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The role of adenosine A_{2A} and A_{2B} receptors in the regulation of TNF-α production by human monocytes

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Abstract

Adenosine is an endogenous nucleoside that regulates many physiological processes through the activation of its four receptors: A_1, A_{2A}, A_{2B} and A_3. Previous studies have identified the involvement of A_2 receptors in the inhibitory activity of adenosine analogues on tumor necrosis factor-α (TNF-α) production by lipopolysaccharide (LPS) activated monocytes, but the relative contributions of A_{2A} versus A_{2B} receptors have not been determined in human primary monocytes. Nor has the role of A_1 and A_3 been clearly identified in the system. The lack of such information impacts on the selection of adenosine receptor agonists for disease intervention. Using LPS-stimulated human primary monocytes, we found that the adenosine receptor agonist, 5′-*N*-ethylcarboxamidoadenosine (NECA) or the A_{2A} receptor agonist, 4-[2-[[6-amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS21680) produced a concentration-dependent inhibition of TNF-α production, with IC_{50}s of 58.4 nM (32.7–104.5 nM, 95% confidence interval) and 49.2 nM (22.7–105.9 nM, 95% confidence interval), respectively. The selective A_{2A} receptor blocker, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylaminso]ethyl)phenol (ZM241385, 30 nM), antagonized the effects of NECA and CGS21680 (pK_B estimates were 8.7 ± 0.1 and 8.9 ± 0.1, respectively), while the selective A_{2B} antagonist, *N*-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide (MRS1754, 100 nM), failed to antagonize the effects of either agonist. Furthermore, neither the A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA) nor the A_3 receptor agonist, 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-*N*-methyl-*b*-D-ribofuranuronamide (2-Cl-IB-MECA) showed significant inhibitory activity at concentrations that effectively bind to their respective receptors. We conclude that A_{2A} receptor activation is predominantly responsible for the inhibitory effects of adenosine receptor agonists on TNF-α production from LPS-stimulated monocytes.

Keywords: Adenosine receptor; A_1; A_{2A}; A_{2B}; A_3; LPS

1. Introduction

Adenosine is an endogenous purine nucleoside released by many cells during normal metabolic function and regulates physiological processes through activation of specific receptors. There are four known adenosine receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3. All four subtypes belong to the superfamily of G protein-coupled receptors, and are linked to a variety of signal transduction systems [1,2].

In many cell types, adenosine secretion is up-regulated in response to injury. Enhanced production of adenosine has been detected in pathological conditions, such as inflammation, hypoxia and ischemia. It is believed that adenosine plays a cytoprotective function as a local regulator of inflammatory responses [3,4]. Early clinical evidence linking adenosine to the immune system comes from studies in patients with abnormally high plasma

Abbreviations: 2-Cl-IB-MECA, 1-[2-chloro-6-[[(3-iodophenyl)methyl]-amino]-9H-purin-9-yl]-1-deoxy-*N*-methyl-b-D-ribofuranuronamide; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS21680, 4-[2-[[6-Amino-9-(*N*-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride; LPS, Lipopolysaccharide; MRS1754, *N*-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide; NECA, 5′-*N*-ethylcarboxamidoadenosine; TNF-α, Tumor necrosis factor-α; ZM241385, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

