective ligands. Because of the lack of selectivity of NECA, cAMP accumulation in peripheral blood cells was evaluated by using the same pharmacological approach described above and previously reported in HMC-1 cells (Feoktistov and Biaggioni, 1998). The stimulatory effect on cAMP levels caused by the A2A receptor was distinguished from an A2B effect through the use of SCH 58261 and MRE 2029-F20 as A2A and A2B selective antagonists, respectively. Functional A2A and A2A subtypes are also present together with A2A and A2B in both neutrophils and lymphocytes (Gessi et al., 2002, 2004). In most cases, a selective blockade of inhibitory adenosine receptors is required to unmask functional stimulatory subtypes (Feoktistov and Biaggioni, 1997). However, under our conditions, as already reported in various glial cells (Fiebich et al., 1996), it was not necessary to block A1 and A2A receptors to observe either A2A or A2B cAMP stimulation. The simultaneous expression of different adenosine subtypes in a single cell is more of a rule than an exception. Because A2B receptors have low affinity for adenosine it is possible that their role become important in pathologic conditions, when adenosine levels increase. However it has to be noted that other intracellular signaling pathways have been associated with the A2B stimulation.

In agreement with literature data showing A2B/Gq coupling in a variety of cells (Feoktistov and Biaggioni, 1995; Strohmeier et al., 1995; Linden et al., 1998, 1999; Gao et al., 1999; Grant et al., 2001; Feoktistov et al., 2002; Rees et al., 2003), we also found that A2B Receptors in hA2BHEK 293 cells activate PLCβ, as demonstrated by inositol phosphate generation and calcium mobilization experiments. NECA induced a significant increase in IP3 production that was counteracted by the A2B antagonist MRE 2029-F20 and also by the PLC inhibitor U73122. In calcium mobilization studies, NECA had an EC50 in the high nanomolar range, in agreement with its affinity in binding experiments, and MRE 2029-F20 was able to potently antagonize this effect. A2B regulation of intracellular calcium increase was dependent on Gq/11 and PLCβ as demonstrated in experiments carried out in the presence of pertussis toxin and U73122. Stimulation of intracellular calcium levels was not observed in wild-type HEK 293 cells, according to Ryzhov et al. (2004), but in contrast with Gao et al. (1999). Again, a different strain of cells might be responsible for these discrepancies, and under our experimental conditions, one could hypothesize that the increase in levels of receptor expression obtained by transfection technique led to a gain in coupling of A2B receptors to PLC signaling pathway. As for neutrophils, both IP3 production and calcium increase were observed after 100 µM NECA stimulation. Because of the presence of A2A receptors in human neutrophils that might be coupled to PLC (Gessi et al., 2002), we verified the role of A2B receptors in the NECA-induced effect. MRE 2029-F20 reduced both IP3 production and intracellular calcium increase, showing a potency in the nanomolar range that was consistent with the involvement of the A2B subtype. The coupling to PLCβ was not observed in human lymphocytes (Mirabet et al., 1999), where the amount of A2B receptors was slightly lower compared with human neutrophils, suggesting that receptor density might be important to establish the signaling pathways activated by receptor agonist stimulation.

In conclusion, we have demonstrated the presence and functional coupling of human A2B adenosine receptors in different peripheral blood cells that play a role in immune and inflammatory processes in which A2B receptors are thought to be involved. This analysis was made possible by [3H]MRE 2029-F20, a new selective and high-affinity antagonist radioligand for human A2B adenosine receptors that has been successfully employed to detect and quantify the amount of this adenosine subtype in native cells. Our data reveal that recombinant A2B Receptors expressed in HEK 293 cells show binding characteristics similar to those presented by A2B subtypes expressed in peripheral blood cells but might differ from these in terms of signal transduction coupling as seen in lymphocytes. Further studies might elucidate other effects mediated by A2B Receptors in neutrophils and lymphocytes, such as their involvement in superoxide anion generation or in cytokine release. The appreciation of the pathways activated by A2B receptors in native cells raises the possibility that selective antagonists may become useful tools for the pharmacological characterization of diseases in which A2B receptors may be involved and possibly a basis for drug development.

References