

RESEARCH PAPER

Reciprocal modulation of anti-IgE induced histamine release from human mast cells by A₁ and A_{2B} adenosine receptors

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BACKGROUND AND PURPOSE

Adenosine is believed to participate in the pathological development of asthma through a mast cell-dependent mechanism. Our study aimed to pharmacologically characterize the functions of adenosine receptor (AR) subtypes (A₁, A_{2A}, A_{2B} and A₃) in primary human cultured mast cells (HCMC).

EXPERIMENTAL APPROACH

HCMC were derived from progenitor stem cells in buffy coat and the effects of adenosine receptor ligands on basal and IgE-dependent histamine release were evaluated.

KEY RESULTS

Adenosine and analogues alone did not induce HCMC degranulation. When HCMC were activated by anti-IgE after 10 min pre-incubation with adenosine, a biphasic effect on histamine release was observed with enhancement of HCMC activation at low concentrations of adenosine (10^{-9} – 10^{-7} mol·L⁻¹) and inhibition at higher concentrations (10^{-6} – 10^{-4} mol·L⁻¹). The potentiating action was mimicked by A₁ AR agonists CCPA and 2'-MeCCPA, and inhibited by the A₁ AR antagonist PSB36. In contrast, the inhibitory action of adenosine was mimicked by the non-specific A₂ AR agonist CV1808 and attenuated by A_{2B} AR antagonists PSB1115 and MRS1760. The non-selective AR antagonist CGS15943 attenuated both the potentiating and inhibitory actions.

CONCLUSIONS AND IMPLICATIONS

We have defined for the first time the contribution of A₁ and A_{2B} ARs, respectively, to the potentiating and inhibitory action of adenosine on human mast cell activation. With reference to the current trend of developing novel anti-asthmatic agents from AR ligands, our results suggest that inhibition of human mast cell activation would be a mechanism for A₁ AR antagonists, but not A_{2B} AR antagonists.

Abbreviations

2-Cl-IB-MECA, 1-[2-chloro-6-[[[3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-b-D-ribofuranuronamide; 2'-MeCCPA, 2-chloro-N-cyclopentyl-2'-methyladenosine; AR, adenosine receptor, BAL, bronchoalveolar lavage; CCPA, 2-chloro-N⁶-cyclopentyladenosine; CGS15943, 9-chloro-2-(2-furanyl)-[1,2,4]triazolo [1,5-c]quinazolin-5-amine; CGS21680, 4-[2-[[6-amino-9-(N-ethyl-b-D-ribofuranuronam-idosyl)-9H-purin-2-yl]amino]ethyl]-benzenepropanoic acid hydrochloride; CV1808, 2-phenylaminoadenosine; Fc ϵ RI (IgE receptor); HCMC, human cultured mast cells; HEMADO, 2-(1-hexynyl)-N-methyladenosine; IMDM, Iscove's modified Dulbecco's medium; MRS1706 N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide; MRS3777, 2-phenoxy-6-(cyclo-hexylamino) purine hemioxalate; NBMPR, 6-S-[[4-nitrophenyl)methyl]-6-thioinosine; NECA, 5'-N-ethylcarboxamidoadenosine; PSB1115, 4-(2,3,6,7-tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzene-sulphonic acid potassium salt; PSB36, 1-butyl-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione; SCH442416, 2-(2-furanyl)-7-[3-(4-methoxy-phenyl)-propyl]-7H-pyrazolo[4,3-e][1,2,4] triazolo[1,5-c]pyrimidin-5-amine

Introduction

Activation of mast cells through cell surface IgE receptors (Fc ϵ RI) initiates the release of chemical mediators, which are pivotal in the pathogenesis of inflammatory disorders such as asthma. The mast cells store some of these mediators preformed in their secretory granules (e.g. histamine, neutral proteases and TNF- α) and synthesize others during activation (e.g. PGD $_2$, leukotrienes and various cytokines). The level of adenosine is increased in both bronchoalveolar lavage (BAL) fluids and exhaled breath condensate of patients with asthma (Livingston *et al.*, 2004), suggesting that this endogenous purine nucleotide is associated with the development of the disease. Moreover, adenosine and its precursor AMP can provoke bronchoconstriction when inhaled by asthmatic individuals, but not in non-asthmatic subjects (Phillips *et al.*, 1990; Forsythe and Ennis, 1999). Mast cells in the airways have been suggested to play a role in the bronchoconstricting effect of exogenous adenosine through the release of strong bronchospastic agents (van den Berge *et al.*, 2007).

Adenosine exerts its cellular effects through four distinct subtypes of adenosine receptor (AR), namely A $_1$, A $_{2A}$, A $_{2B}$ and A $_3$, which belong to the GPCR family (Jacobson and Gao, 2006; Spicuzza *et al.*, 2006). A $_1$ and A $_3$ ARs are G α_i -protein-coupled receptors, and their activation decreases adenylyl cyclase activity and cAMP levels, while PLC activity is increased by the $\beta\gamma$ subunit of the activated G $_i$ protein. In contrast, A $_{2A}$ and A $_{2B}$ ARs are G α_s -protein-coupled receptors and their activation conversely increases cAMP levels. Additionally, A $_1$ ARs have also been reported to couple with G α_o -proteins to activate K $^+$ or inhibit Ca $^{2+}$ conductance, whereas A $_{2B}$ ARs and A $_3$ ARs can also couple to G α_q -proteins and stimulate PLC activity (Jacobson and Gao, 2006; Alexander *et al.*, 2009). Adenosine at nanomolar concentrations can activate A $_1$ and A $_{2A}$ ARs, while sub-micromolar and micromolar concentrations are required for activating A $_3$ and A $_{2B}$ ARs, respectively (Klinger *et al.*, 2002).

Extensive pharmacological studies in rodents revealed that adenosine does not directly activate mast cells but can significantly potentiate mast cell activation induced by a variety of stimuli in an A $_3$ AR-dependent manner (Ramkumar *et al.*, 1993). However, investigations into the interaction between adenosine and human mast cells are less extensive and have produced controversial results. Early studies with dispersed human lung mast cells (HLMC) in general reported that adenosine did not induce histamine release alone but produced opposing effects on histamine release mediated by various secretagogues. Peters *et al.* (1982) and Peachell *et al.* (1991) reported that adenosine enhanced mediator release at low concentrations but exhibited inhibitory actions at high concentrations. However, Hughes *et al.* (1984) reported that adenosine and analogues in general inhibited antigen-induced histamine release dose-dependently when added to HLMC at times up to 5 min before activation. A small potentiation was only observed if adenosine was added 5 min after challenge. The discrepancies between these observations have been suggested to be due to different mast cell dispersion techniques, which modified membrane properties, or the use of impure mast cell preparations containing different degrees of purity in mast cells (Peachell *et al.*, 1991). Pharmacological studies suggested that the responses of HLMC to

adenosine displayed characteristics most closely resembling an A $_2$ AR-linked process (Hughes *et al.*, 1984; Peachell *et al.*, 1991).

