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The role of adenosine receptors in the action of theophylline on human peripheral blood mononuclear cells from healthy and asthmatic subjects

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1 The aim of the present study was to investigate the role of adenosine A2b receptors in the anti-proliferative action of theophylline in human peripheral blood mononuclear cells (HPBMC) from healthy and asthmatic subjects.

2 Theophylline significantly inhibited PHA-induced proliferation of HPBMC from both healthy and asthmatic donors but only at relatively high concentrations at 1 mM ($P<0.05$). Enprophylline, a drug which also acts as a non-selective phosphodiesterase (PDE) inhibitor and is a selective A2b receptor antagonist, had no significant effect on proliferation of cells from either group at concentrations up to 10 μM ($P>0.05$; $n=6$).

3 Adenosine deaminase (2 u ml^{-1}), which metabolizes adenosine, had no significant effect on PHA-induced HPBMC proliferation over a range of concentrations (0–8 μg ml^{-1}) in cells from either healthy or asthmatic subjects.

4 The adenosine receptor agonists N^6-cyclopentyladenosine (CPA, A1-selective) and 5′-N-ethylcarboxamidoadenosine (NECA, A1/A2) produced a small but significant inhibition of PHA-induced proliferation of HPBMC from healthy and asthmatic subjects (10 μM, $P<0.05$; $n=6$). In contrast, 5′-N-ethylcarboxamido-2-[4-(2-]carboxyethyl)phenethyl]adenosine (CGS21680, A2a-selective) was without significant effect ($P>0.05$; $n=6$).

5 The adenosine receptor antagonist alloxazine (A2b-selective) had no significant effect, while 8(3-chlorostyryl)caffeine,(CSC, A2a-selective) significantly inhibited PHA-induced proliferation of HPBMC from both groups ($P<0.05$; $n=6$).

6 Our results suggest that endogenous or exogenous adenosine has little effect on the proliferation of HPBMC obtained from healthy or asthmatic subjects. Thus it would appear that the effect of high concentrations of theophylline is not related to adenosine receptor antagonism.

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Abbreviations: ANOVA, analysis of variance; cyclic AMP, cyclic adenosine monophosphate; CGS 21680, 5′-N-ethylcarboxamido-2-[4-(2-]carboxyethyl)phenethyl]adenosine; CPA, N^6-cyclopentyladenosine; CSC, 8(3-chlorostyryl)caffeine; DMSO, dimethyl sulphoxide; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; FBS, foetal bovine serum; HPBMC, human peripheral blood mononuclear cells; LAR, late asthmatic response; NECA, 5′-N-ethylcarboxamidoadenosine; PDE, phosphodiesterase; PHA, phytohaemagluttinin; TNFα, tumour necrosis factor alpha

Introduction

Theophylline has been widely used in the treatment of airways diseases (Sullivan *et al*., 1994b) although the mechanism of action of this drug remains unknown. Theophylline has well-documented effects on smooth muscle which may be related to its ability to act as a non-selective phosphodiesterase (PDE) inhibitor. However, recent clinical observations have suggested that theophylline can also have significant effects on circulating inflammatory cells at plasma levels well below those required to affect airway smooth muscle (Ward *et al*., 1993; Zocchi *et al*., 1985; Jaffar *et al*., 1996; Sullivan *et al*., 1994a). It has long been recognized that theophylline can influence the behaviour of inflammatory cells *in vitro* (Shore *et al*., 1978), and mononuclear cells have been used to investigate the effects of theophylline as these cells are implicated in the pathogenesis of asthma. However, the mechanism of action of theophylline which underlies this effect on inflammatory cells remains unclear.

One action of theophylline which may contribute to its anti-asthmatic effect is the ability to antagonize adenosine receptors (Persson, 1986). This hypothesis was largely ignored after the discovery that enprophylline, a xanthine with clinical activity similar to theophylline, apparently lacked adenosine receptor antagonism (Persson, 1986). However, the recent observation that enprophylline selectively inhibits adenosine receptor agonist-induced IL-8 secretion by human mast cells *via* A2b receptor antagonism (Feoktistov & Biaggioni, 1995), an effect shared by theophylline, raises the possibility that some of the anti-asthmatic effects observed with theophylline are *via* A2b receptor antagonism (Feoktistov *et al*., 1998).

