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Pharmacological Analysis of Calcium Responses Mediated by the Human A3 Adenosine Receptor in Monocyte-Derived Dendritic Cells and Recombinant Cells

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ABSTRACT

Extensive characterization of adenosine receptors expressed by human monocyte-derived dendritic cells (MDDCs) was performed with quantitative polymerase chain reaction, radioligand binding, and calcium signaling. Transcript for the A3 adenosine receptor was elevated more than 100-fold in immature MDDCs compared with monocyte precursors. A3 receptor transcript was substantially diminished, and A2A receptor transcript increased, by lipopolysaccharide maturation of MDDCs. Saturation binding of N^6-(3-[^{125}I]iodo-4-aminobenzyl)-adenosine-5′-*N*-methyluronamide ([^{125}I]AB-MECA) to membranes from immature MDDCs yielded B_{max} of 298 fmol/mg of protein and K_D of 0.7 nM. Competition against [^{125}I]AB-MECA binding confirmed the site to be the A3 receptor. Adenosine elicited pertussis toxin-sensitive calcium responses with EC_{50} values ranging as low as 2 nM. The order of potency for related agonists was N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide (IB-MECA) ≥ I-AB-MECA > 2Cl-IB-MECA ≥ adenosine > 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxyamidoadenosine (CGS21680). The order of efficacy was adenosine ≥ CGS21680 > IB-MECA ≥ I-AB-MECA > 2Cl-IB-MECA. Calcium responses to 2Cl-IB-MECA and CGS21680, and the lower range of adenosine concentrations, were completely blocked by 10 nM *N*-(2-methoxyphenyl)-*N*-[2-(3-pyridyl)quinazolin-4-yl]urea (VUF5574) but not by 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261) or 8-cyclopentyl-1,3-dipropylxanthine. Pretreatment with 100 nM 2Cl-IB-MECA eliminated responses to CGS21680 but not to monocyte inhibitory protein-1α. For comparison, dose-response functions were obtained from double-recombinant human embryonic kidney 293 cells expressing the human A3 receptor and a chimeric Gαq-i3 protein, which was required to establish A3-mediated calcium signaling. The pharmacological profile of calcium signaling elicited by adenosine-related agonists in the double-recombinant cells was essentially identical to that obtained from immature MDDCs. Our results provide an extensive analysis of A3-mediated calcium signaling and unequivocally identify immature MDDCs as native expressers of the human A3 receptor.

Most of the physiological effects of extracellular adenosine are known to be mediated by at least four G protein-coupled receptors, the A1, A2A, A2B, and A3 receptors (Fredholm et al., 2001a). Adenosine has long been implicated in regulation of the immune system, and a considerable body of evidence has accumulated indicating that the A3 receptor may play an important role in modulation of several leukocytic functions. For example, activation of the A3 receptor with selective agonists has been shown to substantially diminish circulating levels of proinflammatory cytokines in rodent in vivo models of inflammation (Hasko et al., 1996, 1998; Salvatore et al., 2000). Similar anti-inflammatory effects have been attributed to the A3 receptor in human leukocytes and leukocytic cell lines. Selective A3 receptor agonists were found to inhibit release of pro-inflammatory cytokines from monocytic cells (Sajjadi et al., 1996), inhibit *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine-triggered oxidase activity in monocytes (Broussas et al., 1999), inhibit degranulation of eosinophils (Ezeamuzie and Phillips, 1999) and neutrophils (Bouma et al., 1997), and induce apoptosis in peripheral

ABBREVIATIONS: MDDC, monocyte-derived dendritic cells; PCR, polymerase chain reaction; FLIPR, fluorometric imaging plate reader; HEK, human embryonic kidney; CGS21680, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxyamido-adenosine; 2Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide; IB-MECA, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol; MRS1220, 9-chloro-2-(2-furyl)-5-phenylacetylamino[1,2,4]triazolol[1,5-c]quinazoline; VUF5574, *N*-(2-methoxyphenyl)-*N*-[2-(3-pyridyl)quinazolin-4-yl]urea; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; I-AB-MECA, N^6-(3-iodo-4-aminobenzyl)-adenosine-5′-*N*-methyluronamide; AB-MECA, N^6-(4-aminobenzyl)-adenosine-5′-*N*-methyluronamide; MIP, monocyte inhibitory protein; FBS, fetal bovine serum; LPS, lipopolysaccharide; RT, reverse transcription.

blood mononuclear cells (Barbieri et al., 1998). However, the concentrations of agonist used to obtain these effects were well beyond the range of selectivity for the A3 receptor. It has been argued that many of the anti-inflammatory effects of A3 receptor agonists observed in human leukocytes actually are the result of A2A receptor activation (Sullivan and Linden, 1998). Thus, unequivocal association of the human A3 receptor with a specific immunological response has yet to be established.

Defining a clear role for the A3 receptor in the human immune system will largely depend on identifying the leukocytes that express functional A3 receptors, a task that is best accomplished through rigorous pharmacological methods. Recently (Panther et al., 2001), adenosine was reported to elicit pertussis toxin-sensitive calcium signals, chemotaxis, and actin polymerization in human immature MDDCs. Because of the presence of transcript for all four known adenosine receptors and limited pharmacological characterization, the identity of the receptor(s) mediating these responses in MDDCs was not clearly established. We have performed an in-depth investigation by quantitative PCR, radioligand binding, and extensive pharmacological characterization of calcium signaling to determine whether MDDCs express functional A3 receptors. A broad panel of selective adenosine-related agonists and antagonists were tested across concentration ranges using FLIPR, a high-throughput calcium imaging system. The resulting pharmacological profile obtained in MDDCs was then compared with that obtained from a double-recombinant HEK293 cell line engineered to stably express a chimeric G protein that couples the human A3 receptor to calcium signaling. Our results demonstrate strong expression of functional A3 receptors by immature MDDCs. We further show that adenosine is considerably more potent than previously appreciated and that the A2A-selective agonist CGS21680 is a potent full agonist at the human A3 receptor. Finally, we report that the highly selective A3 receptor agonist 2Cl-IB-MECA is a low efficacy partial agonist of A3-mediated calcium signaling. Our approach has unequivocally identified immature MDDCs as one of the few primary cell types that natively express functional A3 receptors and also has yielded important information concerning the pharmacology of the human A3 adenosine receptor.

Materials and Methods

Materials. 2Cl-IB-MECA, IB-MECA, ZM241385, MRS1220, and VUF5574 were purchased from Tocris Cookson Inc. (Ballwin, MO). SCH58261 was obtained from Schering-Plough Corporation (Kenilworth, NJ). I-AB-MECA, CGS21680, 5′-*N*-ethylcarboxamidoadenosine, 2-chloro-N^6-cyclopentyladenosine, 8-cyclopentyl-1,3-dipropylxanthine, adenosine, and pertussis toxin were purchased from Sigma (St. Louis, MO). [^{125}I]AB-MECA (2000 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). MIP-1α and MIP-3β were purchased from R & D Systems (Minneapolis, MN). Dulbecco's phosphate-buffered saline, Hank's balanced saline solution, fetal bovine serum (FBS), and all other cell culture medium and components, unless otherwise noted, were obtained from Invitrogen (Carlsbad, CA).