In order to overcome the lack of a homogeneous population of human mast cells, studies were carried out in the human mast cell like (HMC-1) cell line, and it was demonstrated that the adenosine analogue NECA alone was able to evoke IL-8 release through A $_{2B}$ AR-mediated G α_q -coupled PLC activation (Feoktistov *et al.*, 1995). Because HMC-1 is an immature atypical mast cell-line that lacks Fc ϵ RI, the effect of adenosine on immunological stimulation cannot be truly reflected (Wilson *et al.*, 2009). With the development of culturing techniques, primary human mast cells were developed from progenitors isolated from human umbilical cord-blood, and an early study using these cells observed only an inhibitory action of adenosine (Suzuki *et al.*, 1998). Although the cord-blood derived human mast cells are a better working model for human tissue mast cells than HMC-1 cells, they have different characteristics from adult human mast cells (Inomata *et al.*, 2005; Holm *et al.*, 2008). In contrast, mast cells cultured from progenitors isolated from adult peripheral blood demonstrated a higher degree of resemblance to human tissue mast cells and are more preferred for functional studies (Wang *et al.*, 2006a; Holm *et al.*, 2008). In fact, our preliminary study demonstrated that adenosine produced a biphasic modulatory effect on anti-IgE-induced histamine release from primary mast cells cultured from human peripheral blood progenitors (Yip *et al.*, 2009). This further supports our postulation that these primary human cultured mast cells (HCMC) are suitable for the further characterization of the mast cell modulatory actions of adenosine in humans. The aim of the current study was to provide a more comprehensive pharmacological investigation into the roles of AR subtypes in the activation of primary human mast cells by using peripheral blood derived HCMC.

Methods

Reagents

Adenosine and anti-human IgE antibody (ϵ -chain specific) were purchased from Sigma (St. Louis, MO, USA). Human myeloma IgE and 6-S-[(4-nitrophenyl)methyl]-6-thioinosine (NBMPR) were purchased from Calbiochem (San Diego, CA, USA). AR agonists and antagonists were purchased from Tocris (Bristol, UK). The agonists were 2-chloro-N ^{6} -cyclopentyladenosine (CCPA), 2-chloro-N-cyclopentyl-2'-methyladenosine (2'-MeCCPA), 2-phenylaminoadenosine (CV1808), 4-[2-[[6-amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]-benzenepropanoic acid hydrochloride (CGS21680), 2-(1-hexynyl)-N-methyladenosine (HEMADO), 1-[2-chloro-6-[[3-(iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-b-D-ribofuranuronamide (2-Cl-IB-MECA) and 5'-N-ethylcarboxamidoadenosine (NECA). The antagonists were 1-butyl-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione (PSB36), 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH442416), 4-(2,3,6,7-tetrahydro-2,6-dioxo-1-propyl)-1H-purin-8-yl)-

benzene-sulphonic acid potassium salt (PSB1115), N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS1706), 2-phenoxy-6-(cyclo-hexylamino)purine hemioxalate (MRS 3777) and 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS 15943).

HCMC

Mature primary HCMC were derived from progenitors isolated from fresh buffy coat, as previously described (Wang *et al.*, 2006b). Briefly, isolated progenitors were first cultured with IL-6 and stem cell factor (SCF) (PeproTech Asia, Rehovot, Israel) in serum free methylcellulose (StemCell Technologies, Vancouver, Canada) for 6 weeks with IL-3 added in the first two weeks only. This was followed by 6 weeks of culture in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) supplemented with SCF and IL-6. HCMC were identified by May Grunwald-Giemsa (Merck, Darmstadt, Germany) staining of cytoplasmic granules, as well as immunocytochemical staining of human mast cell tryptase using a monoclonal anti-human tryptase antibody (Chemicon, Billerica, MA, USA) and the alkaline APAAP kit from Dako (Carpinteria, CA, USA).

Histamine release assay

At 12–14th week of culture, HCMC were sensitized with human myeloma IgE ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$), overnight at 37°C in IMDM in a 5% CO_2 incubator. Sensitized HCMC were washed with HEPES-buffered Tyrode solution with 0.03% human albumin and incubated with AR agonists for 0 min or 10 min before challenged with anti-IgE ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$). HCMC were only pre-incubated with adenosine for 10 min when activation with the calcium ionophore, A23187 ($10^{-6} \text{mol}\cdot\text{L}^{-1}$) was investigated. In antagonist studies, HCMC were pre-incubated with antagonists for 10 min before addition of AR agonists, and anti-IgE was added after 10 min. Histamine release was allowed to proceed for 30 min at 37°C following the addition of anti-IgE or A23187, and was stopped by addition of ice-cold buffer followed immediately by centrifugation. The cell pellets and supernatants were collected separately and histamine content was measured spectrofluorometrically using a Bran+Luebbe AutoAnalyzer 3 (Norderstedt, Germany). The percentage of total cellular histamine released into the supernatant was then calculated and expressed as [Histamine release (% total)] in Figures 1A and 2B. For facilitating comparison of the mast cell modulating actions of different AR ligands, all subsequent results were normalized against the anti-IgE-induced histamine release and expressed as the percentage of anti-IgE-induced histamine release (% of anti-IgE).

Statistical analysis

All values are expressed as mean $\pm$ SEM. Statistical analysis was performed where appropriate using ANOVA with Bonferroni's *post-test* by comparing the actual levels of histamine release, with the exception of Figure 2B where Student's *t-test* was used.

Results

Effects of adenosine and NECA on histamine release from HCMC

Incubation of HCMC with adenosine or NECA ($10^{-10} \text{mol}\cdot\text{L}^{-1}$ to $10^{-4} \text{mol}\cdot\text{L}^{-1}$) had no effect on basal histamine release, which was generally less than 7% of total cellular histamine. However, when added to HCMC at the time of anti-IgE challenge, adenosine inhibited histamine release from HCMC in a concentration-dependent manner (Figure 1A). Anti-IgE-induced histamine release was reduced to $45.5 \pm 5.8\%$ of the control level in the presence of $10^{-4} \text{mol}\cdot\text{L}^{-1}$ adenosine ($P < 0.001$). Hughes *et al.* (1984) and our earlier study (Yip *et al.*, 2009) demonstrated that varying the time of adenosine addition, with respect to immunological challenge, changed the modulatory function of adenosine. Accordingly, when HCMC were incubated with adenosine ($10^{-8} \text{mol}\cdot\text{L}^{-1}$) 10 min prior to anti-IgE challenge, anti-IgE-induced histamine release was enhanced by $13.9 \pm 3.3\%$ ($P < 0.01$), while inhibition was only observed at concentrations above $10^{-6} \text{mol}\cdot\text{L}^{-1}$ (Figure 1A). Furthermore, when histamine release was induced by the calcium ionophore A23187, adenosine failed to induce any modulatory action (Figure 1A). The potentiating action of adenosine on anti-IgE-induced histamine release became significant after 2 min pre-incubation with $10^{-8} \text{mol}\cdot\text{L}^{-1}$ adenosine and remained at a similar level even when the pre-incubation period was extended to 15 min (Figure 1B). As for the inhibitory action of $10^{-5} \text{mol}\cdot\text{L}^{-1}$ adenosine, it was observed without pre-incubation and remained at the same level after 15 min pre-incubation. To determine whether the mast cell modulating actions of adenosine were mediated by an interaction with cell surface receptors, the adenosine uptake inhibitor NBMPR was added 10 min before addition of adenosine. As illustrated in Figure 1C, NBMPR failed to affect the adenosine-mediated potentiation and inhibition of anti-IgE-induced histamine release. Similar to adenosine, the non-selective AR agonist NECA also produced a time-dependent biphasic action on anti-IgE-activated HCMC (Figure 2A). When added at the time of anti-IgE activation, NECA demonstrated only an inhibitory effect, and anti-IgE-induced histamine release was reduced to $67.5 \pm 6.2\%$ ($P < 0.01$) in the presence of $10^{-5} \text{mol}\cdot\text{L}^{-1}$ NECA. As seen with responses to adenosine, potentiation of histamine release was observed when HCMC were pre-incubated with $10^{-9} \text{mol}\cdot\text{L}^{-1}$ and $10^{-8} \text{mol}\cdot\text{L}^{-1}$ NECA for 10 min before stimulation with anti-IgE. NECA at $10^{-8} \text{mol}\cdot\text{L}^{-1}$ produced a significant enhancement of anti-IgE-induced histamine release, $29.5 \pm 4.2\%$ ($P < 0.001$), while an inhibitory action was observed at concentrations above $10^{-7} \text{mol}\cdot\text{L}^{-1}$. To investigate whether the biphasic action of non-selective AR agonists is altered by the degree of mast cell activation, we compared the effects of NECA on HCMC that released above 30% of their total cellular histamine with those that released less than 30%. For both groups of HCMC, adenosine produced comparable levels of potentiation and inhibition at $10^{-8} \text{mol}\cdot\text{L}^{-1}$ and $10^{-5} \text{mol}\cdot\text{L}^{-1}$, respectively. As illustrated in Figure 2B, anti-IgE-induced histamine release was increased to $136.8 \pm 7.8\%$ and $128.2 \pm 3.0\%$ of control by $10^{-8} \text{mol}\cdot\text{L}^{-1}$ NECA in the low and high responders, respectively. As for the degree of inhibition produced by

