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

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Running Title: A_{2B} adenosine receptors in recombinant and native cells

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Abbreviations: NECA, 5'-N-ethyl-carboxamidoadenosine; R-PIA, R(-)-N^6(2-phenyl-isopropyl)-adenosine; S-PIA, S(-)-N^6(2-phenylisopropyl)adenosine; IB-MECA, N^6-(3-iodo-benzyl) adenosine-5'-N-methyluronamide; CHA, N^6cyclohexyladenosine; CGS 21680 2-[p-(2-carboxyethyl)-phenetylamino]-5'-*N*-ethylcarboxamidoadenosine. 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 3008F20, 5-*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo [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]; AS 16, 2-(4-benzyloxy-phenyl)-N-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-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-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide]; MRS 1754, 8-[4[((4-cyano-[2,6]-phenyl)carbamoylmethyl)oxy)phenyl]-1,3-di(n-propyl)xanthine; hA_{2B}HEK 293 cells, human embryonic kidney-293 cells transfected with human adenosine A_{2B} receptor; PLC phospholipase C; fMLF (N-formyl-Lmethionyl-L-leucyl-L-phenylalanine); U73122 1-[6-([(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione; ZM 241385 (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,32][1,3,6]triazinyl-amino] ethyl)-phenol).

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ABSTRACT

The present paper compares 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, [^{3}H]-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] ([^{3}H]-MRE 2029-F20), that has high affinity and selectivity for hA_{2B} versus hA_1, hA_{2A} and hA_3 subtypes. [^{3}H]-MRE 2029-F20 bound specifically to the hA_{2B} receptor stably transfected in HEK293 cells with K_D of 2.8±0.2 nM and B_{max} of 450±42 fmol/mg protein. Saturation experiments of [^{3}H]-MRE 2029-F20 binding in human neutrophils and lymphocytes detected a single high affinity binding site with K_D of 2.4 ±0.5, 2.7±0.7 nM and Bmax of 79±10, 54±8 fmol/mg of protein, respectively, in agreement with real-time RT-PCR 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. NECA 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 revealed to be a potent antagonist in counteracting the agonist effect in both signal transduction pathways. In conclusion [^{3}H]-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 have demonstrated their presence and functional coupling in neutrophils and lymphocytes that play a role in inflammatory processes where A_{2B} receptor may be involved.

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Adenosine is an ubiquitous modulator that exerts its physiological functions through the interaction with four G proteins-coupled receptors classified as A_1, A_{2A}, A_{2B}, and A_3 (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 Gs 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 the caecum, large intestine and urinary bladder, but lower levels in the 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 order to understand their role in pathophysiological conditions. The lack of selective pharmacological tools has hampered research in this field and 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 effectivness 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 (Fiebich et al., 1996; Feoktistov and Biaggioni, 1995; 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). Recently, 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 appear implicated in mast-cell secretion (Feoktistov et al., 2001), coronary flow regulation (Talukder et al., 2003), neoangiogenesis (Grant et al., 2001; Feoktistov et al., 2002; Feoktistov et al., 2003; Afzal et al., 2003), cytokine release by bronchial smooth muscle cells

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(Zhong et al., 2004) and nociception (Abo-Salem et al., 2004) 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, untill now, by using low affinity and non selective antagonists like [^{3}H]DPCPX, [^{3}H]ZM 241385 and [^{125}I]ABOPX, that, as a consequence of their low affinity, display a rapid dissociation rate from the receptor. In addition, since these ligands are nonselective their utility in native systems is hampered as 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 radiolabelled compounds with high affinity and selectivity should be synthesized. Recently, high affinity radioligands for A_{2B} receptors, [^{3}H]MRS 1754 and [^{3}H]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-pyrazole xanthine derivatives as potent and selective human A_{2B} adenosine antagonists (Baraldi et al., 2004a,b), and a radiolabelled form of one compound of this series was used as 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, that 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 paper we demonstrate that A_{2B} receptors are strongly linked to adenylyl cyclase in both transfected and native cells, whilst as 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.

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Experimental Procedures

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

Synthesis of [^{3}H]-MRE 2029-F20. The synthesis of [^{3}H]-MRE 2029-F20 was prepared through custom synthesis at Amersham International (Buckinghamshire, UK) 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, Universitat Würzburg, Germany) and subcloned into the expression plasmid pcDNA 3.1 (Invitrogen, Life Technologies). The plasmid was amplified into a competent E. Coli strain and plasmid DNA isolated by using Quiagen Maxiprep columns (Quiagen Plasmid Purification Kit). cDNA was then sequenced on both strands in the University of Padova, DNA Sequence Service CRIBI, and transfected into HEK 293 cells

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by using the calcium phosphate precipitation method (Chen and Okayama 1987). Colonies were selected by growth of cells on 0.8 mg/ml G418. Stably transfected cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12 medium) with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.3 mg/ml G418, at 37° in 5% CO_2/95% air.

Membrane preparation. For membrane preparation the culture medium was removed. The cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized using a Polytron and the homogenate was spun for 30 min at 36,000 x g. The membrane pellet was resuspended in 50 mM Tris HCl buffer, containing 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine, pH 7.4 and incubated with 2 UI/ml of adenosine deaminase for 30 min at 37°C. The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) with bovine 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). Briefly, the blood was centrifuged on Ficoll-Hypaque density gradients. The human peripheral blood mononuclear cells (PBMC) were isolated and removed from the Ficoll-Hypaque gradients. Subsequently, they were washed in 0.02 M phosphate-buffered saline at pH 7.2. Further purification of lymphocytes from PBMC 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. Remaining erythrocytes were lyzed by suspending the cell pellet in 10 ml of distilled water at 4°C under gentle agitation. After 30

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sec, 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 250 g. 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 a ethylenediaminetetraacetic acid (EDTA) anticoagulated tube, diluted in PBS 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 & Sacchi, 1987). Quantitative real-time RT-PCR assay (Higuchi et al., 1993) of A_{2B} mRNA transcript was carried out using gene-specific double fluorescently labelled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington Cheshire, UK). For the real-time RT-PCR of the A_{2B} gene the assays-on-demand^{TM} Gene expression Product accession no. NM0006756 was used 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 β-actin kits was used, and the probe was fluorescent-labeled with VIC^{TM} (Applied Biosystems, Monza, Italy). Moreover a curve of A_{2B} cDNA plasmid standards with a range spanning at least six 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 A_{2B} message in blood cells was

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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 A_1, A_{2A}, and A_3 receptors were performed at 4°C using [^{3}H]-DPCPX, [^{3}H]-ZM 241385 and [^{3}H]-MRE 3008-F20, respectively as described by Varani et al., 2000.

[^{3}H]-MRE 2029-F20 binding assays. Kinetic studies of [^{3}H]-MRE 2029-F20 (2.5 nM) were performed incubating membranes obtained by HEK-293 cells transfected with the human A_{2B} receptors in a thermostatic bath at 4°C. A total assay volume of 250 µl was employed in which final protein levels were 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 ice-cold buffer four times. For the measurement of dissociation rate the samples were incubated at 4° for 90 min, and then 1 µM of the MRE 2029-F20 was added to the mixture. The reaction was terminated at different times from 5 to 100 min. Saturation binding experiments of [^{3}H]-MRE 2029-F20 (0.3 to 30 nM) to hA_{2B} HEK293 cell membranes were performed by incubation for 90 min at 4°C. Competition experiments of 3 nM [^{3}H]-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 $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine pH 7.4, 100 µl of membranes and at least 12-14 different concentrations of typical adenosine receptor agonists and antagonists. Non-specific binding was defined as binding in the presence of 1 µM MRE 2029-F20 and, at the K_D value of the radioligand, was about 30-35% and 40-45% 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

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glass-fiber filters using a Micro-Mate 196 cell harvester (Packard Instrument Company). The filter bound radioactivity was counted with a Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20. K_i values were calculated from IC_{50} values according to Cheng & Prusoff equation $K_i = IC_{50}/(1+[C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant. A weighted non linear least-squares curve fitting program LIGAND (Munson and Rodboard, 1980) was used for computer analysis of saturation and inhibition experiments.

Cyclic AMP assay. hA_{2B}HEK-293 and peripheral blood cells, were suspended in 0.5 ml of Krebs-Ringer phosphate buffer (KRPG) (136 mM NaCl, 5 mM KCl, 0.67 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 3 mM $NaHCO_3$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM HEPES, 10 mM $MgCl_2$, 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 non selective 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 cold 6% trichloroacetic acid. The trichloroacetic acid suspension was centrifuged at 2,000 X 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 the incubation buffer (trizma base 0.1 M; aminophylline 8.0 mM; 2-mercaptoetanol 6.0 mM, pH 7.4) and [^{3}H]-cyclic AMP in a total volume of 0.5 ml. The binding protein, previously prepared from beef adrenals, was added to the samples and incubated at 4° for 150 min. After the addition of charcoal, samples were centrifuged at 2,000 x g for 10 min. The clear

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supernatant (0.2 ml) was mixed with 4 ml of Atomlight and counted in a LS-1800 Beckman scintillation counter.