Cell Culture

MDDCs. Essentially, the method described by Bleharski et al. (2001) for generating immature MDDCs of >95% purity was followed. Human blood was obtained from donors or as buffy coat from a blood bank. EDTA (1 mM) and Rosette Sep (Stem Cell Technologies, Vancouver, BC, Canada) antibody cocktail (50 μl/ml blood) were added to the blood and incubated at room temperature for 10 min. Blood was then diluted with an equal volume of buffer A (Dulbecco's phosphate-buffered saline with 2% FBS, 1 mM EDTA) and then layered over Ficoll (Amersham Biosciences) and centrifuged at 800*g* for 25 min. The resulting monocyte fractions were collected and washed twice with buffer A then resuspended in ammonium chloride/potassium bicarbonate/EDTA buffer (Biosource International, Camarillo, CA and incubated at room temperature for 10 min. The monocyte suspension was then centrifuged and the resulting pellet resuspended in growth medium (RPMI 1640 medium with 10% FBS, penicillin/streptomycin, glutamine, nonessential amino acids, and 2-mercaptoethanol) to a density of 7.5×10^5 cells/ml. The monocyte suspension was then decanted into a culture flask and placed in a humidified incubator (5% CO_2) for 2 h at 37°. After the incubation period, the flask was gently shaken to remove nonadherent cells and the medium was aspirated, then replaced with fresh medium supplemented with 200 ng/ml granulocyte-macrophage colony stimulating factor and 10 ng/ml interleukin-4. Cells remained undisturbed in the incubator until harvested at day 6 of incubation. Immature dendritic cells were differentiated to a mature phenotype by inclusion of LPS (10 ng/ml) in the culture medium for 48 h. Dendritic cell phenotypes were confirmed by fluorescence-activated cell sorting analysis of surface markers CD1a, CD11c, CD14, CD83, CD86, and CD123 and by calcium responses to MIP-1α and MIP-3β. The percentages of immature MDDCs expressing CD1a, CD11c, CD14, CD83, CD86, and CD123 were, respectively, 75, 99, 3, 3, 22, and 67 and for LPS-matured cells, respectively, 37, 48, 10, 87, 91, and 75. Immature MDDCs responded with calcium signals to MIP-1α, but not to MIP-3β; the converse was true for LPS-matured cells.

Recombinant HEK293 Cells. HEK293EBNA cells were obtained from Invitrogen and maintained in DMEM containing 10% FBS. Human A3 adenosine receptor DNA (GenBank accession no. L22607) was cloned into the vector pCEP4 (Invitrogen) and transfected into HEK293EBNA cells by electroporation. Stably transfected human A3 adenosine receptor-expressing cells (hA3/293) were selected by inclusion of 100 μg/ml hygromycin in the culture medium. A chimeric Gα protein (Gαq-i3) was constructed by replacing the five C-terminal residues of human Gαq with the five amino acid residues of human Gαi3 (Conklin et al., 1993) and cloned into the mammalian expression vector pCR3.1 (Invitrogen). The Gαq-i3 construct was transfected into HEK293EBNA and hA3/293 cells by electroporation and stable transfectants (Gαq-i3/293 and hA3/Gαq-i3/293, respectively) were selected by addition of puromycin (1 μg/ml) to the culture medium.

Quantitative PCR

cDNAs were prepared from DNase-treated total RNA isolated from human monocytes, immature MDDCs, and mature MDDCs. cDNA (50 ng; 10 ng/μl) was mixed with 15 μl of 2× Syber Green Mix (Applied Biosystems, Union City, CA) and 10 μl of buffer containing 1 μM concentrations of each of the following human adenosine receptor-specific primer sets: A3, forward primer, 5′- TACATCATTCGGAACAAACTC-3′; reverse primer, 5′-GTCTTGAACTCCCGTCCATAA-3′; A1, forward primer, 5′-TGCGAGTTCGAGAAGGTCATC-3′; reverse primer, 5′-GAGCTGCTTGCGGATTAGGTA-3′; A2A, forward primer, 5′-CGAGGGCTAAGGGCATCATTG-3′; reverse primer, 5′-CTCCTTTGGCTGACCGCAGTT-3′; A2B, forward primer, 5′-CTCTTCCTCGCCTGCTTCGTG-3′; reverse primer, 5′-TTATACCTGAGCGGGACACAG-3′. Adenosine receptor expression was analyzed using real-time quantitative PCR with a 96-well GeneAmp PCR system 5700 (Applied Biosystems). As an internal control for quantification of the total amount of cDNA, a separate set of identical cDNAs was analyzed for the expression of ubiquitin.

Radioligand Binding

A crude membrane preparation was made from cell pellets of immature MDDCs collected from eight donors. Membranes were suspended in buffer B (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 2 U/ml adenosine deaminase) at a concentration of 0.25 mg/ml, then 50 μl of the membrane suspension was placed in each well of a 96-well plate. For saturation binding, a range of [^{125}I]AB-MECA was added to each well in a volume of 50 μl for a final total volume of 100 μl. MRS 1220 (500 nM) was used to determine nonspecific binding. After incubation for 2 h at room temperature, cell membranes were retained and washed on a 96-well filter plate (Unifilter GF/B; PerkinElmer Life Sciences, Boston, MA). Scintillant (20 μl) was added to each well and radioactivity was counted by a scintillation counter (TopCount; PerkinElmer Life Sciences). Similar conditions were used for competition experiments, except that 0.5 nM [^{125}I]AB-MECA was used to label 5 μg of cell membranes in 100 μl of buffer B in the absence or presence of increasing concentrations of nonradioactive ligand. Nonspecific binding accounted for approximately 10% of total [^{125}I]AB-MECA binding in the competition experiments. Data points were fitted by nonlinear regression for saturation isotherms or one-site competitions (Prism 3.02; GraphPad Software, San Diego, CA.

Calcium Signaling

MDDCs and recombinant HEK293 cells were incubated in 11 ml (1×10^6 cells/ml) of their respective culture media containing 2.2 μM Fluo-3AM and 0.022% pluronic acid (Molecular Probes, Eugene, OR) at 37°, 5% CO_2 for 30 to 45 min, mixing briefly after 20 min. After the dye-loading incubation, cells were washed twice by centrifugation and subsequent suspension in buffer C (Hanks balanced saline solution, 25 mM HEPES, 0.1% FBS). The final washed cell pellet was suspended in buffer C at a density of 6×10^5 cell/ml. Cell suspension (40 μl; ~25,000 cells) was added to each well of a 384-well polylysine–coated clear bottom plate (BD Biosciences, Bedford, MA). Plates were then centrifuged at room temperature at approximately 400*g* for 1 min and then left undisturbed for 15 min before being placed into the stage of a FLIPR384 (Molecular Devices Corporation, Sunnyvale, CA). Agonists were added in 20-μl volumes at $t = 0$. Fluorescence measurements (488-nm excitation and 510–570-nm bandpass emission) were recorded once per second for the first 60 s, then every 6 s for the next 120 s. Antagonists were added in 20-μl volumes 90 s before the addition of the agonists. Data were calculated as maximum fluorescence − minimum fluorescence. Dose-response curves were fitted to the data points by nonlinear regression.

Results

Expression of Adenosine Receptors in MDDCs. MDDCs have previously been reported to express adenosine receptors as indicated by RT-PCR (Panther et al., 2001). To better assess the relative expression of the different adenosine receptor subtypes, quantitative measurement of transcript by real-time PCR was performed on monocytes, and immature and mature MDDCs (Fig. 1). Relatively low levels of transcript were detected for A1, A2A, and A3 adenosine receptors in monocytes. Transcript for the A3 receptor was more than 100-fold elevated in immature MDDCs compared with monocytes. In sharp contrast, levels of A3 receptor transcript were greatly diminished and A2A receptor message predominated in LPS-matured MDDCs.