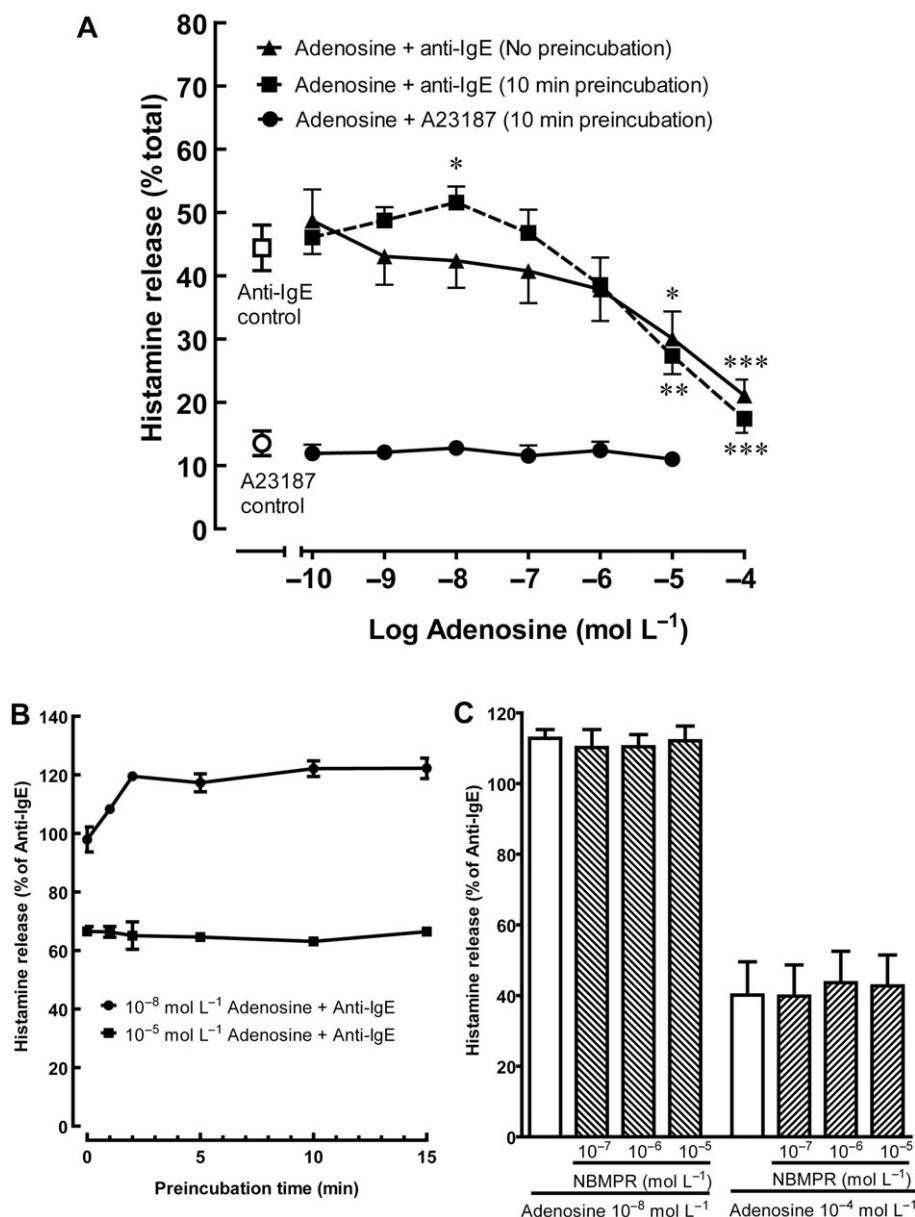


Figure 1

Effects of adenosine on histamine release from activated HCMC. (A) Sensitized HCMC were incubated with adenosine for 0 (no pre-incubation) or 10 min prior to being challenged with anti-IgE ($n = 5$) or calcium ionophore A23187 ($n = 3$) for 30 min. The actual percentage of total cellular histamine released into the supernatant was listed. Significant differences between histamine release induced by anti-IgE alone and in the presence of adenosine are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) Sensitized HCMC were pre-incubated with adenosine (10^{-8} mol·L⁻¹ for potentiation and 10^{-5} mol·L⁻¹ for inhibition) for 0, 1, 2, 5, 10 and 15 min prior to being challenged with anti-IgE ($n = 3$). (C) HCMC were pre-incubated for 10 min with NBMPR and then subsequently incubated with 10^{-8} mol·L⁻¹ or 10^{-4} mol·L⁻¹ adenosine prior to being challenged with anti-IgE ($n = 3$). Results are expressed as a percentage of histamine release induced by anti-IgE alone (30.4–36.1% of total cellular histamine) for (B) and (C) and all values are mean $\pm$ SEM of the number of experiments indicated.

10^{-5} mol·L⁻¹ NECA, it was $65.0 \pm 11.6\%$ and $53.4 \pm 9.7\%$ of control for the low and high responders, respectively.