Ins(1,4,5)P_3 binding assay

IP_3 generation in hA_{2B}HEK 293 and peripheral blood cells was measured by [^{3}H]Ins(1,4,5)P_3 competition assay to IP_3 binding protein according to the method described by Challiss et al., 1990. hA_{2B}HEK 293 cells ($2x10^6$ cells in each tube) and peripheral blood cells ($8x10^6$ cells in each tube) were suspended in KRPG 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 3,000 g for 15 min at 4°C and trichloroacetic acid was extracted with 5x2 vol washes with water-saturated diethyl ether. Finally 125 µl of 30 mM EDTA and 125 µl of 60 mM $NaHCO_3$ 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)P_3 assay standard curve. A 30 µl portion of sample or of trichloroacetic acid-extracted buffer containing standard amounts of Ins(1,4,5)P_3 (0.12-12 pmol) or DL-Ins(1,4,5)P_3 (0.3 nmol to define non specific binding) was added to 30 µl of assay buffer (25 mM Tris HCl, 1 mM EDTA, pH 8) and 30 µl of [^{3}H]Ins(1,4,5)P_3 (7,000 dpm/assay). Then 30 µl (0.4 mg of protein) of the adrenal-cortical binding protein preparation was added and incubation continued for 30 min. Bound and free [^{3}H]Ins(1,4,5)P_3 were separated by rapid filtration through Whatman GF/B glass fibre filters with 4x3 ml washes of ice-cold 25 mM Tris HCl, 1 mM EDTA, 5 mM $NaHCO_3$, pH 8. Scintillator was added to the filter discs, and radioactivity determined after a 12 h extraction period by scintillation counting.

Measurement of cytosolic Ca^{2+} concentration

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$[Ca^{2+}]i$ was evaluated by incubating hA_{2B}HEK 293 cells with the $[Ca^{2+}]$-sensitive fluorescent dye Fura 2-AM (5 μM) in KRPG buffer for 45 min at 37 °C. Alternate excitation at 340 nM and 380 nM was supplied and the F340/F380 emission ratio 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-AM (2 μM) in KRPG buffer, for 30 min at 37 °C, according to Gessi *et al*., 2002. The cells were centrifuged at 1,000 x g 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, Perkin-Elmer, Norwalk, CT spectrofluorimeter, at an excitation wavelength of 340 and 380 nm and emission wavelength of 505 nm, in cuvettes thermostatically controlled at 37 °C and continuously stirred.

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Results

Real-time RT-PCR experiments

HEK 293 cells are recognized as cells expressing native A_{2B} receptors. Therefore the mRNA presence of this adenosine subtype was investigated in both wild type and hA_{2B}HEK 293 cells. Transfection of A_{2B} receptors in HEK 293 cells produced a 350 ± 30 fold increase of A_{2B} mRNA accumulation with respect to the corresponding wild type cells; the expression level of A_{2B} receptors was normalized to the expression level of the endogenous reference (β-actin) in each sample (Fig. 1A and B). In addition, the mRNA level of A_{2B} 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 about 4 fold less mRNA in comparison to the other blood cells (Student's t-test *$P<0.01$ versus monocytes) (Fig. 1C).

Radioligand binding studies in recombinant and native cells

Figure 2A shows that [^{3}H]-MRE 2029-F20 binding to hA_{2B}HEK 293 cells reached equilibrium after approximately 45 minutes and was stable for at least 3 hours. [^{3}H]-MRE 2029-F20 binding was rapidly reversed by the addition of 1 μM MRE 2029-F20 as shown in Figure 2B. Association and dissociation curves were fitted by a one component model significantly better than to a two-component model ($P<0.05$). The rate constants were: k_{obs} = 0.046 ± 0.002 min^{-1} and k_{-1} = 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 [^{3}H]-MRE 2029-F20 to adenosine A_{2B} 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

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with a K_D value of 2.8 ± 0.2 nM and a B_{max} value of 450 ± 42 fmol/mg protein. In contrast to the presence of mRNA message, no specific binding of [^{3}H]-MRE 2029-F20 was detectable in HEK 293 wild type cells (datum not shown). Table 1 shows the affinities, expressed as K_i values, of selected adenosine receptor agonists and antagonists to hA_{2B} receptors expressed in HEK 293 cells using [^{3}H]-MRE 2029-F20. The order of potency in [^{3}H]-MRE 2029-F20 displacement assays for adenosine receptor agonists resulted to be: NECA > R-PIA > CHA > IB-MECA > S-PIA > CGS 21680 (Fig. 4A). The order of potency of the receptor antagonists was: MRE 2029-F20 > MRS 1754 > AS 100 > ZM 241385 > CGS 15943 > AS 16 > DPCPX > enprofylline > theophylline (Figure 4B). The selectivity of MRE 2029-F20 for the human A_{2B} over A_1, A_{2A}, and A_3 receptors was evaluated in radioligand binding assays by using [^{3}H]-DPCPX, [^{3}H]-ZM 241385 and [^{3}H]-MRE 3008-F20, respectively. MRE 2029-F20 displays low affinity for the human A_1 receptor (Ki = 245 ± 31 nM) and no significant affinity for the human A_{2A} and A_3 subtypes (Ki >1,000 nM). This indicates that MRE 2029-F20 is 88 and more than 300 fold selective for the A_{2B} over A_1, A_{2A} and A_3 subtypes, respectively, that means a range of selectivity similar to [^{3}H]-MRS 1754 (selectivity of 210, 260, 290 fold for A_{2B} over A_1, A_{2A} and A_3 subtypes, respectively) and better than [^{3}H]-OSIP339391 (selectivity of 70 fold for A_{2B} over A_1, A_{2A} and A_3 subtypes, respectively) (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_{max} values of 460 ± 50 and 430 ± 48 fmol/mg of protein, respectively, thus suggesting that dissociation constants increased with temperature, while B_{max} data were largely independent of it. This binding behaviour has previously been found to be typical of adenosine A_1, A_{2A} and A_3 subtypes (Borea et al., 1996; Varani et al., 2000). In order 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

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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. Conversely, 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, 2.7 ± 0.7 nM and Bmax of 79 ± 10, 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 being NECA >R-PIA >CHA>IB-MECA>S-PIA>CGS 21680 and MRE 2029-F20> MRS 1754>AS100>ZM 241385>AS16>DPCPX > enprofylline > 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

In order 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}HEK 293 cells and in peripheral blood cells. Our results show that in hA_{2B}HEK 293 cells, NECA showed a potency greater in cAMP (EC_{50} value of 4.5 ± 0.4 nM) than in radioligand binding studies (Ki value of 262 ± 30 nM) (Fig. 6A). Surprisingly this effect was not potently antagonized by MRE 2029-F20 that showed an IC_{50} value of 150 ± 18 nM. Due to 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} mRNAs expression (data not shown) and confirmed by saturation binding experiments of [^{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 not possibly due to a contaminating presence of

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endogenous A_{2B} receptors, as demonstrated by the lack of [^{3}H]-MRE 2029-F20 specific binding in untransfected HEK 293 cells. CGS 21680, an A_{2A} selective agonist, stimulated cAMP levels with an EC_{50} of 410 ± 52 nM but it was less efficacious than NECA. The unstimulated level of cAMP was 15 ± 3 pmol/10^6cells; at concentrations producing maximal effects (10 μM), CGS 21680 induced a four-fold increase, whilst NECA an eight-fold increase in cAMP (to 60 ± 8 and 120 ± 15 pmol/10^6cells, respectively) suggesting the activation in the first case only of A_{2A} receptors and in the second one of both A_{2A} and A_{2B} subtypes (fig. 6A). Therefore to evaluate the potency of MRE 2029-F20 at A_{2B} receptors a Schild analysis of this compound was performed in the presence of the A_{2A} blocker, SCH 58261 (100 nM). The pA_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_D= 6.5 ± 0.7 nM) (Fig. 6B). In human neutrophils and lymphocytes, expressing different adenosine subtypes, the non selective agonist NECA, through the activation of both A_{2A} and A_{2B} receptors, showed an EC_{50} value of 147 ± 16 nM and 220 ± 20 nM, respectively. The unstimulated level of cAMP were 20 ± 5 and 16 ± 4 pmol/10^6cells and were increased by NECA stimulation to 80 ± 15 and 60 ± 9 in neutrophils and lymphocytes, respectively. In order 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 MRE 2029-F20 or 100 nM SCH 58261. In the first case the EC_{50} value, due to the A_{2A} component was 39 ± 6 nM and 70 ± 10 nM, in neutrophils and lymphocytes, respectively. In the second condition the EC_{50} value of NECA, due to the A_{2B} receptor stimulation, was 2,100 ± 220 nM and 2,640 ± 240 nM, in neutrophils and lymphocytes, respectively (Fig. 7 A-B).