[^{125}I]AB-MECA Binding to Membranes from Immature MDDC. Cell pellets of immature MDDCs were pooled from multiple donors and used to make crude membranes. A single high-affinity binding site for the A3/A1-selective radioligand [^{125}I]AB-MECA was detected in the membranes (Fig. 2). Saturation isotherms yielded a K_D of 0.7 nM (95% confidence interval, 0.6–0.9 nM), consistent with the published affinity of [^{125}I]AB-MECA for the human A3 receptor (Varani et al., 1998, 2000), and B_{max} of 298 fmol/mg of membrane protein. The binding site was further characterized by competition of a panel of selective ligands against [^{125}I]AB-MECA binding. Affinity estimates for the competing ligands (Table 1) indicate that the [^{125}I]AB-MECA binding site on immature MDDCs is the A3 receptor (Varani et al., 1998; Baraldi and Borea, 2000; Klotz, 2000).

Calcium Responses in MDDCs. Several selective and nonselective adenosine receptor agonists were tested for their ability to elicit calcium responses from immature MDDCs (Fig. 3). Adenosine elicited robust calcium responses and

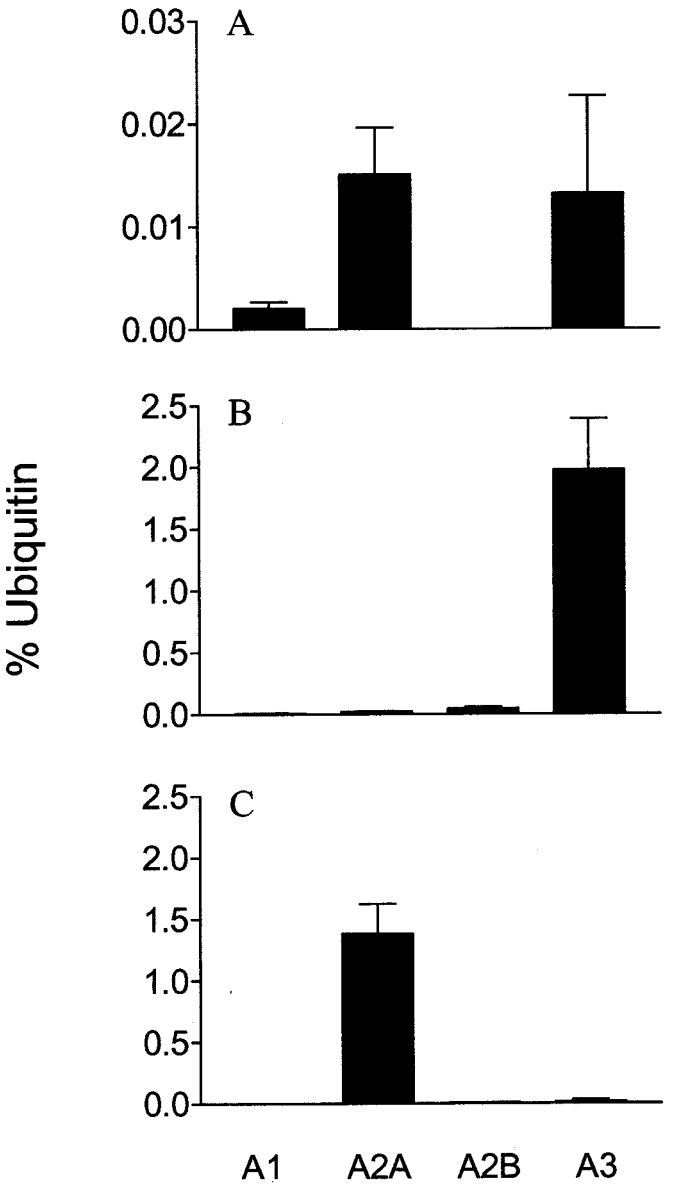

Fig. 1. Expression of adenosine receptor transcript in human MDDCs. Relative amount of transcript from monocytes (note difference in scale) (A), immature MDDCs (B), and LPS-matured MDDCs (C). Monocytes were obtained from individual donors and differentiated to immature MDDCs and LPS-activated MDDCs as described under *Materials and Methods*. cDNAs were obtained from total RNA and used for quantitative PCR. Data are expressed as percent ubiquitin transcript and are the average ± S.E.M. ($n = 6$ donors).

was unexpectedly potent, with EC_{50} values ranging as low as 2 nM among donors (Table 2). The A2A-selective agonist CGS21680 was, depending on the donor, either equally effective or slightly less effective than adenosine, with EC_{50} values ranging between 23 and 175 nM. The A3-selective agonists IB-MECA and 2Cl-IB-MECA were potent agonists. However, the maximal responses to these agonists were at most 50% that of adenosine (Fig. 3, Table 2). The A1-selective agonist 2-chloro-N^6-cyclopentyladenosine was without effect at concentrations below 300 nM. Response to all adenosine-related agonists was abolished by a 4-h pretreatment with 100 ng/ml pertussis toxin (not shown.) Furthermore, A3-mediated calcium signaling was lost upon LPS-induced maturation of MDDCs (not shown).

Effects of Selective Receptor Antagonists on Calcium Responses to Adenosine and Adenosine Analogs. The pharmacological profiles of the agonists used to stimulate calcium responses suggested the involvement of multiple receptors. To determine which receptors were involved in the adenosine-mediated calcium flux, dose-response curves were obtained in the presence of several concentrations of antagonists selective for the various adenosine receptors. It has now been well documented that high-affinity competitive antagonists tend to depress the dose-response functions obtained from calcium flux assays; therefore, traditional null methods for characterizing receptors cannot be applied (Kukkonen et al., 1998; Christopoulos et al., 1999; Lew et al., 2000). Nevertheless, use of multiple fixed concentrations of selective antagonists against dose-response curves still can provide a clear picture of the receptors involved in the calcium response.

The potent A2A-selective antagonists SCH58261 acted as a weak antagonist of both the adenosine- and CGS21680-elicited calcium responses of MDDCs (Fig. 4, A and B). Appreciable shifting of the dose-response curves was not observed until antagonist concentrations of 0.3 to 3 μM were reached. Similar results (not shown) were obtained with another A2A-selective antagonist, ZM241385. Neither SCH58261 (Fig. 4C) nor ZM241385 (not shown) had any effect on 2Cl-IB-MECA dose-response curves. In contrast, low concentrations of the A3-selective antagonist VUF5574 potently shifted the adenosine dose-response curves (Fig. 4, D–F). Consistently among donors, the A3-selective antagonists blocked the lower range of the adenosine dose-response curve but were ineffective at higher adenosine concentrations (Fig. 4D), suggesting the presence of another Ca^{2+}-linked adenosine receptor. However, responses to 2Cl-IB-MECA and, surprisingly, CGS21680 were completely eliminated by low concentrations of VUF5574. Essentially identical results were obtained with MRS1220 (not shown). The high-affinity A1-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (100 nM) had no effect on responses to any of the agonists (not shown).

Desensitization with 2Cl-IB-MECA. The human A3 receptor is a substrate for G protein receptor kinases and readily desensitizes as a result of prolonged agonist occupancy (Ferguson et al., 2000; Trincavelli et al., 2000). Pretreatment with 2Cl-IB-MECA should therefore selectively diminish or eliminate subsequent activity for all agonists

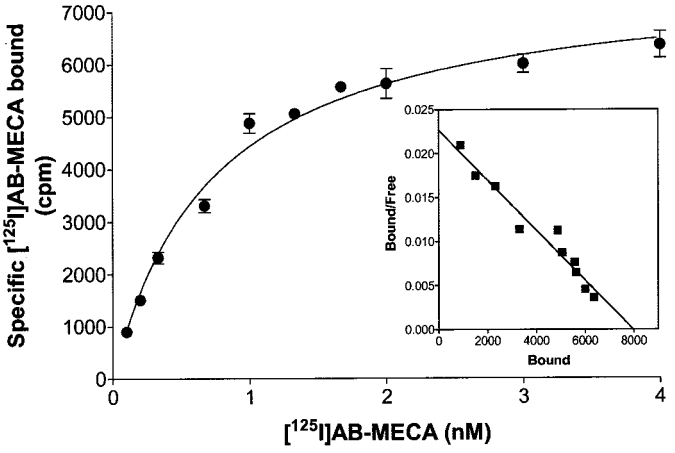

Fig. 2. Saturation of [^{125}I]AB-MECA binding to membranes from human MDDCs. Frozen pellets of MDDCs from 8 donors were thawed and combined to make a crude membrane preparation as described under *Materials and Methods*. Isotherm was fitted, and K_D and B_{max} were derived by nonlinear regression. Data points are mean of triplicate values ± S.E.M. MRS1220 (500 nM) was used to define nonspecific binding. Inset, Scatchard transformation of saturation data. Results representative of two essentially identical experiments are shown.