Mononuclear cells are other circulating inflammatory cells implicated in the pathogenesis of asthma and they proliferate in response to antigen stimulation as part of their role in the immune response. This proliferation can be mimicked *in vitro* by stimulation with mitogens such as phytohaemagluttinin

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(Banner *et al*., 1995) providing a simple model for the investigation of drugs affecting mononuclear cell function. In the present study, we have investigated whether theophylline and enprophylline inhibit the proliferation of mononuclear cells *via* adenosine receptor antagonism and we have also investigated whether endogenous or exogenous adenosine regulate the proliferation of HPBMC.

Methods

Blood donors

Normal healthy donors with no history of asthma, allergic rhinitis or atopic dermatitis were selected and compared to a group of mild asymptomatic asthmatic subjects taking β-agonist therapy only and who had refrained from taking medication for at least 6 h before blood samples were drawn. This study was approved by the Ethics Committee of King's College Hospital, U.K.

Preparation of human peripheral blood mononuclear cells

Peripheral venous blood (25 ml) was collected from healthy and asthmatic subjects ($n=6$ per group) into ethylenediaminetetraacetic acid (EDTA)-coated Vacutainers. Mononuclear cells were prepared by centrifugation of the blood on Histopaque-1077 (Sigma Diagnostics) ($800\times g$, 15 min, 20°C). Following aspiration of the plasma, the fraction containing the mononuclear cells was washed twice ($250\times g$, 10 min, 20°C) and cells resuspended in RPMI 1640 medium, supplemented with 10% foetal bovine serum (FBS) (10^6 ml^{-1}).

Preparation of plates and drugs

All agonists and antagonists were dissolved in DMSO (except theophylline, which was dissolved in saline) at a concentration of 10^{-2} M. Subsequent dilutions were made in RPMI 1640. Mononuclear cells (10^5 well^{-1}) were incubated (5% CO_2, 37°C) with fresh RPMI 1640 containing 10% FBS and with drugs or vehicle (0.1% DMSO). PHA was added to stimulate drug-treated and control wells at a concentration of 2 μg ml^{-1}. For experiments investigating the concentration-response curve to PHA, the concentrations ranged from 0–8 μg ml^{-1} with or without adenosine deaminase (2 u ml^{-1}). Adenosine deaminase (2 u ml^{-1}) was added to wells which received adenosine receptor agonists.

Measurement of proliferation

The 96-well plates were incubated for 24 h (37°C, 5% CO_2) and [^{3}H]-thymidine was then added (0.1 μCi well^{-1}) and plates were incubated for a further 24 h (37°C, 5% CO_2). After this time, cells were harvested onto filter mats (ICN Biomedicals) using a cell harvester and thymidine incorporation was assessed by counting the samples on a beta-counter.

Materials

The following drugs were used: adenosine deaminase (Boehringer Mannheim), N^6-cyclopentyladenosine (CPA), 5′-N-ethylcarboxamidoadenosine (NECA), 5′-N-ethylcarboxamido-2-[4-(2-]carboxyethyl) phenethyl]adenosine (CGS21680), 8(3-chlorostyryl)caffeine, (CSC), alloxazine, enprophylline (all from CalBiochem-Novabiochem (U.K.) Ltd) and theophylline (Sigma, U.K.). Phytohaemagluttinin (PHA) was from Poly Labo, Paris, France. [3H]-thymidine was purchased from Amersham International, U.K. All cell culture reagents were purchased from Life Technologies, U.K. All other reagents were obtained from Sigma, U.K.

Statistical analysis

Results from all experiments are expressed as mean ± s.e.mean. Data are presented as a percentage of [^{3}H]-thymidine incorporation in control wells (wells which received PHA but no drug). The data from the experiments in which a concentration-response curve to PHA in the presence or absence of adenosine deaminase are presented as counts per min. One way repeated measures analysis of variance (ANOVA) followed by Dunnett's test (Wallenstein *et al*., 1980) was used to determine any effect of the drugs on the proliferative response (GraphPad Prism 2.0). Two-way repeated measures ANOVA was used to determine if there were any differences between healthy and asthmatic groups (Minitab version 12). Differences were considered to be statistically significant if $P<0.05$.