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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 pmoles to 15±2 pmoles IP_3 from 1 x 10^6 cells ($P<0.01$, N=3). Pretreatment of the cells with 100 nM MRE 2029-F20 and the aminosteroid PLC inhibitor, 5 μM U73122 antagonized the NECA-mediated IP_3 generation, suggesting the involvement of A_{2B} receptors and PLCβ, respectively (Fig. 8A). When IP_3 generation was evaluated in human lymphocytes, we could not observe any coupling to PLCβ. In contrast in human neutrophils it was possible to detect IP_3 turnover induced by classical stimuli linked to PLC activation such as 10 μM fMLF, and also 100 μM NECA. This dose of NECA induced an increase from 0.45±0.15 to 5.94±0.5 pmoles IP_3 from 1 x 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 pmoles/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 U73122 (5 μM), completely abrogated NECA-induced calcium increase, whilst no changes in calcium levels were observed in the presence of pertussis toxin, that inactivate Gi and Go family G proteins (Fig. 9C). These data suggest that A_{2B} receptors, in hA_{2B}HEK 293 cells, signal through the activation of $Gq_{/11}$ protein and PLCβ enzyme. To

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investigate the coupling of A_{2B} receptors with this pathway also in native cells we performed the same experiments in human lymphocytes and neutrophils. Consistently with IP_3 assay, in human lymphocytes A_{2B} receptors did not appear 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 PLC inhibitor, U73122, indicating the involvement of A_{2B} receptors and PLCβ enzyme (Fig. 10 A-B).

Discussion

In this work, we have compared the pharmacological behaviour and the functional coupling of recombinant A_{2B} adenosine receptors expressed in transfected HEK 293 cells with endogenous receptors present in peripheral blood cells by using the novel radiolabelled receptor antagonist [^{3}H]-MRE 2029-F20. In saturation assays this radioligand shows 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 A_{2B} receptors shows that reference adenosine ligands bound to human A_{2B} receptors with a rank order of potency typical of the A_{2B} subtype. Adenosine antagonists show affinity values similar to those reported in literature. In particular MRE 2029-F20 has affinity and selectivity values comparable to those of MRS 1754 and better than OSIP339391, ZM 241385, and DPCPX. In competition experiments performed in cells transfected with A_1, A_{2A} and A_3 receptors, MRE 2029-F20 displayed a selectivity of 88 and more than 300 fold for A_{2B} than for A_1 (Ki value of 245±31 nM), A_{2A} and A_3 receptors (Ki>1,000 nM), respectively. Taken together these binding characteristics make [^{3}H]-MRE 2029-F20 a useful tool for detection of A_{2B} receptors not only in transfected cells but also in native systems. [^{3}H]-MRE 2029-F20 was used in this work to

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investigate the presence of A_{2B} receptors in peripheral blood cells chosen for the following reasons: i) they represent primary cells expressing endogenous adenosine receptors; ii) they are important inflammatory cells, potentially involved in the exacerbation of asthma and other inflammation conditions, and, finally iii) they are cells containing more than one adenosine receptor subtype and, as a consequence difficult to be studied without the availability of selective radioligands.

First of all, the presence of A_{2B} mRNA message in lymphocytes, neutrophils and monocytes was assessed through real-time RT-PCR assays revealing the presence of A_{2B} transcript with the following increasing rank order, monocytes < lymphocytes ≈ neutrophils. Therefore the A_{2B} message was investigated at the protein level by using [^{3}H]-MRE 2029-F20 binding that shows a single class of high affinity binding sites with a K_D of 2.4±0.5 and 2.7±0.7 nM, and a Bmax of 79±10 and 54±8 fmol/mg of protein in neutrophils and lymphocytes, respectively. The pharmacological profile of A_{2B} ligands in these cells confirmed the data obtained in transfected hA_{2B}HEK 293 cells, with MRE 2029-F20 being the most potent compound followed by MRS 1754, AS 100, ZM 241385 and DPCPX. As for agonists they all confirm a low affinity for the A_{2B} site. In contrast to mRNA presence of A_{2B} receptors, in human monocytes it was not possible to detect any specific binding of [^{3}H]-MRE 2029-F20 and this is in agreement to findings reported by Thiele et al., 2004, showing an increase of A_{2B} message in cultured human monocytes in comparison to freshly isolated cells but not A_{2B} receptor functionality.

In 1999 Linden and collegues reported that in transfected HEK 293 cells A_{2B} receptors appear to be coupled to adenylyl 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

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comparison to natural systems in which they are present at a lower level. Therefore, we compared the intracellular signalling 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 non selective A_{2B} agonist available and MRE 2029-F20 as a highly selective A_{2B} antagonist in HEK 293 cells, neutrophils and lymphocytes. Interestingly, in cAMP accumulation assay, NECA exhibited a potency higher than that observed in binding experiments, according to Linden et al., 1999. As 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 (Bmax 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, that was consistent with its binding affinity. The discrepancy between our and previous data might be due to both a different method used to reveal A_{2A} receptors, or to a different strain 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. Due to 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 et al., 1998). Stimulatory effect on cAMP levels due to the A_{2A} receptor was distinguished from an A_{2B} effect through the use of SCH 58261 and MRE 2029-F20 as A_{2A}

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and A_{2B} selective antagonists, respectively. Functional A_1 and A_3 subtypes are also present together with A_{2A} and A_{2B} 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 in our conditions, as already reported in various glial cells (Fiebich et al., 1996), it was not necessary to block A_1 and A_3 receptors to observe either A_{2A} and A_{2B} cAMP stimulation. The simultaneous expression of different adenosine subtypes in a single cell is more of a rule than an exception. Because A_{2B} 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 A_{2B} stimulation.

In agreement to literature data showing A_{2B}/Gq coupling in a variety of cells (Strohmeier et al., 1995; Feoktistov and Biaggioni 1995; Linden et al., 1998; Linden et al., 1999; Grant et al., 2001; Gao et al., 1999; Feoktistov et al., 2002; Rees et al., 2003) we also found that A_{2B} receptors, in hA_{2B}HEK 293 cells, activate PLCβ, as demonstrated by inositol phosphate generation and calcium mobilization experiments. NECA induced a significant increase in IP_3 production that was counteracted by the A_{2B} antagonist, MRE 2029-F20 and also by the PLC inhibitor U73122. In calcium mobilization studies NECA had an EC_{50} in the high nanomolar range, in agreement with its affinity in binding experiments and MRE 2029-F20 was able to potently antagonize this effect. A_{2B} regulation of intracellular calcium increase was dependent on $G_{q/11}$ 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 wt HEK 293 cells according to Ryzhov et al., (Purines 2004) but in contrast with Gao et al., 1999. Again different strain of cells might be responsible for these discrepancies and in our

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experimental conditions it is possible to hypothesize that the increase in levels of receptor expression obtained by transfection technique led to a gain in coupling of A_{2B} receptors to PLC signaling pathway. As for neutrophils both IP_3 production and calcium increase were observed after 100 μM NECA stimulation. Due to the presence of A_3 receptors in human neutrophils, that might be coupled to PLC (Gessi et al., 2002), we verified the role of A_{2B} receptors in the NECA-induced effect. MRE 2029-F20 reduced both IP_3 production and intracellular calcium increase showing a potency in the nanomolar range that was consistent with the involvement of the A_{2B} subtype. The coupling to PLCβ was not observed in human lymphocytes (Mirabet et al., 1999), where the amount of A_{2B} receptors was slightly lower in comparison to human neutrophils suggesting that receptor density might be important to establish the signaling pathways activated by receptor agonist stimulation.

In conclusion, in this work we have demonstrated the presence and functional coupling of human A_{2B} adenosine receptors in different peripheral blood cells that play a role in immune and inflammatory processes where A_{2B} receptors are thought to be involved. This analysis was made possible by [^{3}H]-MRE 2029-F20, a new selective and high affinity antagonist radioligand for human A_{2B} adenosine receptors which has been successfully employed to detect and quantify the amount of this adenosine subtype in native cells. Our data reveal that recombinant A_{2B} receptors expressed in HEK 293 cells show binding characteristics similar to those presented by A_{2B} 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 A_{2B} receptors in neutrophils and lymphocytes such as their involvement in superoxide anion generation or in cytokine release. The appreciation of the pathways activated by A_{2B} receptors in native cells raises the possibility that selective

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antagonists may become useful tools for the pharmacological characterization of diseases in which A_{2B} receptors may be involved and possibly a basis for drug development.