TABLE 1
Affinities of adenosine receptor ligands determined by competition against 0.5 nM [^{125}I]AB-MECA binding to MDDC membranes.

Ligand	K_i	95% Confidence Interval
	nM	
2Cl-IB-MECA	1.5	1.1–2.0
IB-MECA	1.1	0.9–1.5
VUF5574	0.5	0.2–1.5
MRS1220	1.0	0.5–1.7
CGS21680	52	24–113
DPCPX	251	122–516
ZM241385	>10,000	
SCH58261	>10,000	

DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

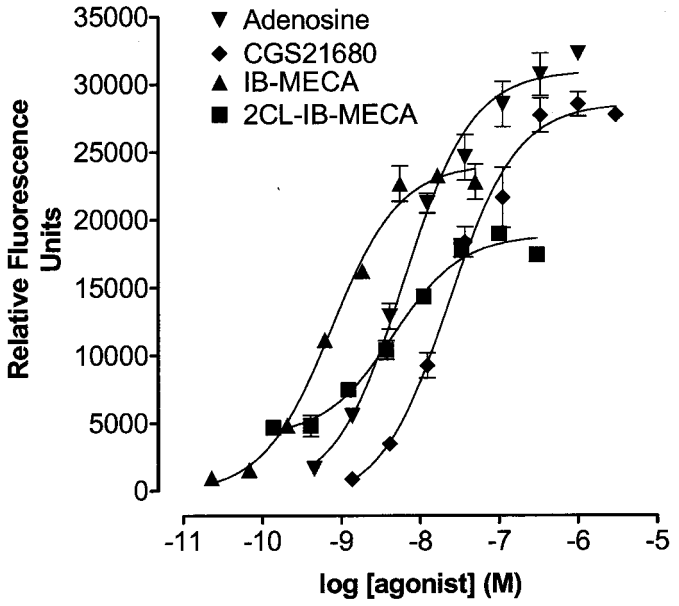

Fig. 3. Calcium responses to adenosine-related agonists in human immature MDDCs. Responses to multiple concentrations of adenosine, CGS21680, IB-MECA, and 2Cl-IB-MECA were obtained by FLIPR as described under *Materials and Methods*. Concentration-effect functions and EC_{50} values for each agonist were obtained by nonlinear regression. Data points are mean of triplicate values ± S.E.M. Data shown are from a single donor and are representative of five similar experiments.

acting through the A3 receptor. Accordingly, immature MDDCs were pretreated for 15 min with 100 nM 2Cl-IB-MECA and then full dose-response curves were obtained for adenosine, CGS21680, and IB-MECA. After the 2CL-IB-MECA pretreatment, calcium responses to lower concentrations of adenosine were eliminated; small responses still were observed at higher adenosine concentrations (Fig. 5A). Responding to CGS21680 (Fig. 5B) and IB-MECA (Fig. 5C) was completely eliminated by the pretreatment. In contrast, responding to MIP1α, an agonist of the CCR1 and CCR5 chemokine receptors, was unaffected by 2Cl-IB-MECA pretreatment (Fig. 5D).

Pharmacology of A3 Receptor-Mediated Calcium Responses in Recombinant Cells. The high potency of adenosine, the agonist activity of CGS21680, and the relatively low maximal responses to 2Cl-IB-MECA were unexpected in the context of what has been reported concerning A3 receptor pharmacology. However, no detailed account of the pharmacology of A3-mediated calcium signaling that could be used for comparison has yet appeared in the literature. We therefore carried out a similar analysis of calcium responses in HEK293 cells transfected with the human A3 receptor. No calcium responses to any of the adenosine-related agonists were observed in single-recombinant hA3/293 cells. The ad-

TABLE 2

Potency and maximal activity (relative to adenosine) of adenosine-related agonists that elicited calcium responses from human immature MDDCs (DC) and hA3/G αq-i3/293 recombinant cells (R)

MDDC values are means from at least five donors; range is given in parentheses. For recombinant cells, EC_{50} values (and in parentheses, 95% confidence interval) are representative of four independent experiments. Max values are means ± S.E.M. from four independent experiments.

Agonist	EC_{50}		Max	
	DC	R	DC	R
	nM		%	%
Adenosine	18 (2–59)	28 (21–36)	100	100
CGS21680	87 (21–175)	81 (61–106)	77 (24–103)	98 ± 10
IB-MECA	0.8 (0.6–3.0)	3 (2–5)	49 (32–67)	60 ± 5
I-AB-MECA	3 (1–7)	3 (2–5)	40 (32–56)	55 ± 6
2Cl-IB-MECA	7 (0.2–19)	15 (6–25)	25 (0–52)	33 ± 6