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levels of adenosine due to defective adenosine deaminase (ADA) expression, resulting in severe combined immunodeficiency (SCID) [5]. Increased levels of adenosine resulting from the defect in ADA are thought to increase thymic apoptosis and to inhibit T-cell antigen receptor-triggered activation in thymocytes [6,7]. A number of reports have also implicated adenosine receptor agonists, and compounds that indirectly augment extracellular adenosine levels, as anti-inflammatory agents. Indeed, the anti-inflammatory actions of methotrexate, commonly used as an anti-rheumatic drug, may be attributed to its induction of cellular adenosine release [8]. Studies using isolated monocytes or macrophages have postulated that the anti-inflammatory properties of adenosine receptor agonists occur, at least in part, via a down-regulation of the release of tumor necrosis factor-α (TNF-α), a cytokine that exhibits deleterious effects in conditions such as septic shock and rheumatoid arthritis [9,10]. In vitro, human monocytes are found to express all four adenosine receptors although the relative levels of expression differ [11]. Intervention with A_2 agonists was found to attenuate the production of TNF-α at the protein level, supporting the possibility that TNF-α inhibition may be an A_2-receptor mediated event [12,13]. However, due to the limited choice of receptor agonists and respective antagonists, previous studies are unable to clarify the relative contribution of A_{2A} and A_{2B} receptor activation to the inhibition of TNF-α release by lipopolysaccharide (LPS)-stimulated monocytes. The identification of which receptor (A_{2A} or A_{2B}) mediates this effect may have important consequences for the design of novel anti-inflammatory drugs. A_{2A} and A_{2B} receptors are both coupled to G_s proteins and stimulate adenylyl cyclase activity [14,15]. However, activation of A_{2B} receptors may facilitate the release of allergic and pro-inflammatory mediators [16,17], while activation of A_{2A} receptors may protect tissues from ischemia reperfusion injury by reducing inflammation [18]. A_3 receptors are also implicated in the inhibition of TNF-α production by macrophages [19], but since the study was carried out in U937 cells, the relevance of this finding to human primary monocytes is uncertain. Thus, the aim of this study was to differentiate the relative contribution of A_{2A} and A_{2B} receptors in the modulation of TNF-α release from human primary monocytes and to clarify whether or not A_1 and A_3 receptor activation could also contribute to the anti-TNF-α activity observed in response to adenosine agonists.

2. Materials and methods

2.1. Materials

All cell culture reagents were purchased from Invitrogen Ltd. 5′-(*N*-ethylcarboxamido)-adenosine (NECA), 2-Chloro-N^6-cyclopentyladenosine (CCPA), dimethyl sulfoxide (DMSO), LPS from *Escherichia coli* (serotype 0127:B8), disodium ethylenediaminetetraacetate dihydrate (EDTA) and tripotassium ethylenediaminetetraacetate dihydrate (K_3EDTA) were obtained from Sigma–Aldrich Company Ltd. 4-[2-[[6-Amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS21680), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]-ethyl)phenol (ZM241385) and 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-*b*-D-ribofuranuronamide (2-Cl-IB-MECA) were purchased from Tocris Cookson Ltd. *N*-(4-Cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide (MRS1754) was synthesized in house. Ficoll–Hypaque was obtained from Amersham Biosciences. The TNF-α DuoSet ELISA Development System was purchased from R&D Systems (Europe) Ltd.

2.2. Isolation of monocytes from whole blood

Peripheral venous blood was collected from healthy volunteers into 50 ml Falcon tubes with K_3EDTA as an anti-coagulant. The donors had provided informed consent, and had refrained from taking anti-inflammatory medication for 48 h previously. The study was approved by the local research ethics committee.

The whole blood was diluted with an equal volume of Hank's balanced salt solution, overlaid on Ficoll–Hypaque and centrifuged at $370 \times g$ for 30 min at 20 °C. The mononuclear cell fractions at the plasma–Ficoll interface were collected and washed three times in ice-cold PBS containing 0.02% EDTA. The cells were finally resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM) and antibiotics (penicillin–streptomycin at 100 U/ml and 100 μg/ml, respectively) (complete medium). Monocytes were then isolated from this preparation by 1 h adherence to plastic flasks and recovered using a cell scraper. The monocytes had greater than 90% viability as determined by trypan blue exclusion. The monocytes were finally resuspended in the complete medium.

2.3. Treatment of monocytes with adenosine analogues

All A_2 adenosine receptor agonists and antagonists were dissolved in DMSO at a concentration of 10 mM. The A_1 agonist, CCPA was solubilized in DMSO to 4.5 mM and the A_3 agonist, 2-Cl-IB-MECA, was dissolved in DMSO to 1 mM. All subsequent dilutions were prepared in complete medium. The maximal concentration of DMSO applied to cells in culture did not exceed 1% and was found to have no significant effects in the assay.