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Figure Legends

Figure 1 - Representative amplification plots for the A_{2B} receptors mRNA in transfected (■) and untransfected (□) HEK 293 cells (A) as compared with the respective β-actin mRNA (B). (C) Expression of A_{2B} receptors mRNA in monocytes, lymphocytes and neutrophils. The amount of product in blood cells was expressed as the ratio of mRNA (μg) determined by interpolation from standard curve of Ct values generated from the plasmid dilution series and total RNA (μg) 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).

Figure 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 which 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_{+1}= 0.0084 ± 0.0009 min^{-1} nM^{-1} - (B) Kinetic of [^{3}H]-MRE 2029-F20 binding to human A_{2B} adenosine receptors with dissociation curve representative of a single experiment. Inset, first-order plot of [^{3}H]-MRE 2029-F20 binding. Dissociation rate constant was: k_{-1}= 0.025 ± 0.003 min^{-1}.

Figure 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 is shown. K_D value was 2.8 ± 0.2 and B_{max} value was 450 ± 42 fmol/mg of protein. Experiments were performed as described in Experimental Procedures.

Figure 4 - Competition curves of specific [^{3}H]-MRE 2029-F20 binding to human A_{2B} adenosine receptors in HEK 293 cells by adenosine agonists (A) and antagonists (B). Curves

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are representative of a single experiment from a series of four independent experiments. Competition experiments were perfomed as described in Experimental Procedures.

Figure 5 – (A) Saturation of [^{3}H]-MRE 2029-F20 binding to A_{2B} adenosine receptors in human neutrophils (■) and lymphocytes (▲). (B) Scatchard plots of the same data are shown. K_D values were 2.4 ± 0.5 and 2.7 ± 0.7 nM and B_{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 in Experimental Procedures.

Figure 6 - (A) Effect of increasing concentrations of NECA and CGS 21680 on cAMP accumulation in hA_{2B}HEK 293 cells (EC_{50}= 4.5 ± 0.4 and 410 ± 52 nM, respectively). The data are normalized to the maximal NECA response. - (B) Dose-response curves for cAMP accumulation in the absence (■) and in the presence of increasing concentrations of MRE 2029-F20 (30 (•), 300 (▼), 3000 (♦)nM) and with a fixed concentration of SCH 58261 (100 nM). The inset is a Schild plot giving a pA_2 of 7.81 ± 0.10 and a slope of 0.82 ± 0.25. C, concentration of MRE 2029-F20 (in M); CR, ratio of the IC_{50} values of NECA in the presence and absence of MRE 2029-F20. Results are presented as the mean ± S.E.M. of three independent experiments.

Figure 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; 2,100 ± 220; 39 ± 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; 2,640 ± 240; 70 ± 10 nM, respectively). 100% represents the

% maximal response for each curve. Results are presented as the mean ± S.E.M. of five independent experiments.

Figure 8 – (A) Effect of 1 μM NECA on IP_3 production in hA_{2B}HEK 293 cells and antagonism by 100 nM MRE 2029-F20 and 5 μM U73122. (B) Effect of 10 μM fMLF (added for 30 sec) 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).

Figure 9- (A) Effect of increasing concentrations of NECA on intracellular Ca^{2+} levels in hA_{2B}HEK 293 cells (EC_{50}= 312 ± 30 nM). [Ca^{2+}] 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-fifty 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) (upper traces, bold and light, respectively) and effect of U73122 5 μM (lower trace). The results are typical of four experiments.

Figure 10 – (A) Effect of 100 μM NECA, on intracellular Ca^{2+} levels in human neutrophils in the absence (upper trace) and in the presence (lower 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.

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TABLE 1

K_i values from competition experiments with [^{3}H]-MRE 2029-F20 of selected adenosine receptor agonists and antagonists for A_{2B} receptors in hA_{2B} HEK 293 cells, human neutrophils and lymphocytes.

Results are presented in nM as the mean ±S.E.M. of four independent experiments; NT, not tested

Agonists	hA_{2B} HEK 293 (K_i) nM	Human neutrophils (K_i) nM	Human lymphocytes (K_i) nM
NECA	262 ± 30	315 ± 37	340 ± 42
R-PIA	3,500 ± 440	4,800 ± 550	5,300 ± 570
S-PIA	8,500 ± 920	9,200 ± 950	9,700 ± 990
CHA	4,200 ± 450	5,100 ± 530	5,600 ± 600
IB-MECA	7,600 ± 800	8,300 ± 870	9,000 ± 980
CGS 21680	> 10,000	> 10,000	> 10,000
Antagonists			
MRE 2029-F20	3.0 ± 0.15	3.5 ± 0.6	4.0 ± 0.7
MRS 1754	3.5 ± 0.20	4.6 ± 0.4	5.2 ± 0.5
AS 16	22 ± 1.5	35 ± 5	40 ± 6
AS 100	3.8 ± 0.31	5.0 ± 0.4	5.5 ± 0.8
CGS 15943	9.8 ± 0.9	NT	NT
ZM 241385	9.0 ± 0.8	22 ± 4	18 ± 2
DPCPX	35 ± 5	41 ± 5	45 ± 6
Enprofylline	5,500 ± 600	6,200 ± 700	6,800 ± 750
Theophylline	6,700 ± 800	7,500 ± 800	7,900 ± 900