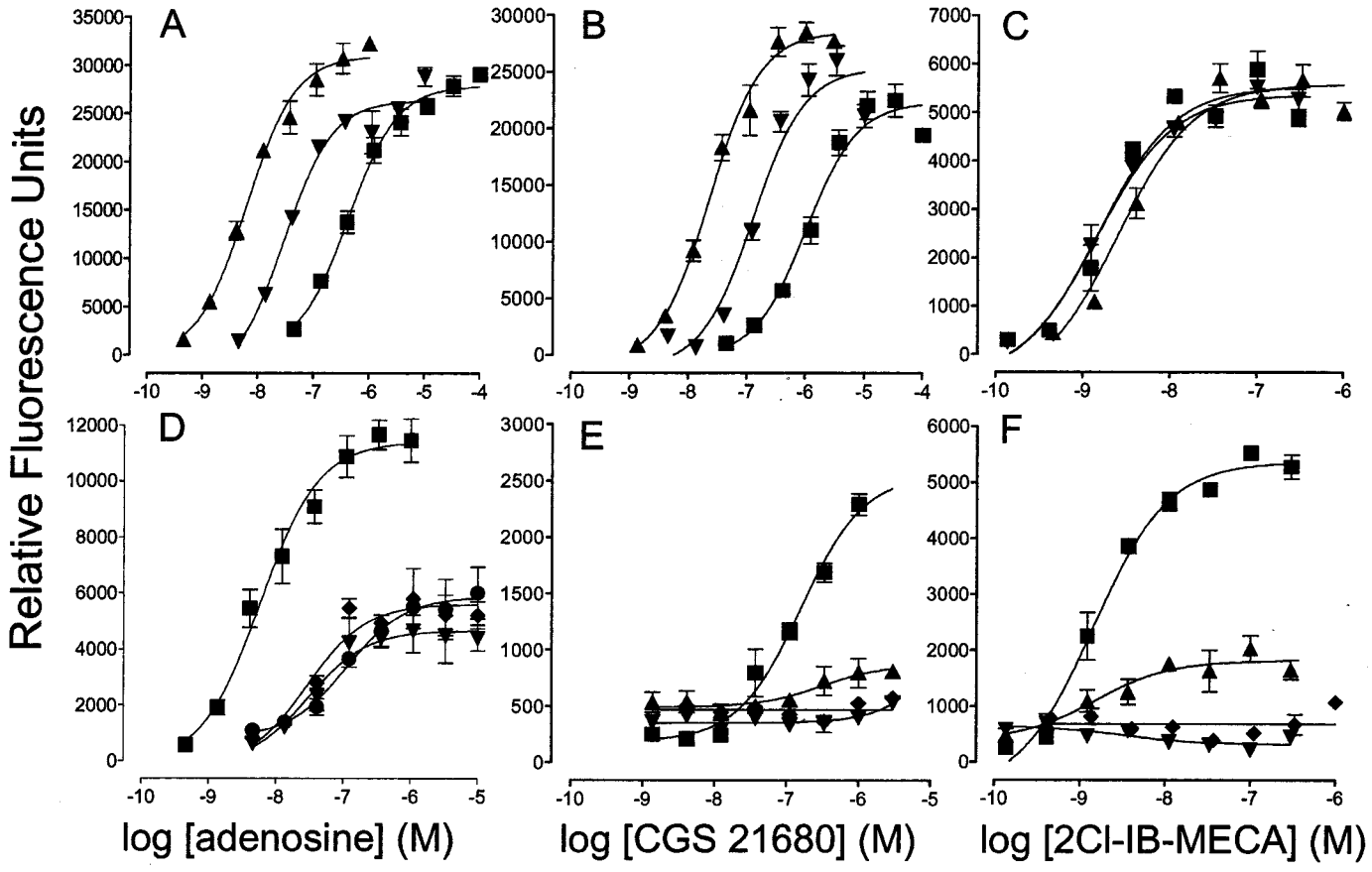

Fig. 4. Selective antagonism of calcium responses to adenosine-related agonists in human immature MDDCs. Effect of SCH 58261 (A–C) and VUF5574 (D--F) on calcium responses to adenosine (A and D), CGS21680 (B and E), and 2Cl-IB-MECA (C and F) in human immature MDDCs. Concentrations of SCH58261 are 0 (▲), 300 (▼), and 3000 (■) nM. Concentrations of VUF5574 are 0 (■), 10 (♦), 100 (▼), and 1000 (●) nM in D and 0 (■), 1 (▲), 10 (♦), and 100 (▼) nM in E and F. Concentration-effect functions were obtained by nonlinear regression. Data points are mean of triplicate values ± S.E.M. Data in each are from a single donor and are representative of at least three similar experiments. Ranges of relative fluorescence varied considerably across donors.

ditional transfection of a chimeric G protein, Gαq-i3, was required to couple the human A3 receptor to calcium signaling in HEK293 cells. All agonists were effective at eliciting calcium responses from double-recombinant hA3/Gαq-i3/293 cells (Fig. 6). Only adenosine, at concentrations of 3 μM and above, produced calcium signals in single-recombinant Gαq-i3/293 cells (not shown).

The pharmacology of the A3-mediated calcium responses in double-recombinant hA3/Gαq-i3/293 cells was strikingly similar to that of the immature MDDCs (Table 2). Adenosine was potent and maximally effective. CGS21680 produced responses that were comparable in magnitude with those of adenosine. IB-MECA, I-AB-MECA, and 2Cl-IB-MECA were all potent but produced submaximal responses compared with those elicited by adenosine or CGS21680. Similar to the results obtained with MDDCs, SCH 58261 had little effect, whereas low concentrations of VUF5574 strongly antagonized responses to adenosine-related agonists (Fig. 7, A–C).

2Cl-IB-MECA Is a Partial Agonist of A3-Mediated Calcium Signaling. The relatively low maximal effect of 2Cl-IB-MECA observed in both the recombinant cells and in MDDCs suggested that 2Cl-IB-MECA was a partial agonist. Low-efficacy partial agonists are capable of antagonizing the responses to higher efficacy full agonists (Kenakin, 1997b). We therefore tested the effects of 2Cl-IB-MECA simultaneously added with CGS21680 on the calcium responses of MDDCs. Increasing concentrations of 2Cl-IB-MECA progressively depressed the CGS21680 dose-response curve (Fig. 8A). In the presence of 100 nM 2Cl-IB-MECA, the maximal response to CGS21680 was no greater than that of 2Cl-IB-MECA alone. In contrast, the effects of 2Cl-IB-MECA where additive with MIP-1α, resulting in an upward shift in the MIP-1α dose-response curves (Fig. 8B).

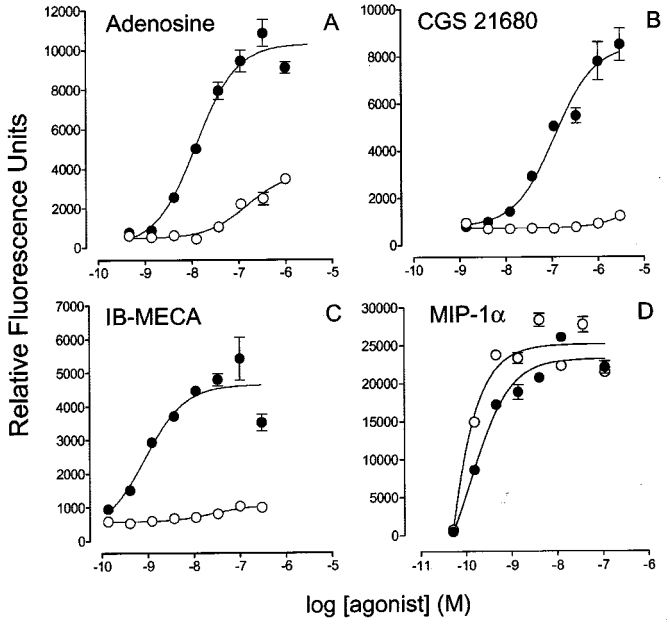

Fig. 5. Effect of 2Cl-IB-MECA pretreatment on subsequent calcium responses to adenosine-related agonists and MIP-1α in immature MDDCs. Responses to agonists are shown as control (●) or as pretreated for 15 min with 100 nM 2Cl-IB-MECA (○). Data points are mean of triplicate values ± S.E.M. Data shown are representative of three similar experiments.

Discussion

A key step in establishing a role for the A3 receptor in the regulation of human immune system function is identification of the cells that express the receptor. To date, few human primary cells or tissues have been identified as expressing the A3 receptor. Presence of transcript has been demonstrated by nonquantitative RT-PCR in various human cell types. However, RT-PCR can amplify negligible amounts of transcript, resulting in misleading conclusions about the likelihood of the presence of receptor protein on the surface of cells. Evidence for expression of A3 receptor protein by radioligand binding previously has been shown only for eosinophils (Kohno et al., 1996) and neutrophils (Gessi et al., 2002).

We now show that, in addition to eosinophils and neutrophils, MDDCs are native expressers of the A3 receptor. Immature MDDCs express relatively high levels of A3 receptor as evinced both by quantitative PCR and by radioligand binding. Expression of the receptor seems to be highly dependent on the state of differentiation, because A3 transcript is considerably lower in monocyte precursors and in MDDCs pushed to a mature phenotype by LPS-activation. In contrast to previous results (Panther et al., 2001), our quantitative PCR data indicate that the A3 receptor is the predominant receptor expressed by immature MDDCs, with much lower levels of A1, A2A, and A2B transcript. The apparent discrepancy is likely to have resulted from our use of quantitative real-time PCR instead of nonquantitative RT-PCR. Data generated by quantitative PCR give a better indication of relative amounts of transcript and, consequently, the likelihood of protein expression. Similarly, Broussass et al. (1999) have provided evidence of A3 receptor expression in human monocytes by RT-PCR; however, our results show that the level of A3 receptor transcript in monocytes is quite low compared with that of immature MDDCs, suggesting that the actual