Results

Effect of adenosine deaminase on PHA-induced HPBMC proliferation

Stimulation of HPBMC with increasing concentrations of PHA (0.125–8 μg ml^{-1}) resulted in a typical bell-shaped concentration-response curve with a maximal response obtained at 2 μg ml^{-1}. The addition of adenosine deaminase (2 u ml^{-1}) had no significant effect on proliferation of cells

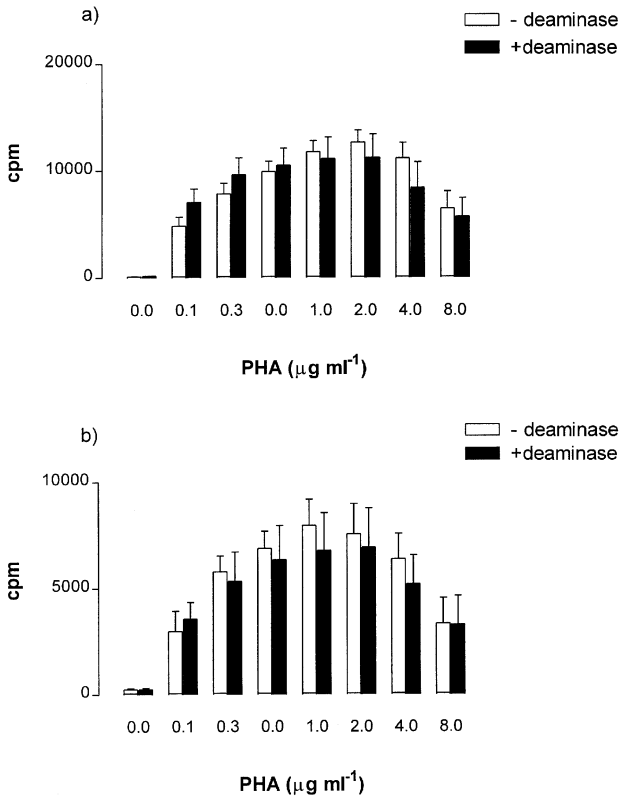

Figure 1 Effect of adenosine deaminase on PHA-induced proliferation HPBMC from (a) healthy and (b) asthmatic subjects. Data are expressed as counts per minute. Each point represents mean ± s.e.mean. ($n=6$ for both groups).

from either healthy (Figure 1a) or asthmatic (Figure 1b) subjects ($P>0.05$).

Effect of adenosine receptor agonists on PHA-induced HPBMC proliferation

NECA (A1/A2) significantly inhibited proliferation of HPBMC from healthy subjects ($P<0.05$ ANOVA) but not asthmatic subjects ($P>0.05$ ANOVA). CPA (A1) significantly reduced proliferation of HPBMC from asthmatic subjects ($P<0.05$ ANOVA) but not healthy subjects ($P>0.05$ ANOVA). CGS21680 (A2a) had no significant effect on proliferation from HPBMC from healthy or asthmatic subjects ($P>0.05$ ANOVA).

When compared to control wells which had received PHA and adenosine deaminase, it was found that NECA (10 μM) produced a small but significant attenuation of PHA-induced HPBMC proliferation of cells from healthy (control 96 ± 5 versus drug treated $81\pm6\%$; $P<0.05$) but not asthmatic (control 86 ± 6 versus drug treated $74\pm13\%$; $P>0.05$) subjects ($n=6$ per group; Figure 2a). Conversely, CPA (10 μM) produced a significant inhibition of HPBMC proliferation of cells from asthmatic (control 85 ± 6 versus drug treated $60\pm8\%$; $P<0.05$) but not healthy (control 84 ± 0 versus drug treated $66\pm8\%$; $P>0.05$) individuals ($n=6$ per group; Figure 2b). CGS21680 (10 μM) had no significant effect on proliferation of HPBMC from either healthy (control 88 ± 11 versus drug treated $86\pm6\%$) or asthmatic (control 86 ± 3 versus drug treated $79\pm11\%$) subjects ($P>0.05$; $n=6$ per group; Figure 2c). There was no significant difference between healthy and asthmatic groups with any of the agonists used ($P>0.05$ ANOVA).