Concentration–effect curves for adenosine receptor agonists were produced by plating freshly isolated, viable monocytes onto 96-well plates (1×10^5 cell/well) and pre-treating for 1 h (37 °C/5% CO_2) with agonists. Drug-treated and control monocytes were then stimulated

Table 1
Binding affinity of agonists and antagonists at human adenosine receptors

Compound	A_1	A_{2A}	A_{2B}	A_3	Reference
CCPA	0.8 (0.55–1.26)	2300 (2000–2700)	40100 (±16400)	42 (32–56)	[23] [22]
NECA	14 (6.4–29)	20 (12–35)	330 (±60) 360 (±120)	6.2 (3.5–11)	[23] [22] [24]
CGS21680	290 (230–360)	27 (12–59)	361000 (±21000)	67 (50–90)	[23] [22]
Cl-IB-MECA	115 (114–116)	2100 (1700–2500)		11 (9.4–13)	[25]
MRS1754	400 (±190)	500 (±11)	2.0 (±0.31)	570 (±180)	[21]
ZM241385	260 (190–390)	0.8 (0.7–1.0)	32 (±6)	>10000	[20] [22]

K_i values (nM) with 95% confidence intervals or ±S.E.M. in parentheses.

with LPS at a sub-maximal concentration of 100 ng/ml for a further 24 h (37 °C, 5% CO_2). This LPS concentration was identified through experiments investigating the concentration–response relationship to LPS in human monocytes (data not shown). It was also found that in the absence of LPS stimulation, the basal levels of TNF-α release by resting monocytes were below 15 pg/ml.

A_2 adenosine receptor antagonist studies were performed by pre-treating the cells for 15 min in the presence of either the adenosine A_{2A} receptor antagonist, ZM241385 (30 nM) [20] or the adenosine A_{2B} receptor antagonist, MRS1754 (100 nM) [21] prior to the addition of adenosine receptor agonists. Concentrations of ZM241385 and MRS1754 were chosen based on the reported affinities of these compounds for human recombinant adenosine receptors as summarized in Table 1, such that selective antagonism of an A_{2A} or A_{2B} mediated response, respectively, would be anticipated. The use of ZM241385 was important in dissecting out the relative importance of A_{2A} versus A_{2B} receptors. Based on the known affinity of this antagonist for these two receptor subtypes (Table 1), a concentration (30 nM) was selected which would be predicted to cause a 30–40-fold rightward displacement of the agonist concentration–response curves if the agonists act via A_{2A} receptors but only a two-fold rightward displacement should the agonists act via A_{2B} receptor stimulation. All treatments were carried out in duplicate. Control cultures were performed under identical conditions employing compound vehicles. After 24 h, the assay plates were centrifuged to obtain cell-free supernatants that were stored at −20 °C until analysis.

2.4. Cytokine determination

TNF-α production was quantified using the TNF-α DuoSet ELISA Development System according to the manufacturer's instructions. The optical density was determined at 450 nM with a reference wavelength of 570 nM by using a spectrophotometer (SpectraMax plate reader, Molecular Devices).

2.5. Data analysis

All results are expressed as mean ± standard error of the mean (S.E.M.), with the exception of IC_{50} values which are presented as geometric mean with 95% confidence interval. The concentration–effect curves were analyzed using nonlinear regression (GraphPad Prism® Version 3.02), and sigmoidal concentration–effect curves (three parameter logistic curve fit) were plotted to analyze the effect of the agonists (in the absence or presence of antagonists) on TNF-α release. The pK_B estimate was calculated as: $pK_B = \log_{10}$ (concentration ratio -1) $-\log_{10}$ [antagonist concentration used], where concentration ratio is the ratio between the IC_{50} of an agonist in the presence of an antagonist and the IC_{50} of the same agonist in the absence of the antagonist.

3. Results

3.1. Role of adenosine A_1 and A_3 receptors in modulating TNF-α production by monocytes

The effects of serial concentrations of the adenosine A_1 and A_3 agonists, CCPA and 2-C1-IB-MECA, respectively, on LPS-stimulated TNF-α release by human monocytes were investigated. As Fig. 1 shows, neither compound significantly inhibited TNF-α production at concentrations up to and including 100 nM. At concentrations in excess of 100 nM, both compounds caused inhibition of TNF-α release, with respective IC_{50}s of 844 nM (30.4 nM–23.4 μM, 95% confidence interval) for CCPA and 1.6 μM (152.3–16.3 μM, 95% confidence interval) for 2-C1-IB-MECA.