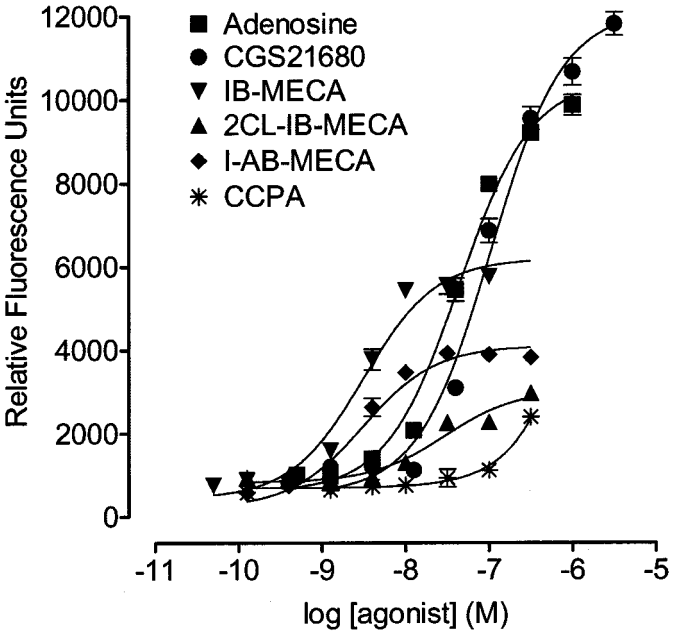

Fig. 6. Calcium responses to adenosine-related agonists in hA3/Gαq-i3/293 double-recombinant cells. Data shown are representative of 4 similar experiments and each point is the mean of triplicate values ± S.E.M.

expression of receptor protein is negligible. Definitive substantiation of receptor expression is obtained through radioligand binding and functional pharmacology.

Unequivocal evidence of cell surface expression of the A3 receptor by immature MDDCs was obtained from [^{125}I]AB-MECA binding experiments. Saturation isotherms indicated a B_{max} of approximately 300 fmol/mg membrane protein, and competition of the radioligand with a variety of adenosine receptor ligands categorically identified the binding site as the A3 receptor. The B_{max} obtained with the agonist radioligand [^{125}I]AB-MECA is likely to underestimate the actual receptor concentration, because agonist binding is highly sensitive to the coupling state of the A3 receptor (Varani et al., 2000). Therefore, expression of the A3 receptor by immature MDDCs seems to be relatively robust.

The highly selective A3 receptor agonist 2Cl-IB-MECA has been used in a variety of cell types to demonstrate functional coupling of the human A3 receptor to calcium signaling (Kohno et al., 1996; Gessi et al., 2001, 2002; Merighi et al., 2001). However, the concentrations of 2Cl-IB-MECA used for eliciting calcium responses in these reports typically exceeds 10 μM, which is well beyond the range of selectivity for the A3 receptor (Klotz, 2000; Fredholm et al., 2001a). Therefore, association of the A3 receptor with calcium signaling in any cell type remained unsubstantiated. Ours is the first article to describe detailed pharmacological characterization of calcium signaling mediated through the human A3 receptor. Because no such analysis previously existed, we generated a recombinant system to serve as a standard for comparing the data we obtained from MDDCs. Although our recombinant hA3/HEK293 cells express high amounts of human A3 receptors (3 pmol/mg of membrane protein; data not shown), 2Cl-IB-MECA (up to 10 μM) failed to elicit calcium signals from these cells. As evinced by our double-recombinant hA3/Gαq-i3/293 cells, A3-mediation of calcium signaling required the additional transfection of a chimeric Gα protein, Gαq-i3, that has been used extensively to establish a link between G_i-coupled receptors and calcium signaling (Conklin et al., 1993). Evidently, all the necessary components for constituting calcium signaling through the A3 receptor exist naturally in MDDCs. The magnitude of MDDC calcium signaling varied considerably across donors. Some of this variability seems to have resulted from donor-dependent differences in the ability of the MDDCs to take up and maintain Fluo-3. Because Fluo-3 is not a ratiometric calcium indicator, differences in loading of dye can greatly affect the quantum output. Response magnitude was relatively constant for a given agonist across experiments when using the double-recombinant hA3/Gαq-i3/293 cells. We have also observed donor-dependent differences in the magnitude of calcium signaling of MDDCs loaded with the ratiometric dye Fura-2 (R. K. Palmer, unpublished observations) that are likely to be more physiological in origin.

In agreement with previous findings (Panther et al., 2001), we were able to completely abolish A3-mediated calcium signaling in immature MDDCs with pertussis toxin pretreatment. This result and the requirement of the chimeric G protein for calcium signaling in the hA3 recombinant cells strongly indicate that the human A3 receptor does not normally couple to Gq.

The FLIPR-based analysis has revealed several previously unrealized aspects of A3 receptor pharmacology. First, adenosine is much more potent at the A3 receptor than has been appreciated. Generally, adenosine has been regarded as a low potency agonist of the A3 receptor, with apparent affinities ranging from ~300 nM to 1 μM (Fredholm et al., 2001a). We have demonstrated that adenosine can activate the A3 receptor at concentrations in the low nanomolar range. A variety of enzymatic activities, such as adenosine deaminase (Franco et al., 1997) and uptake mechanisms (Fredholm et al., 2001b), are known to tightly regulate extracellular adenosine concentrations and are likely to account for the low potency of adenosine commonly observed. For example, inclusion of the uptake inhibitor nitrobenzylthioinosine in a cAMP accumulation assay using recombinant A3 receptor-expressing Chinese hamster ovary cells was found to decrease the EC_{50} of adenosine from ~300 to 60 nM (Fredholm et al., 2001b). Cellular activities that effectively decrease the concentration of exogenous adenosine should have greater impact on assays requiring longer periods of time. In this regard, measurement of calcium signals has an advantage over most biochemical assays of functional pharmacology because the calcium responses are detected within seconds

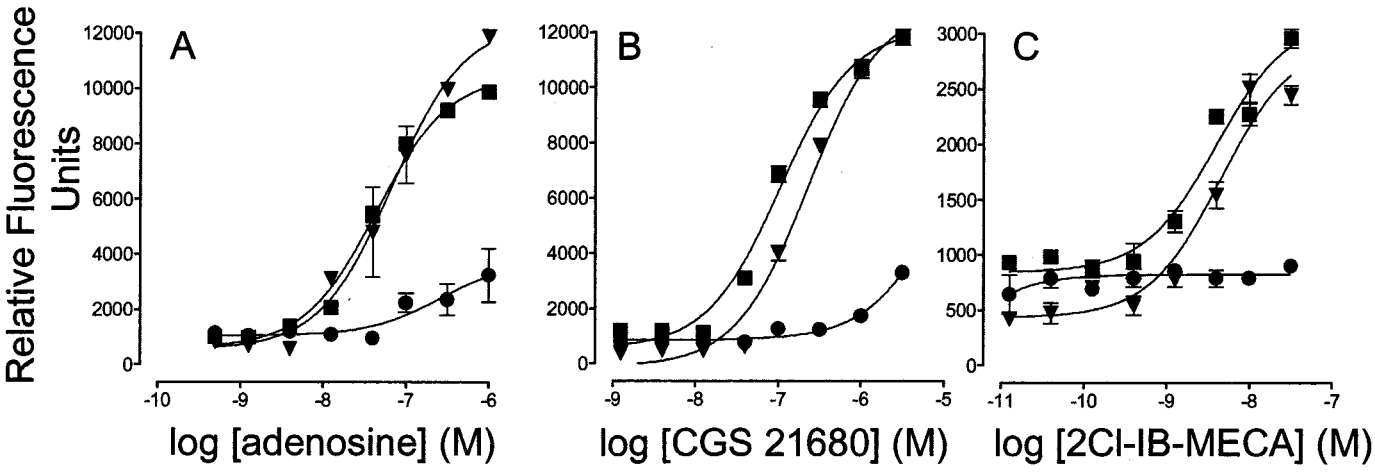

Fig. 7. Selective antagonism of calcium responses to adenosine-related agonists in hA3/Gαq-i3/293 double-recombinant cells. Effect of 100 nM SCH 58261 (▼) and 100 nM VUF5574 (●) on calcium responses to adenosine (A), CGS21680 (B), and 2Cl-IB-MECA (C). ■, data for agonist in the absence of antagonist. Data shown are representative of three similar experiments and each point is the mean of triplicate values, ± S.E.M.

after addition of agonist, leaving little time for enzymatic modification of exogenous adenosine.