Figure 1

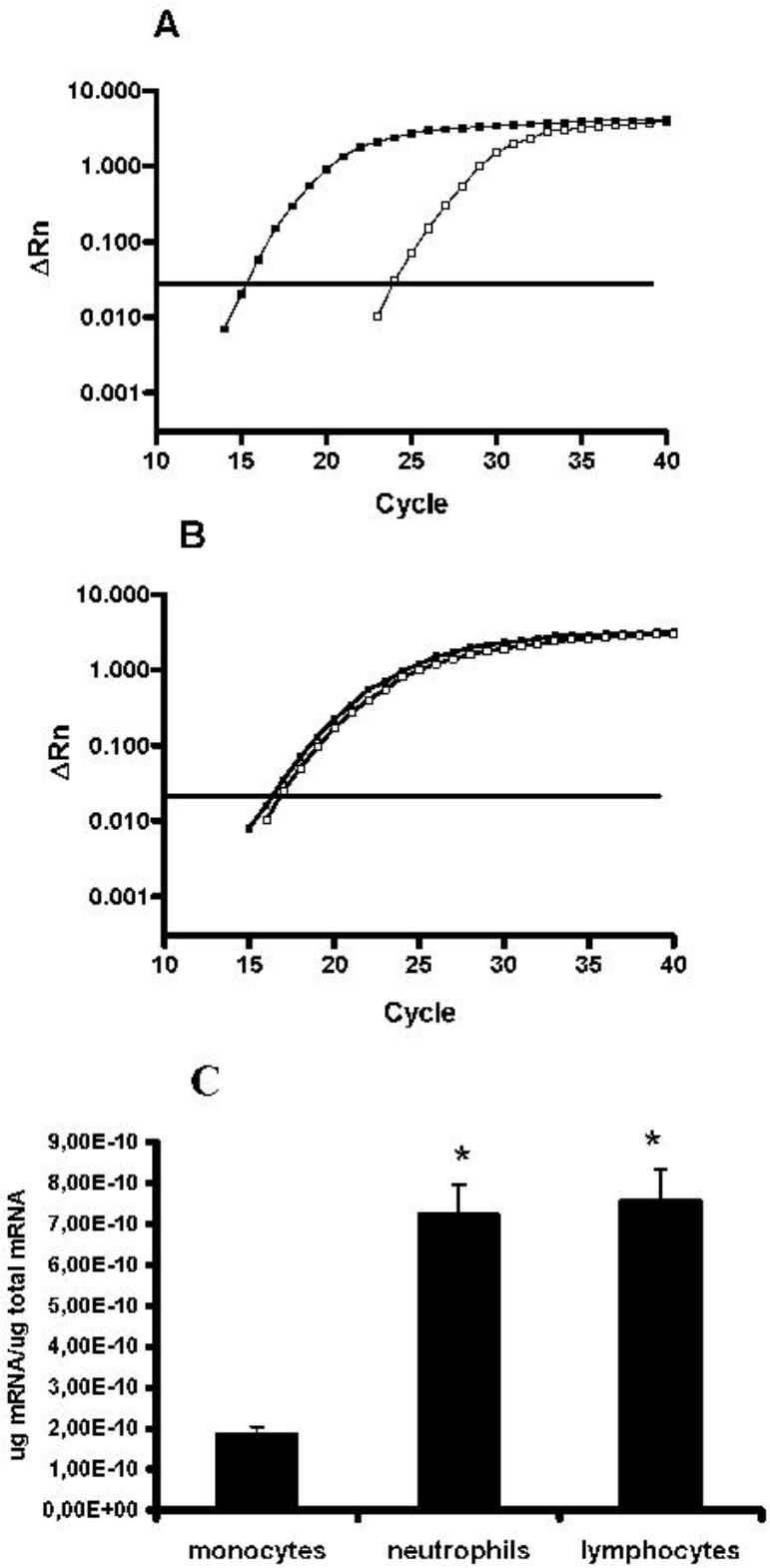

A
10.000
1.000
0.100
0.010
0.001
ΔRn
10
15
20
25
30
35
40
Cycle
B
10.000
1.000
0.100
0.010
0.001
ΔRn
10
15
20
25
30
35
40
Cycle
C
9,00E-10
8,00E-10
7,00E-10
6,00E-10
5,00E-10
4,00E-10
3,00E-10
2,00E-10
1,00E-10
0,00E+00
ug mRNA/ug total mRNA
*
*
monocytes
neutrophils
lymphocytes

Figure 2

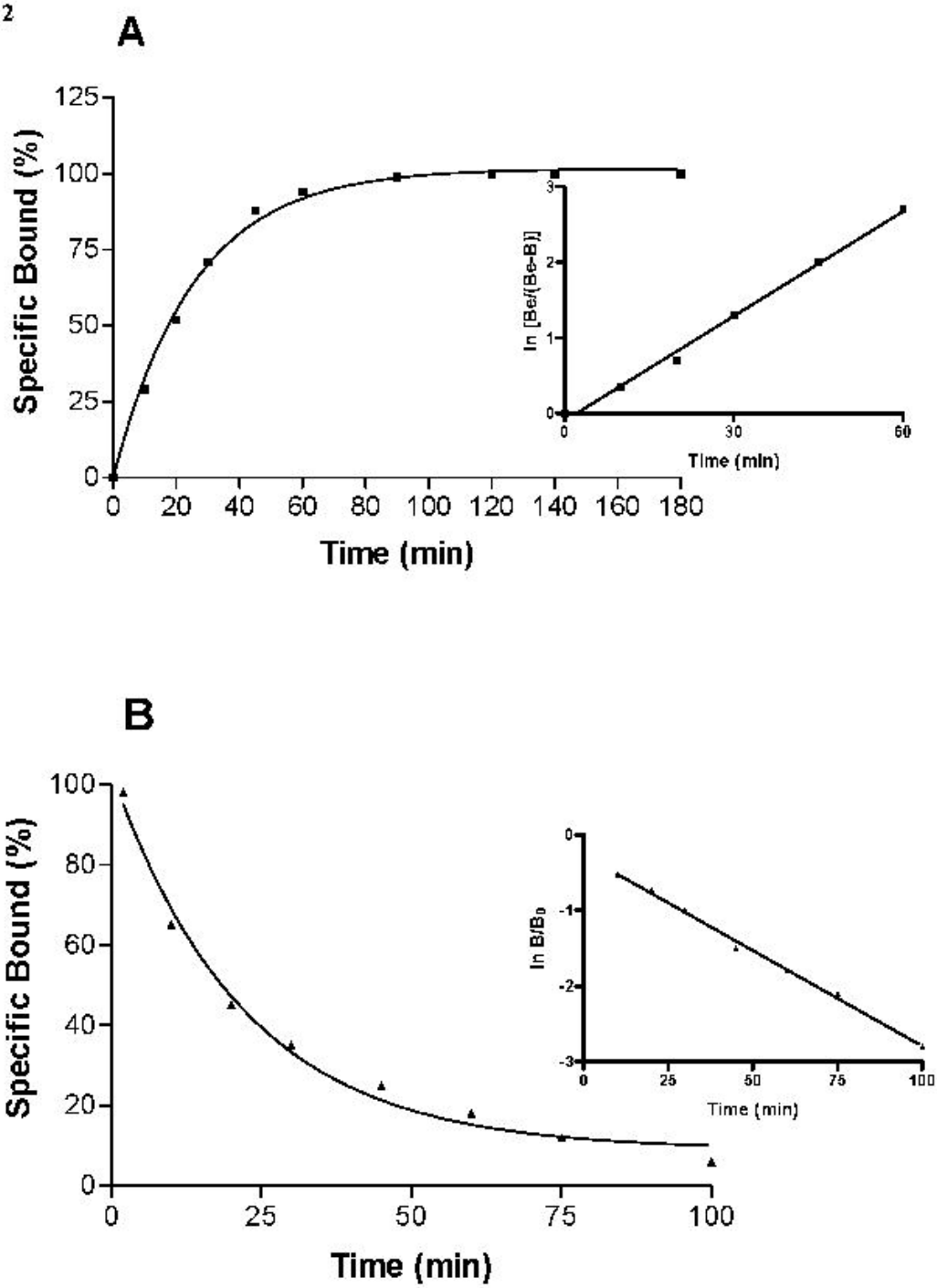

A
Specific Bound (%)
125
100
75
50
25
0
0 20 40 60 80 100 120 140 160 180
Time (min)
ln [Be/(Be-B)]
3
2
1
0
0 30 60
Time (min)
B
Specific Bound (%)
100
80
60
40
20
0
0 25 50 75 100
Time (min)
ln B/B0
0
-1
-2
-3
0 25 50 75 100
Time (min)

Figure 3

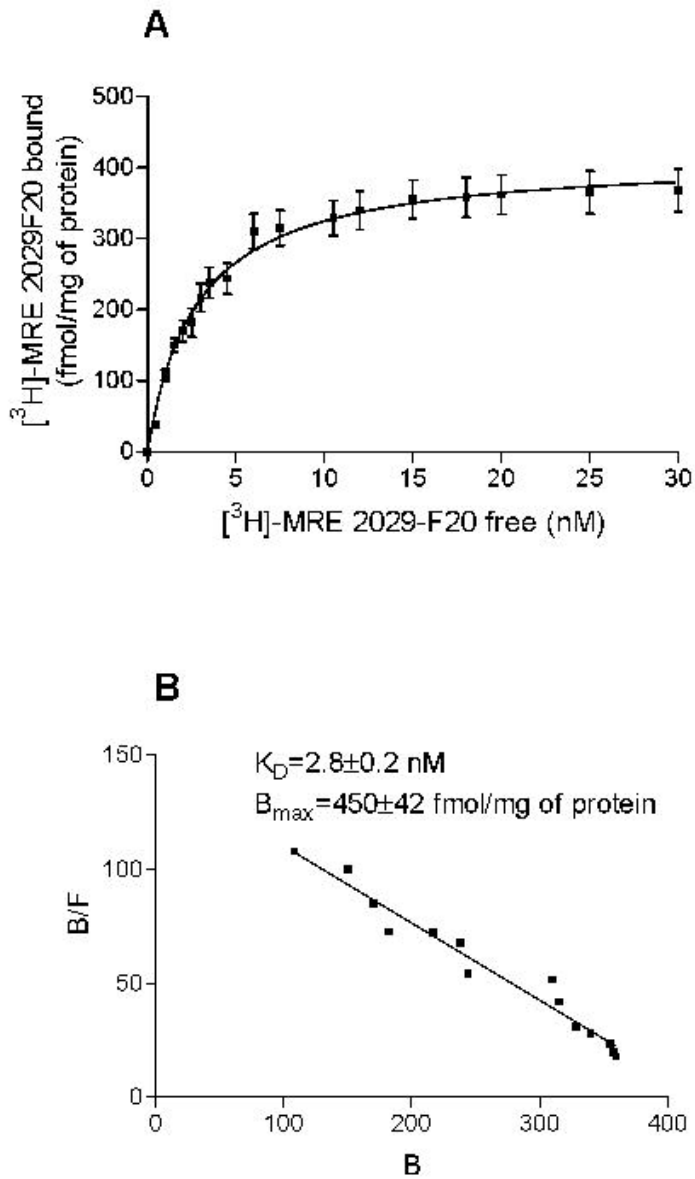

A
[3H]-MRE 2029F20 bound (fmol/mg of protein)
500
400
300
200
100
0
0
5
10
15
20
25
30
[3H]-MRE 2029-F20 free (nM)
B
KD=2.8±0.2 nM
Bmax=450±42 fmol/mg of protein
B/F
150
100
50
0
0
100
200
300
400
B