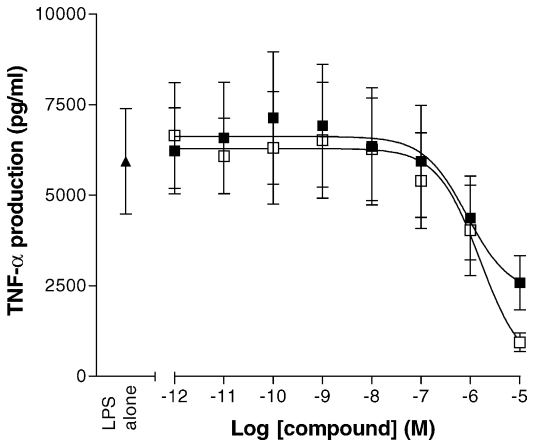

Fig. 1. Effects of the A_1 agonist, CCPA (■) and the A_3 agonist, 2-C1-IB-MECA (□) on TNF-α production by LPS (100 ng/ml) stimulated monocytes. Data are expressed as mean ± S.E.M. of three donors.

3.2. Role of adenosine A_{2A} and A_{2B} receptors in modulating TNF-α production by monocytes

CGS21680 is an adenosine receptor agonist, which is highly selective for A_{2A} versus A_{2B} receptors (Table 1), while NECA is a non-selective adenosine receptor agonist. Both compounds were tested on LPS-stimulated TNF-α secretion by human monocytes. As Fig. 2 shows, both CGS21680 and NECA concentration-dependently inhibited TNF-α secretion, with IC_{50} values of 49.2 nM (22.7–105.9 nM, 95% confidence interval) for CGS21680 and 58.4 nM (32.7–104.5 nM, 95% confidence interval) for NECA. The maximal inhibition of LPS-stimulated TNF-α production was approximately 70% at the highest concentration evaluated (10 μM) for both compounds. The adenosine receptor agonists had minimal effects on unstimulated monocytes, which produced only small amounts of TNF-α (<100 pg/ml) (data not shown).

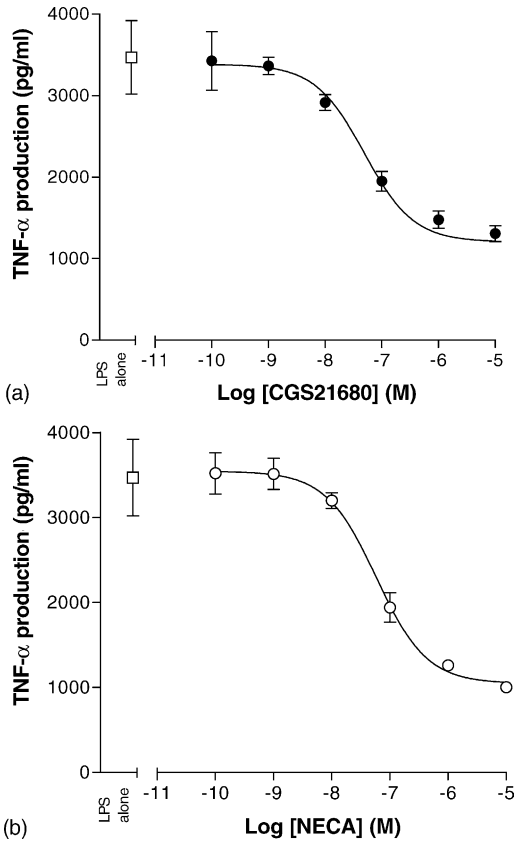

Fig. 2. Effects of CGS21680, an agonist that is more selective to A_{2A} than to A_{2B} receptors (a) and the non-selective adenosine receptor agonist, NECA and (b) on the production of TNF-α by LPS-stimulated human monocytes. Data are expressed as mean ± S.E.M. of three donors.

3.3. Effect of A_{2A} and A_{2B} antagonists on agonist induced TNF-α inhibition

To further elucidate the role of A_2 adenosine receptor subtypes in TNF-α suppression, antagonists selective for A_{2A} or A_{2B} receptors were evaluated for their ability to block the inhibitory effects evoked by CGS21680 and NECA. Pre-treatment of monocytes with MRS1754