CGS21680, regarded as selective for the A2A receptor, has been used extensively to identify A2A-mediated responses. However, recent evidence has indicated appreciable affinity of CGS21680 for the human A3 receptor, with reported K_i values of 67.1 (Klotz et al., 1998) and 82 nM (Murphree et al., 2002). Our binding data are in complete agreement with these reports, and we further show that CGS21680 is a strong agonist at the human A3 receptor. These results underscore the notion that CGS21680 can no longer be considered useful for distinguishing among the human adenosine receptor subtypes.

A final unexpected aspect of the pharmacology of the A3-mediated calcium signaling was the lower intrinsic activity of 2Cl-IB-MECA relative to that of CGS21680 and adenosine. The maximal responses to 2Cl-IB-MECA varied across donors, ranging from nonexistent to 50% of the responses to adenosine or CGS21680. These results suggest that 2Cl-IB-MECA is a partial agonist at the human A3 receptor. Consistent with this notion is the finding that simultaneous addition of 2Cl-IB-MECA depressed the maximal calcium responses to CGS21680 in MDDCs. No evidence has yet appeared in the literature indicating that 2Cl-IB-MECA is a partial agonist. It is possible that the lower efficacy of 2Cl-IB-MECA is specific to calcium responses, occurring as a result of less efficient coupling between the A3 receptor and phospholipase C (Kenakin, 1997a; Yang and Lanier, 1999). A structurally related compound, N^6-(4-amino-3-iodobenzyl) adenosine, also has been characterized as a partial agonist of the human A3 receptor (Salvatore et al., 1993). Similarly, our results suggest that IB-MECA and I-AB-MECA are probably partial agonists as well. Relatively low intrinsic efficacy at the human A3 receptor therefore might be a general property of N^6-benzyl-substituted adenosine derivatives. Alternatively, differences in the observed maximal activity could result from the activation of other adenosine receptors. It is important to note that the nonselective agonists adenosine and CGS21680 generated the largest responses. Small responses to high concentrations of adenosine in the immature MDDCs were not blocked by the A3-selective antagonist VUF5574, indicating the presence of an additional calcium-linked adenosine receptor. Also, SCH 58261 weakly antagonized both adenosine and CGS21680 responses but was ineffective against 2Cl-IB-MECA. Although VUF5574 completely antagonized the adenosine and CGS21680 dose-response functions in the double-recombinant hA3/Gαq-i3/293 cells, we cannot yet rule out the possibility that simultaneous activation of endogenous cAMP-linked A2B receptors (Cooper et al., 1997) could influence the magnitude of the A3-mediated calcium signals.

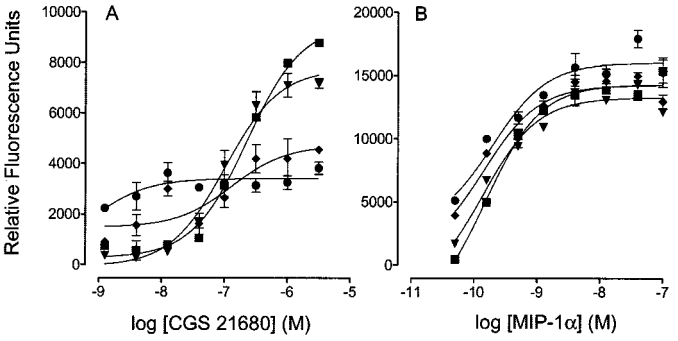

Fig. 8. Effect of simultaneous addition of 2Cl-IB-MECA with CGS21680 or MIP-1α in human MDDCs. Dose-response functions for CGS21680 (A) and MIP-1α (B) in the absence (■) or presence of 1 (▼), 10 (♦), and 100 (●) nM 2Cl-IB-MECA. Data points are mean of triplicate values, ± S.E.M. Data shown are from a single experiment using cells from a single donor and are representative of three similar experiments.

Dendritic cells play a central role in directing the activities of the immune system (Banchereau et al., 2000). They are potent antigen-presenting cells; a single dendritic cell has the capacity to activate hundreds or even thousands of T cells (Banchereau and Steinman, 1998). Furthermore, dendritic cells are known to participate in a variety of important activities, such as elimination of self-recognizing T cells in the thymus (Zal et al., 1994) and activation of B cells (Dubois et al., 1999) and natural killer cells (Siegal et al., 1999). Various subsets of dendritic cells reside in tissues as an immature phenotype, where they serve as sentinels efficiently taking up antigens (Banchereau et al., 2000; Lipscomb and Masten, 2002). Antigen capture and other signals can initiate changes in phenotype that result in a mature antigen-presenting dendritic cell capable of migrating to lymphatic tissues to interact with T cells (Caux et al., 2000). The immature phenotype expresses the A3 receptor. Panther et al. (2001) demonstrated that adenosine can initiate actin polymerization and can also serve as a mild chemotactic agent. Our data indicate that these activities are probably mediated through the A3 receptor.

The A3 receptor was previously thought to be active only under conditions in which high extracellular concentrations of adenosine would be expected, such as sites of inflammation, ischemia, or tissue damage. We have shown that the human A3 receptor can be stimulated by low nanomolar concentrations of adenosine that are likely to be encountered by the receptor under normal conditions. Adenosine, therefore, could act in a paracrine or autocrine manner on dendritic cells, possibly serving a homeostatic function. The strict dependence of A3 receptor expression on the immature cells suggests that A3 also could be involved in the maintenance of the immature phenotype, and its abrupt disappearance may be crucial for transition to a fully activated dendritic cell.

In summary, we have firmly established the expression of functional A3 adenosine receptors by human immature MDDCs and have presented the first detailed pharmacological analysis of calcium signals mediated through the human A3 receptor. Our results show that adenosine and CGS21680 are potent full agonists, and that 2Cl-IB-MECA is a low-efficacy partial agonist at the human A3 adenosine receptor.

Acknowledgments

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References

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, and Palucka K (2000) Immunobiology of dendritic cells. *Annu Rev Immunol* **18:**767–811.

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. *Nature (Lond)* **392:**245–252.

Baraldi PG and Borea PA (2000) New potent and selective human adenosine A3 receptor antagonists. *Trends Pharmacol Sci* **21:**456–459.