Figure 4

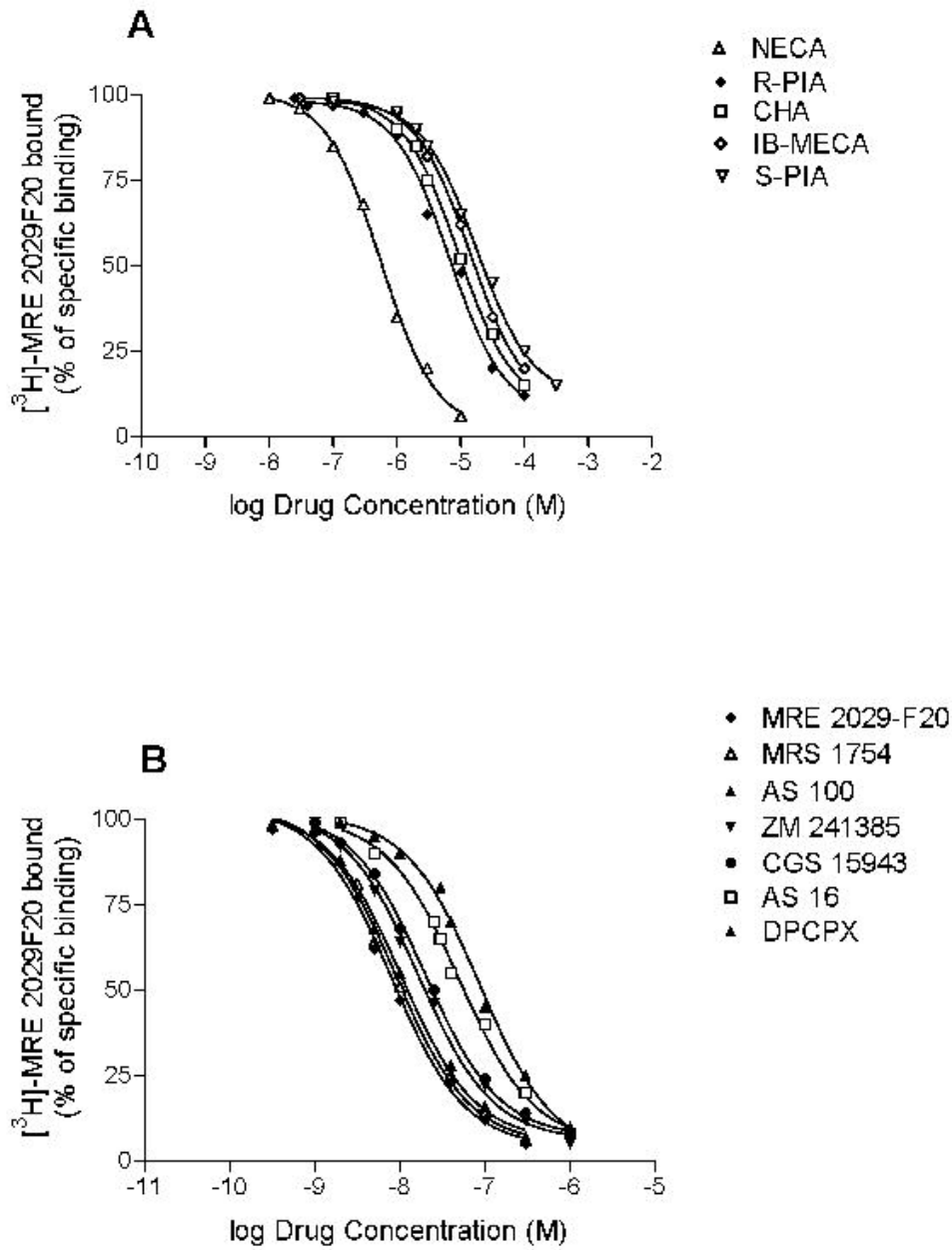

A
[3H]-MRE 2029F20 bound (% of specific binding)
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
log Drug Concentration (M)
NECA
R-PIA
CHA
IB-MECA
S-PIA
B
[3H]-MRE 2029F20 bound (% of specific binding)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log Drug Concentration (M)
MRE 2029-F20
MRS 1754
AS 100
ZM 241385
CGS 15943
AS 16
DPCPX

Figure 5

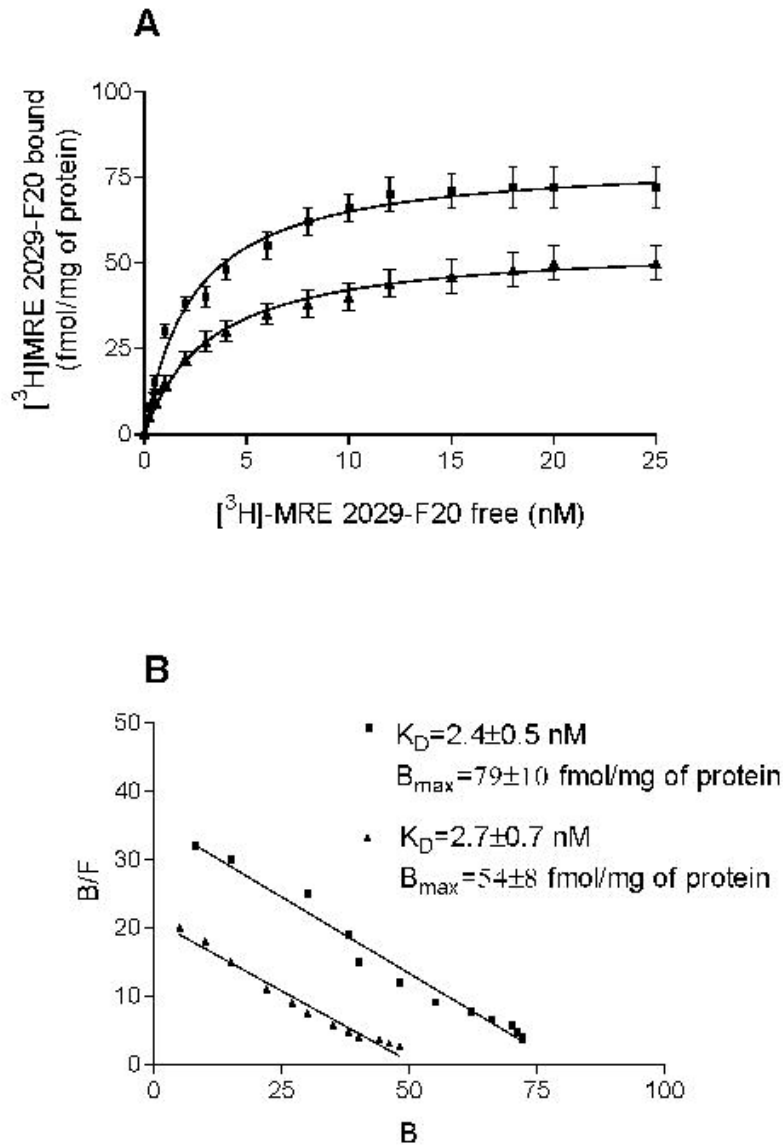

A
[3H]MRE 2029-F20 bound (fmol/mg of protein)
100
75
50
25
0
0
5
10
15
20
25
[3H]-MRE 2029-F20 free (nM)
B
B/F
50
40
30
20
10
0
0
25
50
75
100
B
KD=2.4±0.5 nM
Bmax=79±10 fmol/mg of protein
KD=2.7±0.7 nM
Bmax=54±8 fmol/mg of protein