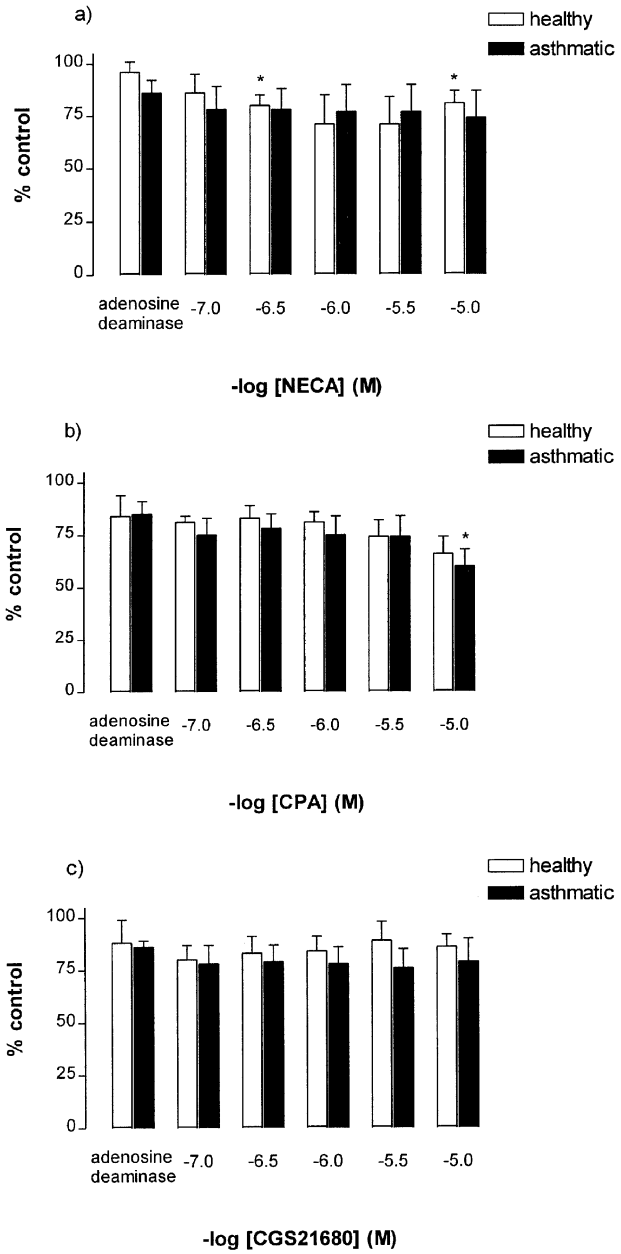

Figure 2 Effect of (a) NECA, (b) CPA and (c) CGS21680 on PHA-induced proliferation of HPBMC from healthy and asthmatic subjects. Data are expressed as per cent control (stimulated cells with no drug added). Each point represents mean$\pm$s.e.mean. ($n=6$ for all groups).

Effect of adenosine receptor antagonists on PHA-induced HPBMC proliferation

CSC (A2a; $P<0.05$ ANOVA) but not alloxazine (A2b; $P>0.05$ ANOVA) and enprophylline ($P>0.05$ ANOVA) significantly inhibited proliferation of HPBMC from both healthy and asthmatic subjects. Compared to control values (wells which received only PHA), CSC (10 μM) induced a small but significant inhibition of PHA-induced HPBMC proliferation of cells isolated from healthy ($74\pm4\%$) and asthmatic ($79\pm7\%$) subjects ($P<0.05$; $n=6$ per group; Figure 3a). Neither alloxazine (Figure 3b) nor enprophylline (Figure 4a) inhibited PHA-induced HPBMC proliferation in either the healthy or asthmatic population ($P>0.05$). Theophylline (10 μM) did not reduce proliferation of HPBMC from asthmatic ($98\pm5\%$) or healthy ($97\pm5\%$) subjects ($P>0.05$)