Effects of AR agonists on anti-IgE-induced histamine release from HCMC

The AR agonists tested in the current study were selected according to their reported affinity or potency at the various AR subtypes, as listed in Table 1. All the agonists when tested

alone did not affect basal histamine release from HCMC. Among them, only high concentrations of the A₁ AR agonists, CCPA and 2'MeCCPA, induced a significant enhancement of HCMC activation when they were pre-incubated with HCMC for 10 min before anti-IgE challenge (Figure 3). Anti-IgE-induced histamine release was increased by $22.9 \pm 4.5\%$ ($P < 0.01$) and $27.7 \pm 4.1\%$ ($P < 0.001$) in the presence of CCPA and 2'MeCCPA (10^{-5} mol·L⁻¹), respectively (Figure 3A and B).

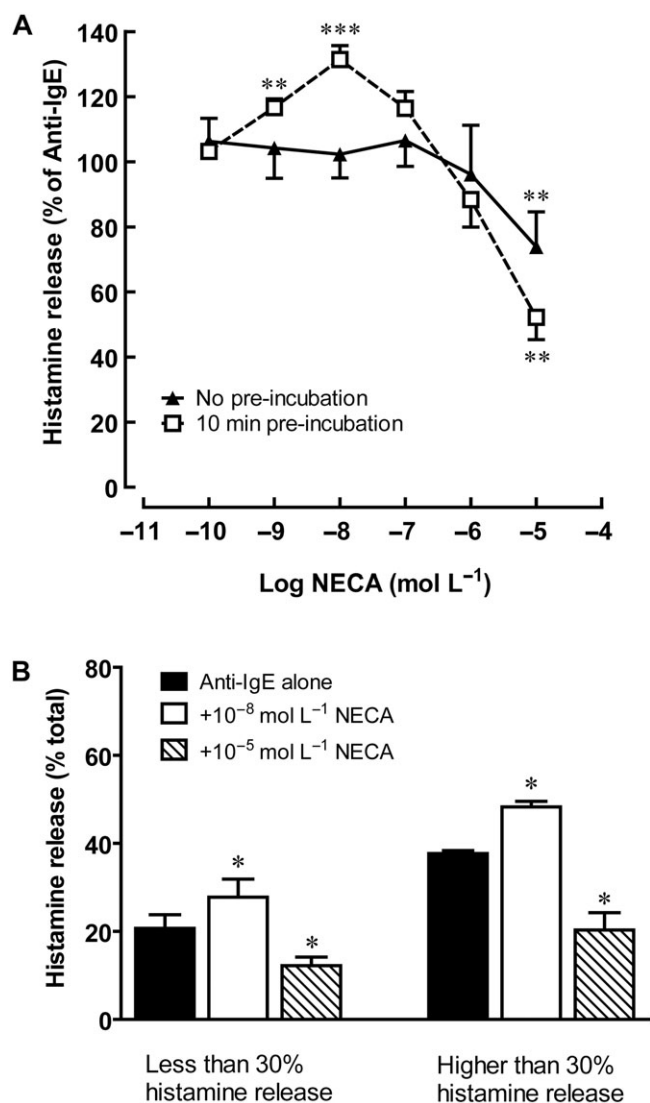


Figure 2

Effects of NECA on anti-IgE-induced histamine release from HCMC. (A) Sensitized HCMC were incubated with NECA for 0 (no pre-incubation) or 10 min prior to being challenged with anti-IgE for 30 min. Results are expressed as a percentage of histamine release induced by anti-IgE alone (28.8–43.8% of total cellular histamine). Significant differences between histamine release induced by anti-IgE alone and in the presence of NECA are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 5$). (B) The degree of modulation produced by 10 min pre-incubation with NECA was compared in low-responding HCMC ($20.7 \pm 3.1\%$ histamine release, $n = 6$) and high-responding HCMC ($37.7 \pm 0.7\%$ histamine release, $n = 5$). The actual percentage of total cellular histamine released into the supernatant was listed. * $P < 0.05$ indicates significant differences between histamine release induced by anti-IgE alone and in the presence of NECA.

As with adenosine and NECA, this enhancing effect was not observed when the agonists were added to HCMC at the time of anti-IgE stimulation. In a separate experiment involving three independent samples from different donors, the potentiating action of 10^{-5} mol·L⁻¹ 2'MeCCPA (increased anti-IgE-

induced histamine release to $126.7 \pm 5.7\%$ of control, $P < 0.05$ vs. anti-IgE control) was significantly attenuated ($105 \pm 3.9\%$ of anti-IgE-induced histamine release) in the presence of 10^{-6} mol·L⁻¹ PSB36, an A₁ AR-specific antagonist, but not by 10^{-6} mol·L⁻¹ of the A₃ AR-specific antagonist MRS 3777.

In contrast to the A₁ AR agonists, the non-selective A₂ AR agonist CV1808 demonstrated an inhibitory effect on anti-IgE-induced activation at 10^{-4} mol·L⁻¹, which was not observed with the A_{2A} AR agonist CGS21680 (Figure 3C and D). Although the A₃ AR agonist 2-Cl-IB-MECA significantly reduced anti-IgE-induced histamine release from HCMC at 10^{-6} mol·L⁻¹ (Figure 3E), the other A₃ AR agonist HEMADO did not demonstrate any significant mast cell modulatory effect (Figure 3F). In a separate set of experiments involving three independent samples from different donors, the inhibitory action of 10^{-5} mol·L⁻¹ 2-Cl-IB-MECA (reduced anti-IgE-induced histamine release to $73.8 \pm 6.7\%$ of control, $P < 0.05$ vs. anti-IgE control) was blocked by 10^{-6} mol·L⁻¹ of the specific A_{2B} AR antagonist, PSB 1115, with anti-IgE-induced release restored to $90.3 \pm 4.8\%$ of control, but not by 10^{-6} mol·L⁻¹ of the A₃ AR specific antagonist, MRS 3777.

Effects of AR antagonists on adenosine and NECA-mediated effects on HCMC activation

The antagonists tested in the current study were selected according to their reported affinity for the various AR subtypes, as listed in Table 1. None of the antagonists alone affected basal or anti-IgE-induced histamine release. Instead of studying their effects on the whole concentration-response curves of adenosine or NECA, the effects of the antagonists on the potentiating action produced by 10^{-4} mol·L⁻¹ adenosine or 10^{-5} mol·L⁻¹ NECA and the inhibitory action produced by 10^{-8} mol·L⁻¹ of adenosine or NECA on anti-IgE-induced histamine release following 10 min pre-incubation with these agonists were investigated. Both actions of adenosine and NECA on activated HCMCs were significantly attenuated by the non-selective adenosine AR antagonist CGS15943, in a concentration-dependent manner (Figures 4A and 5A). The potentiating actions of adenosine and NECA, were concentration-dependently antagonized by the A₁ AR antagonist, PSB36, but not by the A_{2A} antagonist, SCH442416 and the A_{2B} AR antagonists, PSB1115 and MRS1706 (Figure 4B–E). The potentiation of histamine release was almost completely abolished at the highest concentration of PSB36 tested (Figure 4B). Although the A₃ AR antagonist MRS3777 slightly reduced the enhancing actions of adenosine and NECA, the effect was not statistically significant (Figure 4F).

The inhibitory effects of adenosine and NECA were prevented by the selective A_{2B} AR antagonists, PSB1115 and MRS1706 (Figure 5D and E). The level of anti-IgE-induced histamine release was restored to 80–90% of that produced by anti-IgE alone in the presence of either A_{2B} antagonist at 10^{-6} mol·L⁻¹. In contrast, the A₁, A_{2A} and A₃ AR antagonists all failed to attenuate the inhibitory effect of adenosine and NECA (Figure 5B, C and F, respectively).