Figure 6

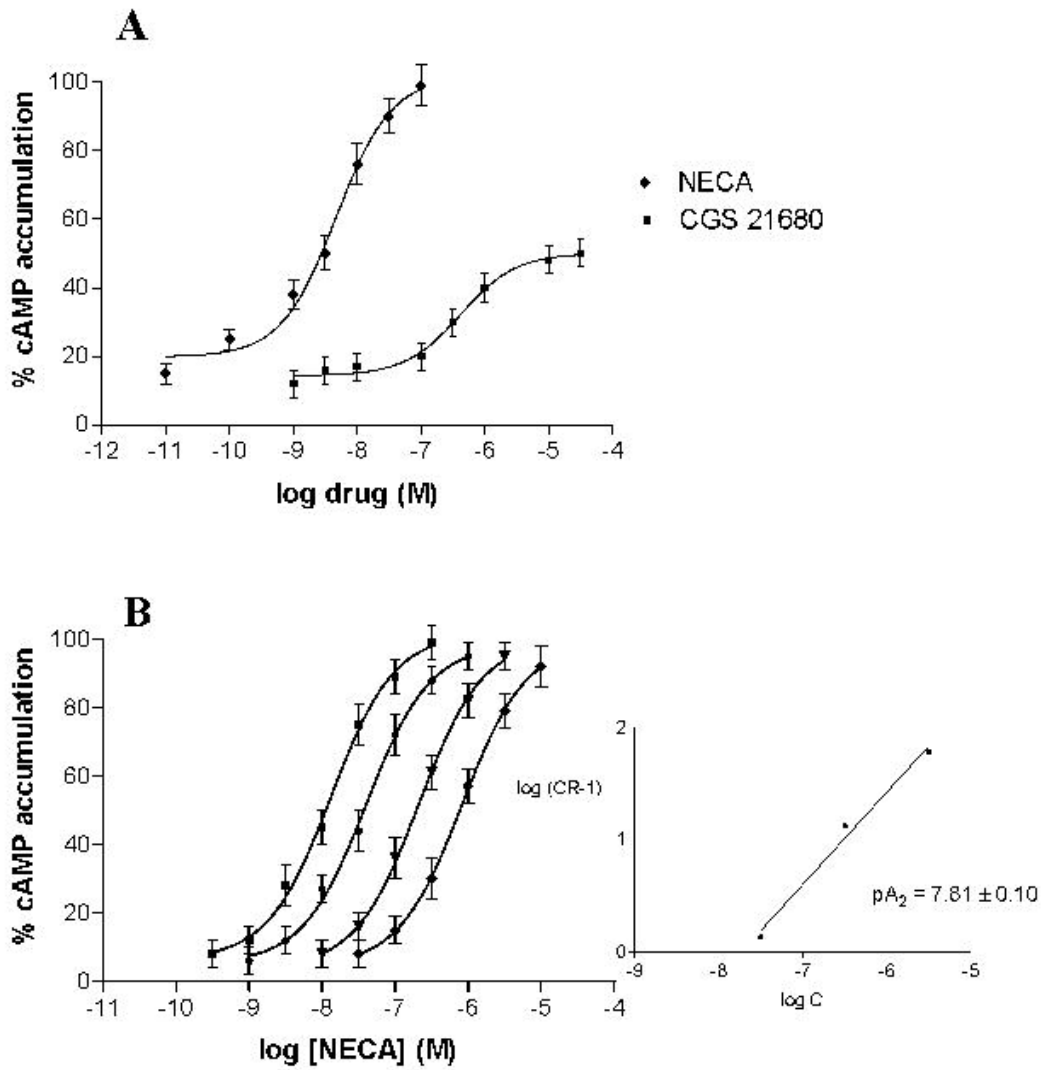

A
% cAMP accumulation
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
log drug (M)
NECA
CGS 21680
B
% cAMP accumulation
log [NECA] (M)
log (CR-1)
2
1
0
-9
-8
-7
-6
-5
log C
pA2 = 7.81 ±0.10

Figure 7

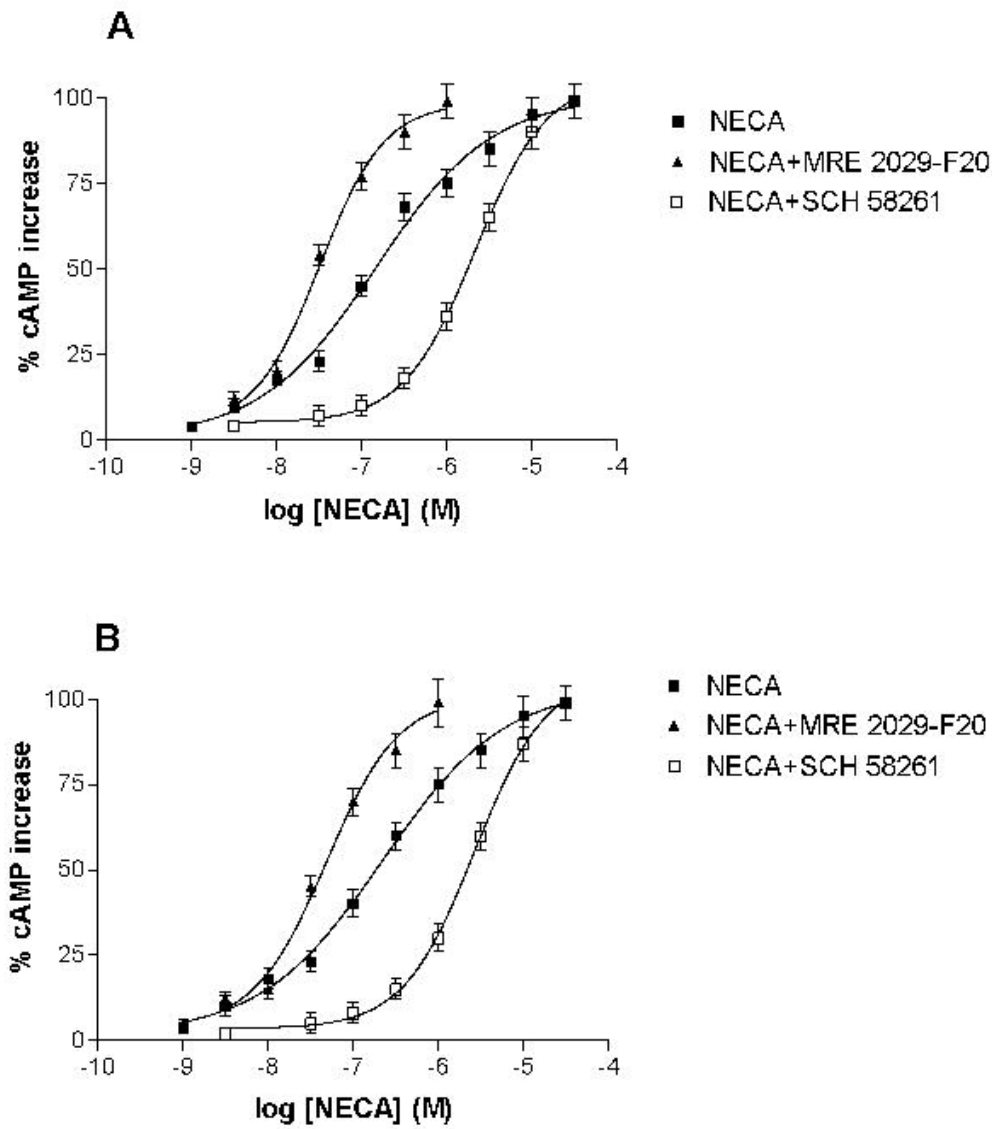

A
100
75
50
25
0
% cAMP increase
-10
-9
-8
-7
-6
-5
-4
log [NECA] (M)
NECA
NECA+MRE 2029-F20
NECA+SCH 58261
B
100
75
50
25
0
% cAMP increase
-10
-9
-8
-7
-6
-5
-4
log [NECA] (M)
NECA
NECA+MRE 2029-F20
NECA+SCH 58261