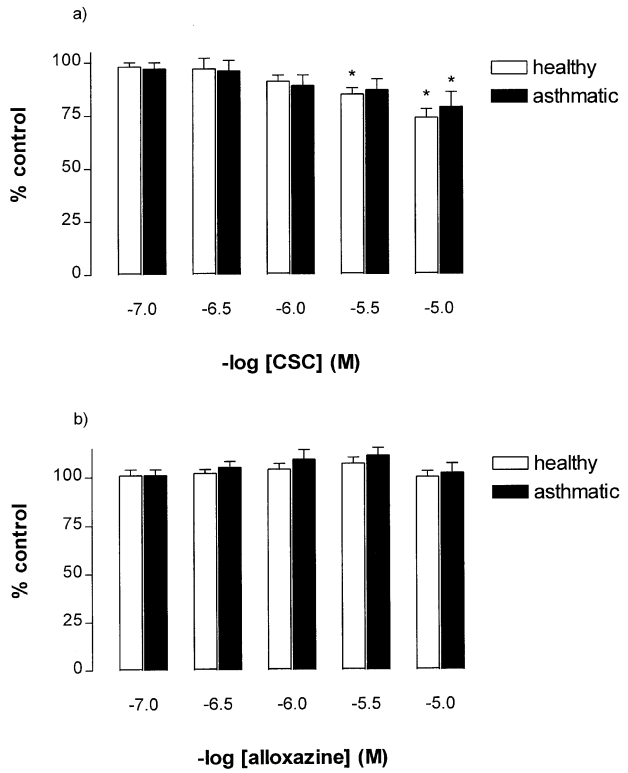

Figure 3 Effect of (a) CSC and (b) alloxazine on PHA-induced proliferation of HPBMC from healthy and asthmatic subjects. Data are expressed as per cent control (stimulated cells with no drug added). Each point represents mean$\pm$s.e.mean. ($n=6$ for all groups).

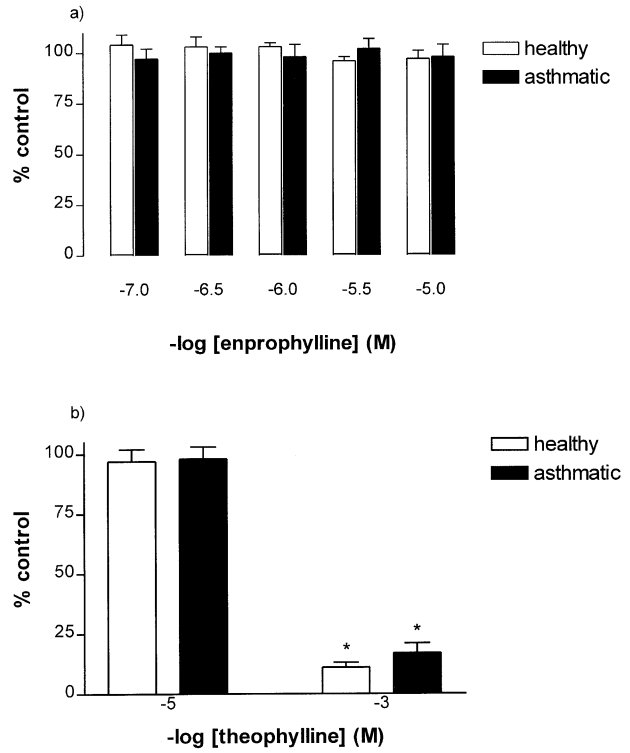

Figure 4 Effect of (a) enprophylline and (b) theophylline on PHA-induced proliferation of HPBMC from healthy and asthmatic subjects. Data are expressed as per cent control (stimulated cells with no drug added). Each point represents mean ± s.e.mean. ($n=6$ for all groups).

but significantly abrogated HPBMC proliferation of both healthy (11 ± 2%) and asthmatic (17 ± 4%) groups at a concentration of 1 mM ($P<0.05$; $n=6$ per group; Figure 4b). There was no significant difference between healthy and asthmatic subjects with any of the adenosine receptor antagonists used ($P>0.05$ ANOVA).

Discussion

Theophylline and enprophylline equipotently suppress NECA-induced IL-8 release from the human mast cell line HMC-1 *via* A2b receptor antagonism (Feoktistov & Biaggioni, 1995). The dissociation constant for the antagonist-receptor complex (K_B: 7 μM) falls within the therapeutic plasma levels of theophylline and enprophylline (5–25 μmol L^{-1}) that have recently been shown to influence the activation of inflammatory cells (Sullivan *et al*., 1994a), suggesting that these drugs could produce some of their clinical effects *via* adenosine antagonism. However, theophylline only inhibited HPBMC proliferation at higher concentrations than are required to antagonize A2b receptors, indicating that its anti-proliferative effect *in vitro* is unlikely to be due to adenosine receptor antagonism. This conclusion is corroborated by the lack of anti-proliferative effect of the A2b receptor-selective antagonist, alloxazine. Furthermore, enprophylline did not have a significant anti-proliferative effect at relevant concentrations (10 μM), suggesting that the ability of this drug to act as a selective A2b antagonist in human mast cells does not extend to human mononuclear cells. Whilst the anti-proliferative effect of theophylline occurs at concentrations higher than achieved in plasma clinically, these effects may still be of relevance to our understanding of the effect of theophylline on circulating inflammatory cells since several such studies have reported that such effects of theophylline occur after chronic treatment, perhaps reflecting a requirement of the cells to be exposed to higher levels of drug (Nielson *et al*., 1986; Zocchi *et al*., 1985).