Discussion

We have previously reported preliminary results, which demonstrated that adenosine was able to produce a biphasic

Table 1

Affinity values of agonists and antagonists at human adenosine receptor subtypes

	A ₁	A _{2A} K _i (nmol·L ⁻¹)	A _{2B}	A ₃	References
Agonist					
Adenosine	310 ^{aa}	730 ^{aa}	23 500 ^{aa}	290 ^{aa}	(Fredholm <i>et al.</i> , 2001b)
NECA	14	20	2 400 ^{aa}	6.2	(Klotz, 2000)
CCPA	0.83	2 270	18 800	38	(Jacobson and Gao, 2006)
2'MeCCPA	3.3	9580	37 600	1 150	(Cappellacci <i>et al.</i> , 2005)
CGS21680	290	27	88 800 ^{aa}	67	(Klotz, 2000)
CV1808	N.D.	76 ^a	N.D.	1 450 ^b	^a (Dionisotti <i>et al.</i> , 1997) ^b (Varani <i>et al.</i> , 1998)
2-Cl-IB-MECA	220	5 360	>100 000	0.33	(Jacobson and Gao, 2006)
HEMADO	327	1 230	>100 000	1.1	(Klotz <i>et al.</i> , 2007)
Antagonists					
CGS15943	3.5	4.2	16	51	(Fredholm <i>et al.</i> , 2001a)
PSB36	0.12	552	187	2 300	(Weyler <i>et al.</i> , 2006)
SCH442416	1 111	0.048	>10 000	>10 000	(Moro <i>et al.</i> , 2006)
PSB1115	>10 000	24 000	53.4	>10 000	(Moro <i>et al.</i> , 2006)
MRS1706	403	503	2	570	(Moro <i>et al.</i> , 2006)
MRS3777	>10 000	>10 000	>10 000	47	(Jacobson and Gao, 2006)

Data were collected from various references listed. ND = Not determined.

^{aa}EC₅₀ values of agonists at human adenosine receptors were determined in a functional assay using changes in cAMP formation in CHO cells stably transfected with the human A₁, A_{2A}, A_{2B}, and A₃ receptors.

K_i values were obtained from binding experiments at recombinant human A₁, A_{2A}, A_{2B} and A₃ adenosine receptors.

modulation of anti-IgE-induced histamine release from HCMC (Yip *et al.*, 2009). Our current results extend these observations to include the effect of the more potent non-selective AR agonist NECA. Furthermore, we have demonstrated that both adenosine and NECA at concentrations above 10⁻⁶ mol·L⁻¹ produced a predominantly inhibitory effect on HCMC activation induced by anti-IgE challenge. A potentiating effect was observed at 10⁻⁸ mol·L⁻¹ only after HCMC were pre-incubated with these non-selective AR agonists for 10 min prior to anti-IgE challenge. Although the inhibitory action required no prior incubation with AR agonists, the development of the potentiating effect was time-dependent; optimal potentiation was reached after 2 min pre-incubation with adenosine prior to anti-IgE-induced activation (Figure 1B). Furthermore, neither the degree of potentiation nor inhibition was affected by the level of anti-IgE-induced histamine release, as demonstrated in Figure 2B. The effects of adenosine and NECA observed in our current study are similar to those previously reported for highly purified dispersed HLMC in which NECA demonstrated a biphasic action on anti-IgE-induced histamine release (Peachell *et al.*, 1991), and thus, further support our previous observations that our cultured human mast cells are functionally similar to primary HLMCs (Wang *et al.*, 2006a). As histamine release induced by the calcium ionophore A23187 was not affected by adenosine, the mast cell modulatory action of adenosine is specific for the IgE receptor-mediated signalling that precedes the influx of calcium. The concentration-

dependent biphasic effect on anti-IgE-induced histamine release induced by both adenosine and NECA suggests the activation of AR subtypes with different affinity for these ligands (Calabrese, 2001). The inhibition of the biphasic action of adenosine by the non-selective AR antagonist CGS15943 confirms that adenosine specifically interacts with ARs in HCMC (Figures 4A and 5A). The failure of the adenosine uptake blocker NBMPR to effect the mast cell modulatory actions of adenosine (Figure 1C) and the fact that NECA is not a substrate of the adenosine transporter (Meester *et al.*, 1998) suggest that these ARs are located in the cell membrane of HCMC. In our earlier study (Yip *et al.*, 2009), we proposed that A₁ and A_{2B} ARs are involved in the modulatory action of adenosine on human mast cells. This hypothesis is now confirmed in the current investigation in which the effects of more agonists over a wider concentration range were studied and selective antagonists used.

The significant enhancement of HCMC activation by the A₁ AR agonists, CCPA and 2'MeCCPA (Figure 3A and B), and the suppression of the effects of adenosine and NECA by the A₁ AR antagonist PSB36 (Figure 4B), suggest that adenosine potentiates anti-IgE-induced histamine release from HCMC through activation of A₁ ARs. Although the A₃ AR antagonist MRS3777 also suppressed the potentiating action of adenosine, this effect was not statistically significant and required high concentrations of this antagonist (Figure 4F). The ineffectiveness of the two specific A₃ AR agonists to enhance anti-IgE-induced histamine release further rules out the