Figure 8

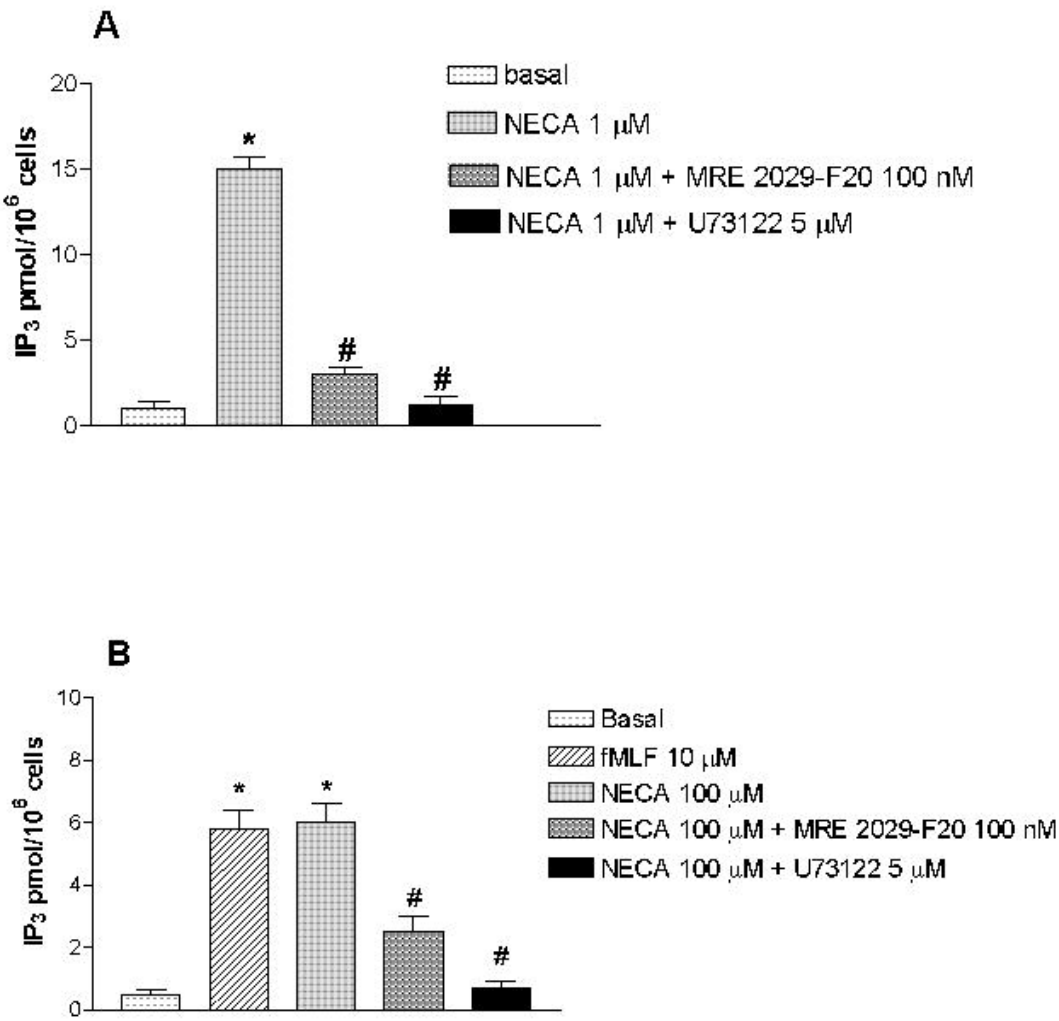

A
IP3 pmol/10^6 cells
20
15
10
5
0
*
#
#
basal
NECA 1 μM
NECA 1 μM + MRE 2029-F20 100 nM
NECA 1 μM + U73122 5 μM
B
IP3 pmol/10^6 cells
10
8
6
4
2
0
*
*
#
#
Basal
fMLF 10 μM
NECA 100 μM
NECA 100 μM + MRE 2029-F20 100 nM
NECA 100 μM + U73122 5 μM

Figure 9

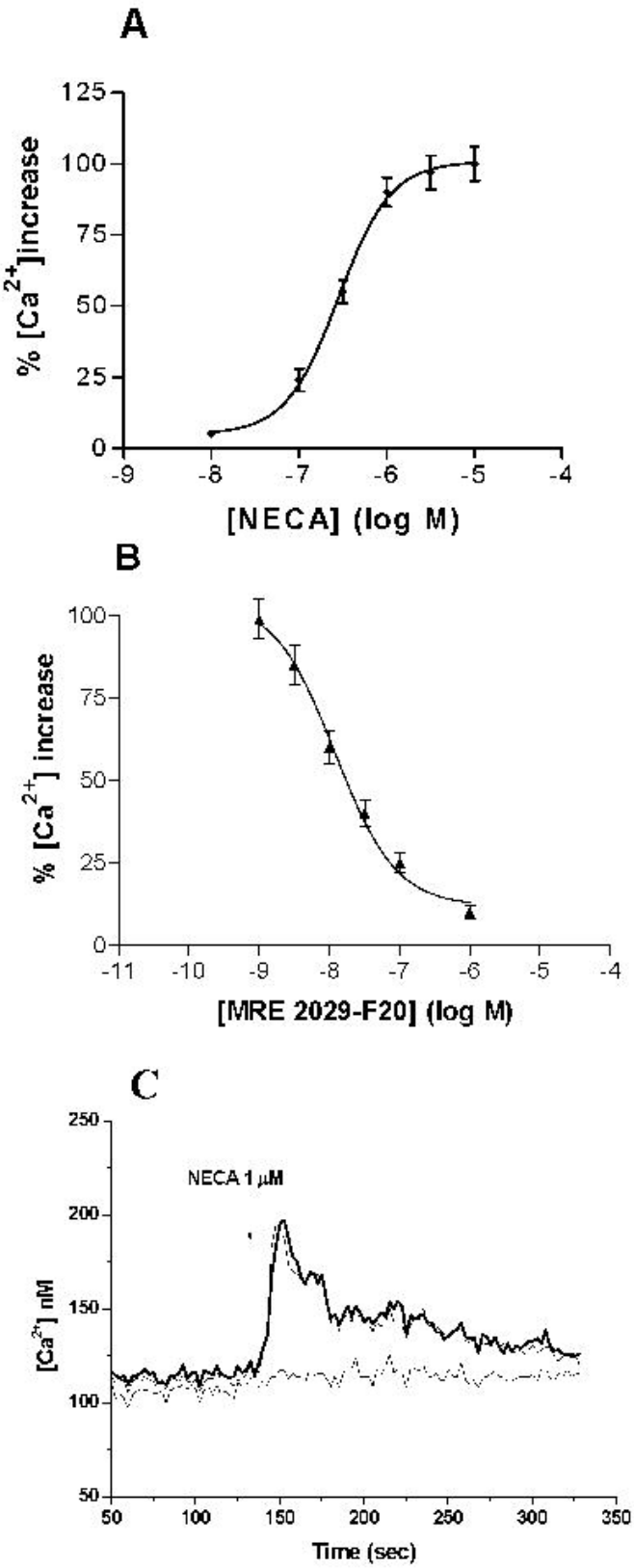
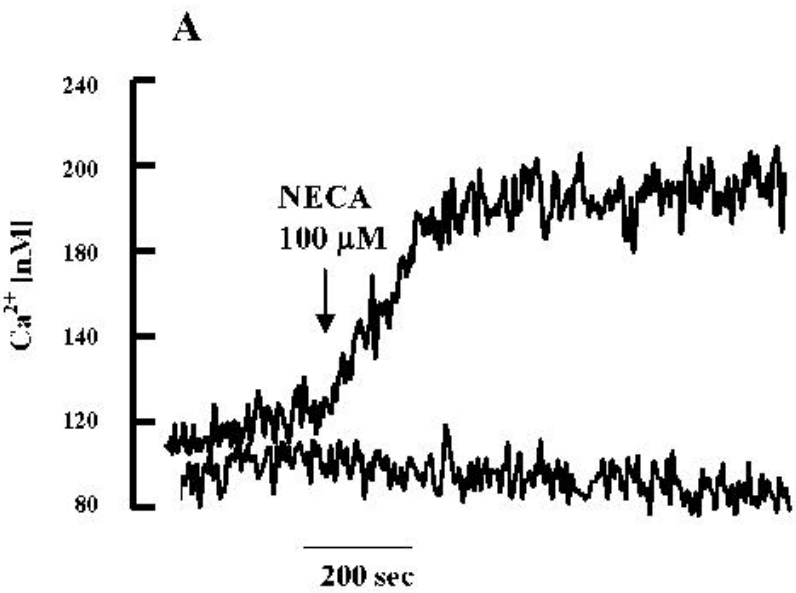
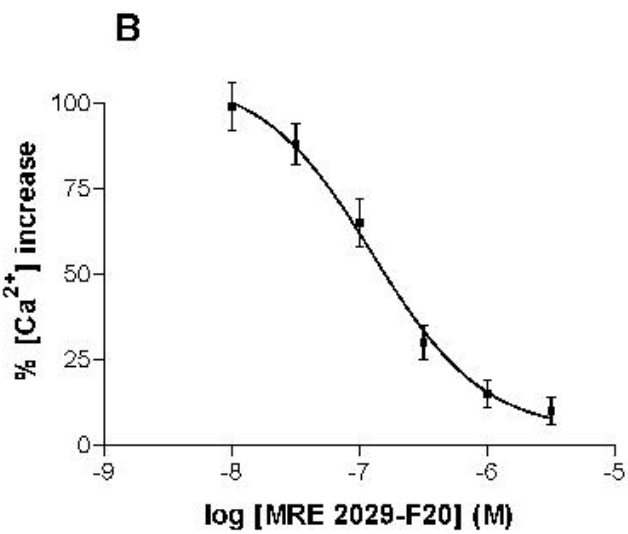

A
% [Ca2+]increase
125
100
75
50
25
0
-9
-8
-7
-6
-5
-4
[NECA] (log M)
B
% [Ca2+] increase
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
-4
[MRE 2029-F20] (log M)
C
NECA 1 μM
[Ca2+] nM
250
200
150
100
50
50
100
150
200
250
300
350
Time (sec)

Figure 10


A
240
200
180
140
120
80
Ca^{2+} [nM]
NECA
100 μM
200 sec


B
100
75
50
25
0
% [Ca^{2+}] increase
-9
-8
-7
-6
-5
log [MRE 2029-F20] (M)