The mechanism for this anti-proliferative effect of theophylline is not known, but could be *via* inhibition of PDE isoenzymes. In support of this is the finding that, whilst theophylline potentiated the release of superoxide from human and guinea-pig eosinophils at clinically relevant concentrations (1–10 μM), superoxide generation was inhibited at higher concentrations (1 mM). In contrast, the related molecule 8-phenyltheophylline (an adenosine antagonist which lacks the ability to inhibit PDE activity) dose-dependently augmented superoxide release (Yukawa *et al*., 1989).

However, recent work has demonstrated that the effects of theophylline on inflammatory cells may be *via* a mechanism of action distinct from inhibition of PDE enzymes. Theophylline promotes apoptosis of human granulocytes while the selective PDE4 inhibitor rolipram promotes the survival of these cells (Yasui *et al*., 1997). Of more relevance to the current study is the observation that the related xanthine pentoxifylline can exert anti-proliferative effects independent of cyclic AMP generation (Peterson *et al*., 1998).

The role of adenosine in the control of cell proliferation has previously been investigated in a number of cell types, yielding conflicting results. Adenosine stimulates proliferation of cultured mesangial cells, an effect which is reversed by the addition of theophylline (MacLaughlin *et al*., 1997). Conversely, adenosine and selective A1 and A2 receptor agonists inhibit proliferation of Sertoli-like TM4 cells which can be prevented by methylxanthines (Shaban *et al*., 1995). The importance of endogenous adenosine has also been investigated. Proliferation of human umbilical vein endothelial cells is significantly decreased by the addition of adenosine deaminase, an effect which is reversed by NECA, CPA and CGS21680 (Sexl *et al*., 1995). In the same study it was demonstrated that a xanthine amine congener (also an adenosine receptor antagonist) inhibited proliferation of these cells, though proliferation could be restored when NECA was added. Thus, there is no consistent role for adenosine in the control of cell proliferation *in vitro*.

Several studies have been conducted investigating the effect of adenosine on the function of purified human monocytes and lymphocytes. It has been shown that production of tumour necrosis factor-alpha (TNFα), IL-6 and IL-8 from LPS-stimulated human monocytes is inhibited by adenosine, NECA and, to a lesser extent, CPA, thus showing that cytokine production is predominantly an A2 receptor-mediated event (Bouma *et al*., 1994). Similarly, it has been demonstrated that adenosine deaminase activity increases 3 fold in PHA-stimulated human peripheral blood lymphocytes after 24 h, before the onset of DNA synthesis (Hovi *et al*., 1976). When adenosine deaminase was inhibited by M coformycin, thymidine incorporation was significantly reduced and this effect was increased synergistically by the addition of adenosine (Hovi *et al*., 1976). However, our results suggest that adenosine deaminase does not play a significant role in regulating HPBMC proliferation *in vitro* as there was no significant effect when it was added to the culture medium. A possible explanation for the discrepancy between our findings and previous studies is the use of a mixed mononuclear cell population in our studies which could alter the way the cells respond to adenosine since any action of adenosine appears to be dependent on cell type.

Furthermore, our results do not support a major role for adenosine in modulating mononuclear cell proliferation since NECA (an A1/A2 agonist), CPA (an A1 agonist) and CSC (an A2a antagonist) only caused modest inhibition of HPBMC proliferation, and only at the highest concentrations of these drugs. In addition, we did not observe any major difference between the response of HPBMC isolated from asthmatic and healthy donors to any of the drugs used, suggesting that while adenosine may play an important role in asthma (Bjorck *et al*., 1992), there is no altered sensitivity of HPBMC to this mediator.

In summary, our results suggest that exogenous and endogenous adenosine have little impact on HPBMC proliferation, since various adenosine receptor agonists and antagonists, and adenosine deaminase had little or no effect on HPBMC from healthy and asthmatic subjects. While it remains plausible that adenosine may modulate other functions of HPBMC which could be influenced by theophylline, the anti-proliferative effect of this drug only occurs at higher concentrations than are required to significantly antagonize A2b receptors.

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