Barbieri D, Abbracchio MP, Salvioli S, Monti D, Cossarizza A, Ceruti S, Brambilla R, Cattabeni F, Jacobson KA, and Franceschi C (1998) Apoptosis by 2-chloro-2′-

deoxy-adenosine and 2-chloro-adenosine in human peripheral blood mononuclear cells. *Neurochem Int* **32:**493–504.
Bleharski J, Niazi K, Sieling P, Cheng G, and Modlin R (2001) Signaling lymphocytic activation molecule is expressed on CD40 ligand-activated dendritic cells and directly augments production of inflammatory cytokines. *J Immunol* **167:**3174–3181.
Bouma MG, Jeunhomme TM, Boyle DL, Dentener MA, Voitenok NN, van den Wildenberg FA, and Buurman WA (1997) Adenosine inhibits neutrophil degranulation in activated human whole blood: involvement of adenosine A2 and A3 receptors. *J Immunol* **158:**5400–5408.
Broussas M, Cornillet-Lefebvre P, Potron G, and Nguyen P (1999) Inhibition of fMLP-triggered respiratory burst of human monocytes by adenosine: involvement of A3 adenosine receptor. *J Leukoc Biol* **66:**495–501.
Caux C, Ait-Yahia S, Chemin K, de Bouteiller O, Dieu-Nosjean MC, Homey B, Massacrier C, Vanbervliet B, Zlotnik A, and Vicari A (2000) Dendritic cell biology and regulation of dendritic cell trafficking by chemokines. *Springer Semin Immunopathol* **22:**345–369.
Christopoulos A, Parsons AM, Lew MJ, and El-Fakahany EE (1999) The assessment of antagonist potency under conditions of transient response kinetics. *Eur J Pharmacol* **382:**217–227.
Conklin BR, Farfel Z, Lustig KD, Julius D, and Bourne HR (1993) Sunstitution of three amino acids switches receptor specificity of Gqα to that of Giα. *Nature (Lond)* **363:**274–276.
Cooper J, Hill S, and Alexander SPH (1997) An endogenous A2B adenosine receptor coupled to cyclic AMP generation in human embryonic kidney (HEK 293) cells. *Br J Pharmacol* **122:**546–550.
Dubois B, Bridon JM, Fayette J, Barthelemy C, Banchereau J, Caux C, and Briere F (1999) Dendritic cells directly modulate B cell growth and differentiation. *J Leukoc Biol* **66:**224–230.
Ezeamuzie C and Phillips E (1999) Adenosine A3 receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. *Br J Pharmacol* **127:**188–194.
Ferguson G, Watterson KR, and Palmer TM (2000) Subtype-specific kinetics of inhibitory adenosine receptor internalization are determined by sensitivity to phosphorylation by G protein-coupled receptor kinases. *Mol Pharmacol* **57:**546–552.
Franco R, Casado V, Ciruela F, Saura C, Mallol J, Canela EI, and Lluis C (1997) Cell surface adenosine deaminase: much more than an ectoenzyme. *Prog Neurobiol* **52:**283–294.
Fredholm B, IJzerman A, Jacobson K, Klotz K-N, and Linden J (2001a) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53:**527–552.
Fredholm B, Irenius E, Kull B, and Schulte G (2001b) Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem Pharmacol* **61:**443–448.
Gessi S, Varani K, Merighi S, Cattabriga E, Iannotta V, Leung E, Baraldi PG, and Borea PA (2002) A3 adenosine receptors in human neutrophils and promyelocytic HL60 cells: a pharmacological and biochemical study. *Mol Pharmacol* **61:**415–424.
Gessi S, Varani K, Merighi S, Morelli A, Ferrari D, Leung E, Baraldi PG, Spalluto G, and Borea PA (2001) Pharmacological and biochemical characterization of A3 adenosine receptors in Jurkat T cells. *Br J Pharmacol* **134:**116–126.
Hasko G, Nemeth Z, Vizi ES, Salzman A, and Szabo C (1998) An agonist of adenosine A3 receptors decreases interleukin-12 and interferon-γ production and prevents lethality in endotoxemic mice. *Eur J Pharmacol* **358:**261–268.
Hasko G, Szabo C, Nemeth Z, Kvetan V, Pastores SM, and Vizi ES (1996) Adenosine receptor agonists differentially regulate IL-10, TNFα and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J Immunol* **157:**4634–4640.
Kenakin T (1997a) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18:**456–464.
Kenakin T (1997b) *Pharmacologic Analysis of Drug-Receptor Interaction,* 3rd ed, pp 366–369, Lippincott-Raven Publishers, Philadelphia.
Klotz K-N (2000) Adenosine receptors and their ligands. *Naunyn-Schniedeberg's Arch Pharmacol* **362:**382–391.
Klotz K-N, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, and Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes—characterization of stably transfected receptors in CHO cells. *Naunyn-Schniedeberg's Arch Pharmacol* **357:**1–9.
Kohno Y, Ji X, Mawhorter SD, Koshiba M, and Jacobson KA (1996) Activation of A3 adenosine receptors on human eosinophils elevates intracellular calcium. *Blood* **88:**3569–3574.
Kukkonen JP, Nasman J, Rinken A, Dementjev A, and Akerman KE (1998) Pseudononcompetitive antagonism of M1, M3 and M5 muscarinic receptor-mediated Ca^{2+} mobilization by muscarinic antagonists. *Biochem Biophys Res Commun* **243:**41–46.
Lew MJ, Ziogas J, and Christopoulos A (2000) Dynamic mechanisms of non-classical antagonism by competitive AT1 receptor antagonists. *Trends Pharmacol Sci* **21:**376–381.
Lipscomb MF and Masten BJ (2002) Dendritic cells: immune regulators in health and disease. *Physiol Rev* **82:**97–130.
Merighi S, Varani K, Gessi S, Cattabriga E, Iannotta V, Ulouglu C, Leung E, and Borea PA (2001) Pharmacological and biochemical characterization of adenosine receptors in the human malignant melanoma A375 cell line. *Br J Pharmacol* **134:**1215–1226.
Murphree LJ, Marshall MA, Reiger JM, MacDonald TL, and Linden J (2002) Human A2A adenosine receptors: high-affinity agonist binding to receptor-G protein complexes containing Gβ4. *Mol Pharmacol* **61:**455–462.
Panther E, Idzko M, Herouy Y, Rheinen H, Gebicke-Haerter PJ, Mrowietz U, Dichmann S, and Norgauer J (2001) Expression and function of adenosine receptors in human dendritic cells. *FASEB J* **15:**1963–1970.
Sajjadi F, Takabayashi K, Foster A, Domingo R, and Firestein G (1996) Inhibition of TNF-α expression by adenosine: role of A3 adenosine receptors. *J Immunol* **156:**3435–3442.
Salvatore C, Jacobson M, Taylor H, Linden J, and Johnson R (1993) Molecular cloning and characterization of the human A3 adenosine receptor. *Proc Natl Acad Sci USA* **90:**10365–10369.
Salvatore C, Tilley A, Latour A, Fletcher D, Koller B, and Jacobson M (2000) Disruption of the A3 adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *J Biol Chem* **275:**4429–4434.
Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, and Liu YJ (1999) The nature of the principal type 1 interferon-producing cells in human blood. *Science (Wash DC)* **284:**1835–1837.
Sullivan GW and Linden J (1998) Role of A2A adenosine receptors in inflammation. *Drug Dev Res* **45:**103–112.
Trincavelli ML, Tuscano D, Cecchetti P, Falleni A, Benzi L, Klotz KN, Gremigni V, Cattabeni F, Lucacchini A, and Martini C (2000) Agonist-induced internalization and recycling of the human A3 adenosine receptors: role in receptor desensitization and resensitization. *J Neurochem* **75:**1493–1501.
Varani K, Cacciari B, Baraldi PG, Dionisotti S, Ongini E, and Borea PA (1998) Binding affinity of adenosine receptor agonists and antagonists at human cloned A3 adenosine receptors. *Life Sci* **63:**PL 81–87.
Varani K, Merighi S, Gessi S, Klotz KN, Leung E, Baraldi PG, Cacciari B, Romagnoli R, Spalluto G, and Borea PA (2000) [^{3}H]MRE 3008F20: a novel antagonist radioligand for the pharmacological and biochemical characterization of human A3 adenosine receptors. *Mol Pharmacol* **57:**968–975.
Yang Q and Lanier SM (1999) Influence of G protein type on agonist efficacy. *Mol Pharmacol* **56:**651–656.
Zal T, Volkmann A, and Stockinger B (1994) Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J Exp Med* **180:**2089–2099.

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