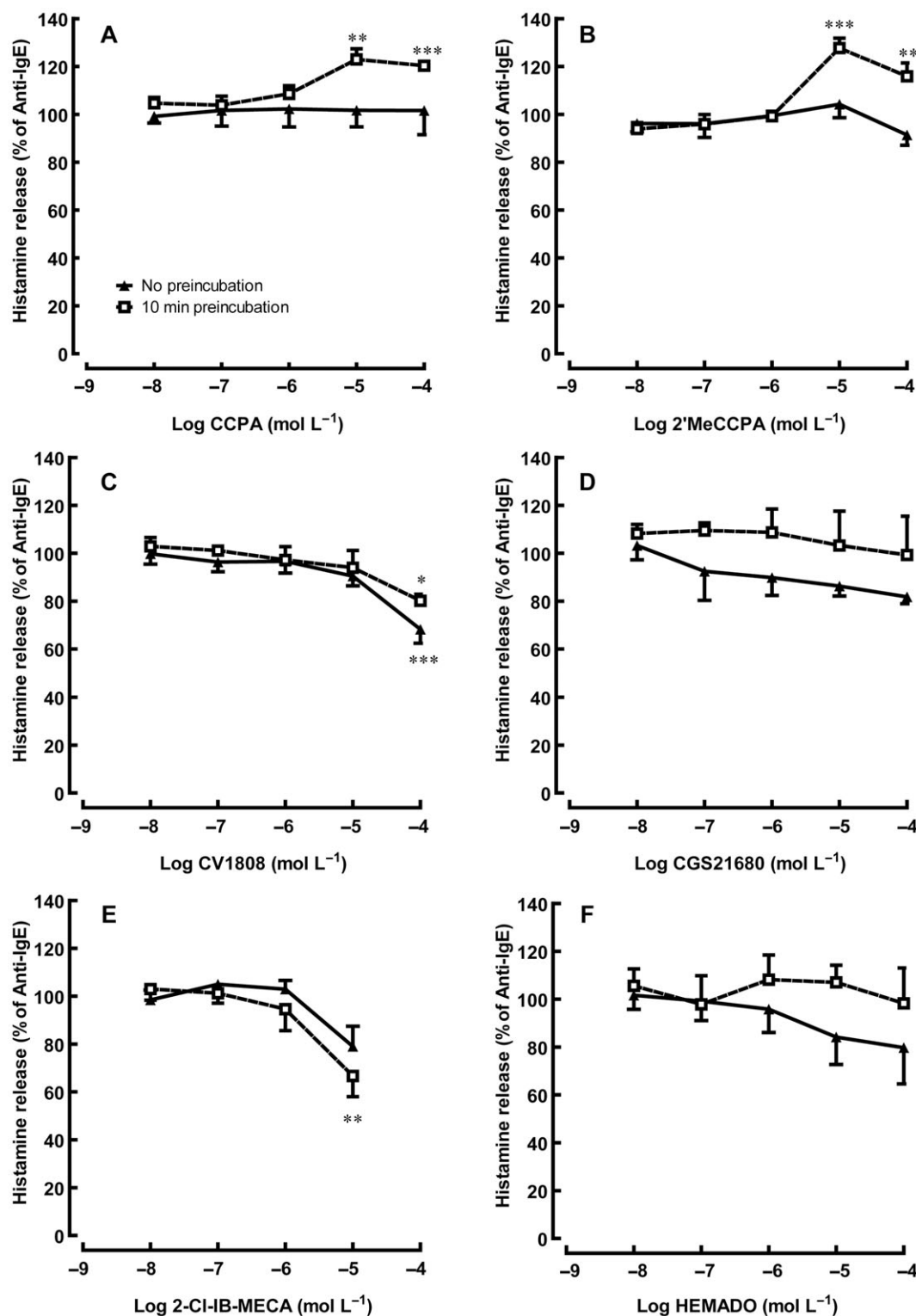


Figure 3

Effects of adenosine receptor agonists on anti-IgE-induced histamine release from HCMC. Sensitized HCMC were incubated with an adenosine receptor agonist for 0 (no pre-incubation) or 10 min prior to being challenged with anti-IgE for 30 min. Adenosine agonists used were: (A, B) selective A₁ adenosine receptor agonists CCPA and 2'MeCCPA (C) A₂ adenosine receptor agonists CV1808 (D) selective A_{2A} adenosine receptor agonist CGS21680 and (E, F) selective A₃ adenosine receptor agonists 2-CI-IB-MECA and HEMADO. Significant differences between the histamine release induced by anti-IgE alone and in the presence of adenosine receptor agonists are indicated by asterisks: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Results are expressed as a percentage of histamine release induced by anti-IgE alone (28.6–41.6% of total cellular histamine) and all values are mean ± SEM of four experiments.

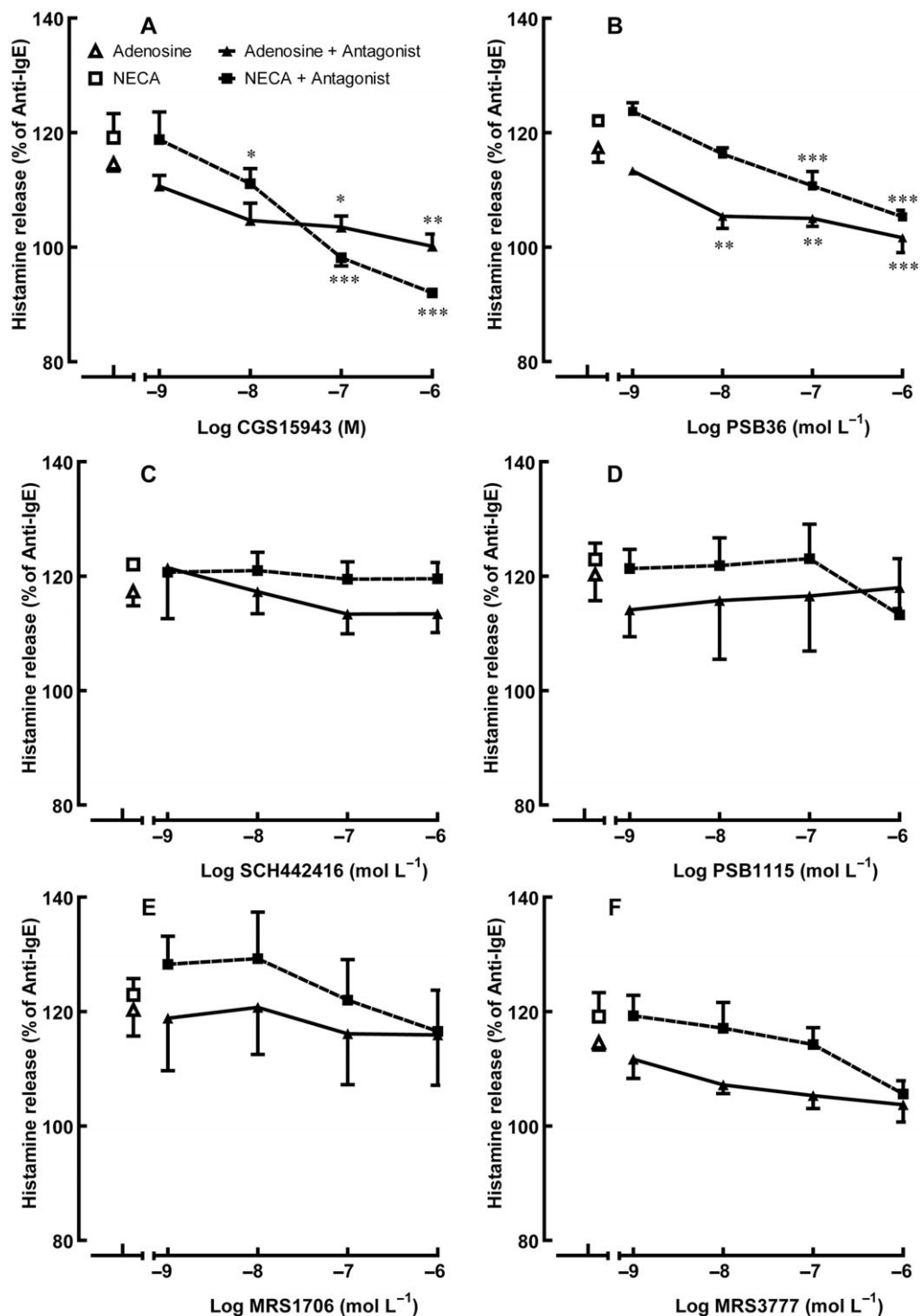


Figure 4

Effect of adenosine receptor antagonists on adenosine/NECA-mediated potentiation of anti-IgE-induced histamine release from HCMC. Sensitized HCMC were pre-incubated with an adenosine receptor antagonist for 10 min and then subsequently with 10^{-8} mol·L $^{-1}$ adenosine or 10^{-8} M NECA for 10 more min prior to being challenged with anti-IgE for 30 min. Adenosine receptor antagonists used were: (A) the non-selective adenosine receptor antagonist CGS15943 (B) the selective A_1 adenosine receptor antagonist PSB36 (C) the selective A_{2A} adenosine receptor antagonist SCH442416 (D, E) selective A_{2B} adenosine receptor antagonists PSB1115 and MRS1706, respectively, and (F) the selective A_3 adenosine receptor antagonist MRS3777. Significant differences between the percentage of anti-IgE-induced histamine release in adenosine/NECA alone and in the presence of adenosine/NECA with adenosine receptor antagonists are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Results are expressed as a percentage of histamine release induced by anti-IgE alone (28.5–48.3% of total cellular histamine) and all values are mean $\pm$ SEM of four experiments.