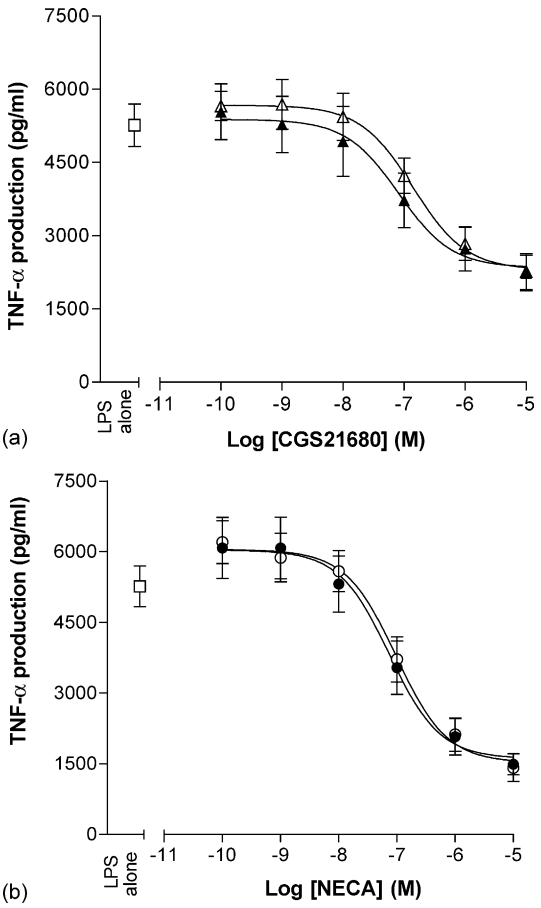

Fig. 3. Effects of the A_{2B} antagonist, MRS1754 (100 nM) on adenosine analogue-mediated inhibition of TNF-α production by LPS-stimulated human monocytes. (a) TNF-α production was inhibited by CGS21680 alone (▲) but little antagonistic effect could be observed in the presence of MRS1754 (△). (b) TNF-α production was inhibited by NECA alone (●), but the presence of MRS1754 (○) had little antagonistic effect. Data are expressed as mean ± S.E.M. of three donor samples.

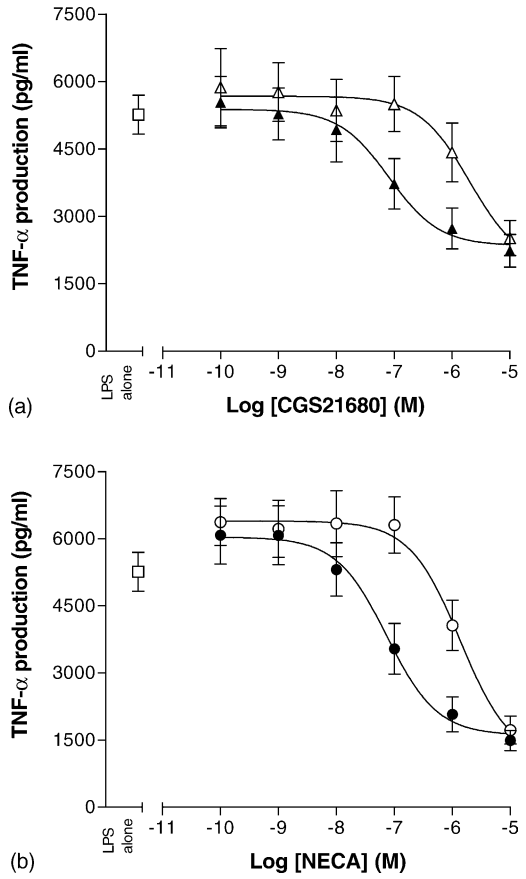

Fig. 4. Effects of the A_{2A} antagonist, ZM241385 (30 nM) on adenosine analogue mediated inhibition of TNF-α production by LPS-stimulated human monocytes. (a) TNF-α production was inhibited by CGS21680 alone (▲). In the presence of ZM241385 (△), a significant rightward shift could be seen. (b) TNF-α production was inhibited by NECA alone (●). In the presence of ZM241385 (○), a significant rightward shift could be seen. Data are expressed as mean ± S.E.M. of three donor samples.

(100 nM), an A_{2B} adenosine receptor antagonist, failed to antagonize the effects of either CGS21680 or NECA on TNF-α production (Fig. 3). However, the A_{2A} antagonist, ZM241385 at 30 nM caused a significant rightward shift in the location of both the NECA and CGS21680 concentration–response curves, giving pK_B estimates of 8.9 ± 0.1 against CGS21680 and 8.7 ± 0.1 against NECA (Fig. 4).