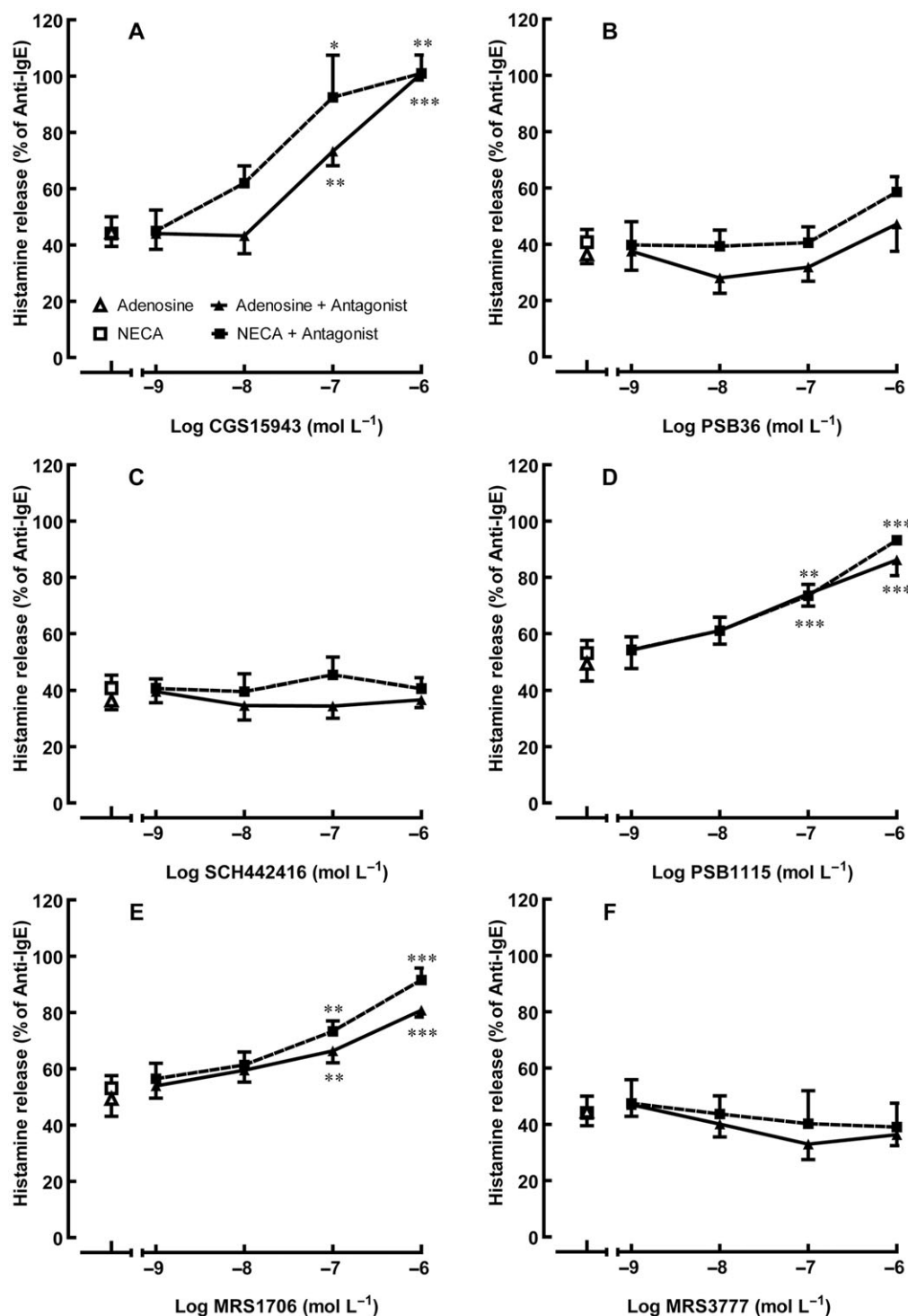


Figure 5

Effect of adenosine receptor antagonists on adenosine/NECA-mediated inhibition of anti-IgE-induced histamine release from HMCs. Sensitized HMCs were pre-incubated with an adenosine receptor antagonist for 10 min and then subsequently with 10^{-4} mol·L⁻¹ adenosine or 10^{-5} mol·L⁻¹ NECA for 10 more minutes prior to being challenged with anti-IgE for 30 min. Adenosine receptor antagonists used were: (A) the non-selective adenosine receptor antagonist CGS15943 (B) the selective A₁ adenosine receptor antagonist PSB36 (C) the selective A_{2A} adenosine receptor antagonist SCH442416 (D, E) selective A_{2B} adenosine receptor antagonists PSB1115 and MRS1706, respectively, and (F) the selective A₃ adenosine receptor antagonist MRS3777. Significant differences between the percentage of anti-IgE-induced histamine release in adenosine/NECA alone and in the presence of adenosine/NECA with adenosine receptor antagonists are indicated by asterisks: * P < 0.05, ** P < 0.01, *** P < 0.001. Results are expressed as a percentage of histamine release induced by anti-IgE alone (28.5–48.3% of total cellular histamine) and all values are mean $\pm$ SEM of four experiments.

involvement of the A₃ AR (Figure 3E and F). A direct correlation between A₁ AR and human mast cell activation has not been previously reported, but A₁ AR was suggested to promote adenosine-induced hyper-responsiveness and allergen-induced early and late phase allergic responses in a hyper-responsive rabbit model of asthma (Ali *et al.*, 1994; Obiefuna *et al.*, 2005). Moreover, A₁ AR expression was found to be markedly up-regulated in bronchial tissue obtained from subjects with asthma who are responsive to AMP challenge (Brown *et al.*, 2008a). Stimulation of the A₁ AR leads to inhibition of adenylyl cyclase activity through G α -protein and activation of PLC through the $\beta\gamma$ subunit (Jacobson and Gao, 2006). It is well known that activation of PLC generates metabolites that trigger the mobilization of intracellular Ca²⁺ stores and subsequently opening of Ca²⁺ channels in the plasma membrane. The increase in intracellular calcium consequently initiates the degranulation process of mast cells (Bradding, 2005). As calcium ionophores directly transport extracellular calcium into the cytosol, the failure of adenosine to affect histamine release induced by A23187 supports the involvement of such early calcium elevation mechanisms, which are bypassed by A23187, in the activation of A₁ AR. These signalling events are capable of up-regulating IgE receptor-mediated mast cell activation and may help to explain the observed potentiating effect of A₁ AR agonists on HCMC.