4. Discussion

In the present study, we have confirmed previous observations that the inhibition of TNF-α production by LPS-stimulated human primary monocytes by adenosine agonists is primarily an A_2-receptor mediated event [12,13]. In addition, we have extended those findings by demonstrating that the inhibition of TNF-α by adenosine agonists is mediated via the A_{2A} receptor rather than the A_{2B} receptor.

Both CGS21680 and NECA have been found to decrease TNF-α production by LPS-activated monocytes in a concentration-dependent manner with virtually identical IC_{50}s in the nanomolar range. This is consistent with A_2 receptor activation, and is in agreement with A_2 receptor expression by monocytes [11], and previous reports on the involvement of these receptors in TNF-α inhibition. Furthermore, since CGS21680 is highly selective for A_{2A} versus A_{2B} receptors (Table 1), the potency of this compound in inhibiting TNF-α release from monocytes points to a potentially important role for A_{2A} receptors in mediating this activity. This was further corroborated through antagonist studies. Thus, the inhibitory activities of NECA and CGS21680 on TNF-α production were competitively antagonized in the presence of ZM241385, the A_{2A} selective antagonist, but not in the presence of MRS1754, the A_{2B} selective antagonist. In addition, the pK_B values determined for ZM241385 against the A_2 agonists, NECA and CGS21680 are consistent with the published affinity of this antagonist for the human recombinant A_{2A} receptor (Table 1). Taken together, the potency of ZM241385 in inhibiting this response, thus implicating a receptor of the A_{2A} sub-type, coupled to the lack of effect of the A_{2B} receptor antagonist MRS1754, indicate that A_{2A} is the predominant receptor responsible for the inhibitory activity observed in response to NECA and CGS21680.

Both A_1 and A_3 receptors are expressed by freshly isolated human monocytes [11]. It has also been postulated that the A_3 receptor may be involved in the inhibitory activity of adenosine to TNF-α production by LPS-stimulated U937 cells, a monocytic cell line derived from histiocytic lymphoma [19]. We, therefore, investigated the possible contribution of A_1 and A_3 receptors to modulation of TNF-α release in this monocyte assay. We selected CCPA, a potent A_1 receptor agonist and C1-IB-MECA, a potent A_3 receptor agonist for this investigation and interestingly, neither agonist showed inhibitory activity at concentrations well above the K_i value for their respective receptors (Table 1). Indeed, at 100 nM, both compounds are able to interact with both A_1 and A_3 receptors but no inhibitory activity was observed (Fig. 1). In contrast, when the concentration–effect curve was extended to 1 and 10 μM, where both agonists would be predicted to bind to the A_{2A} receptors (Table 1), some inhibitory activity could be observed (Fig. 1). Further detailed antagonist studies would be required to rule out a role of A_1 and/or A_3 receptors, however, based on the current data it seems unlikely that they play a major role in the inhibition of TNF-α production by adenosine agonists in LPS-stimulated human primary monocytes.

Our findings may have significant implications for the design of novel drugs, which inhibit inflammatory processes via adenosine receptor stimulation. Although the anti-inflammatory potential of adenosine A_2 receptor stimulation is well recognized [26], the relative importance of A_{2A} and A_{2B} receptors in this activity has for some time remained unclear. The recent discovery of selective antagonists for both these receptor subtypes is at last allowing clarification of this issue. Thus, while previous

workers using cell lines suggested a potential role of the A_{2B} receptor in modulation of monocyte TNF-α production [27], our study using primary monocytes has confirmed that A_{2A}, rather than A_{2B} receptors, play a significant role. Moreover, recent work by other researchers has demonstrated several adverse and pro-inflammatory roles attributable to A_{2B} receptor activities, such as stimulating the production of MCP-1 and IL-6 by bronchial smooth muscle cells [17] and increasing cell activation and IL-8 release by human mast cells [16,28,29]. Thus, anti-inflammatory therapeutic strategies using adenosine analogues should target A_{2A} receptor agonists [30,31] while perhaps antagonists of adenosine A_{2B} receptors may represent a possible beneficial approach to asthma [32].

In summary, adenosine receptor agonists can down-regulate TNF-α production by LPS-activated human peripheral blood monocytes and this effect is primarily mediated via A_{2A} receptors. Selective adenosine A_{2A} receptor agonists may represent an attractive therapeutic approach to various inflammatory diseases.

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