Both adenosine and NECA bind to the A₁ ARs with high affinity and this corresponds to the low concentrations required for producing the potentiating action observed in the current study. However, the requirement of at least 10⁻⁵ mol·L⁻¹ of the specific A₁ AR agonists, CCPA and 2'MeCCPA (both with K_i values of around 10⁻⁹ mol·L⁻¹ for A₁ AR), to produce a significant potentiating effect, is unexpected. Despite these high concentrations, the potentiating effect demonstrated by the A₁ AR agonists were confirmed to be mediated through A₁ AR as this effect was totally suppressed by the A₁ AR specific antagonist PSB36 (Figure 4B), but not by the A₃ AR antagonist MRS3777 (Figure 4F). It is also interesting to note that the inhibitory potency of CV1808 (Figure 3C) and the inhibitory tendency of high concentrations of CGS21680 and HEMADO (Figure 3D and E, respectively) were less apparent in cells that were activated after 10 min pre-incubation with these agonists. At concentrations above micromolar, these AR agonists would be non-selective and are likely to induce a time-dependent activation of A₁ AR, which would produce a potentiating effect that would counteract the more dominant suppressive action mediated at the same time by the A_{2B} AR.

The lower potency of the two A₁ AR agonists than that anticipated and the time-dependent characteristic of the A₁ AR-mediated action may be due to the location of the receptors in the cell membrane. In cardiomyocytes, A₁ ARs have been found to be localized in lipid rafts/caveolae under basal conditions (Lasley *et al.*, 2000; Cavalli *et al.*, 2007). Upon the addition of agonists, A₁ ARs were shown to translocate out of the caveolae and disperse into the general plasma membrane (Lasley *et al.*, 2000). The exact function of this translocation process is not known, but it can be prevented by prior incubation of the myocytes with the A₁ AR antagonist, 8-cyclopentyl-1,3-dipropylxanthine. The lack of potentiating action of adenosine, NECA or the specific A₁ AR agonists

when added to HCMC at the time of anti-IgE-induced activation may thus be due to the time required for the A₁ ARs to translocate from the caveolae to the plasma membrane, where they can functionally interact with the IgE receptor-mediated signalling pathways. As for the lower potency of the two A₁ agonists, it may be caused by difficulties of these ligands in reaching the A₁ ARs in the caveolae. Obviously, further studies are required to confirm this hypothesis. The physiological concentration of adenosine in tissues is reported to be in the range of 0.5–2 × 10⁻⁷ mol·L⁻¹ (Latini *et al.*, 2001), and activation of the A₁ AR on human mast cells at the initiation of an allergic reaction could serve the purpose of accentuating the IgE receptor-mediated activation to provide a more substantial stimulation of the immunological response against the potentially harmful exogenous allergen.

Elevation of intracellular cAMP leads to the suppression of human mast cell activation (Peachell *et al.*, 1988), suggesting a role for G α _s coupled A₂ AR in the inhibitory actions of adenosine and NECA. The A_{2B} AR subtype is likely to be involved, as the high concentrations of adenosine and NECA required to significantly suppress anti-IgE-induced histamine release correlate with the low affinity of these ligands for A_{2B} AR. This hypothesis was supported by the observation that significant inhibition of anti-IgE-induced histamine release was induced only by the non-selective A₂ AR agonist CV1808 (Figure 3C), but not by the A_{2A} AR selective agonist CGS21680 (Figure 3D) even at 10⁻⁴ mol·L⁻¹. NECA is currently the most potent agonist of A_{2B} AR, while CV1808 and CGS21680 have been shown to be 108- and 626-fold less potent than NECA, respectively, in a study investigating the potency of A₂ AR agonists in guinea pig (Gurden *et al.*, 1993). It is, thus, not surprising that significant inhibition of anti-IgE-induced histamine release by CV1808 was observed only at 10⁻⁴ mol·L⁻¹ (Figure 3C), whereas 10⁻⁶ mol·L⁻¹ NECA (Figure 2A) was required to produce a similar effect. The definitive evidence for the involvement of A_{2B} AR comes from the observation that only the selective A_{2B} AR antagonists, PSB1115 and MRS1706 (Figure 5D and E), but not the selective A_{2A} AR antagonist SCH442416 (Figure 5C), demonstrated concentration-dependent attenuation of the inhibitory effect of adenosine and NECA. The reduction of anti-IgE-induced histamine release by 10⁻⁵ mol·L⁻¹ of the A₃ AR agonist 2-Cl-IB-MECA (Figure 3E) was also mediated through the A_{2B} AR, as this effect was attenuated by 10⁻⁶ mol·L⁻¹ PSB 1115.

Our results demonstrate and confirm for the first time that the A_{2B} AR is associated with an inhibitory action of adenosine in human mast cells. However, our findings contrast with those from an earlier study in cord blood derived human mast cells (CBMC) in which it was reported that the inhibitory action of adenosine was mediated through the A_{2A} AR (Suzuki *et al.*, 1998). The discrepancy may be due to intrinsic differences between mast cells cultured from these two sources. CBMC are regarded as less suitable than peripheral blood derived mast cells as a tool for studying physiological and pathological characteristics of human mast cells due to their lower content of histamine and incomplete expression of IgE receptors (Andersen *et al.*, 2008). Moreover, the significance of the CBMC study is questionable as the conclusion was based on only two independent experiments employing limited pharmacological tools. Although it was

reported that the A_{2A} AR antagonist, ZM241383, could attenuate adenosine-induced inhibition of human mast cell activation at concentrations above 10^{-8} mol·L⁻¹, this effect could still be mediated through A_{2B} ARs as the pA_2 of this antagonist at the A_{2B} AR is around 7.5 (Prentice *et al.*, 1997).

According to studies employing primary human tissue or cultured mast cells, adenosine does not directly induce release of mediators. In contrast, studies with the mast cell like HMC-1 cell line have demonstrated that adenosine can directly induce IL-8 release through $G\alpha_q$ protein coupled signalling pathways (Feoktistov *et al.*, 1995; 1999). HMC-1 cells are derived from a highly malignant, undifferentiated human mastocytoma cancer and are regarded as atypical mast cells as they do not possess functional IgE receptors. The relevance of the direct activating action of adenosine in HMC-1 cells to the roles of adenosine in IgE immunologically-sensitized human mast cells in allergic asthma is thus doubtful (Wilson *et al.*, 2009). Although some studies using bronchoalveolar (BAL) fluid attempted to provide indirect evidence to support the hypothesis that mast cells in the BAL fluid of atopic asthmatic patients are particularly sensitive to the stimulating action of adenosine due to the presence of $G\alpha_q$ coupled A_{2B} ARs (Forsythe *et al.*, 1999), the BAL fluid contains other cells in addition to mast cells, and an additional signal from these contaminating cells might have acted to promote mast cell stimulation by adenosine (Brown *et al.*, 2008b). Further studies are required to confirm the existence of $G\alpha_q$ coupled A_{2B} ARs in purified BAL mast cells of atopic asthmatic patients and to explain why BAL mast cells of normal subjects do not have this property; inhaled adenosine does not induce bronchoconstriction in them.

The suppression of mast cell activation by adenosine through G_s -coupled A_{2B} ARs has recently also been demonstrated in mice (Hua *et al.*, 2007). Murine bone marrow derived mast cells obtained from A_{2B} AR-deficient mice were reported to have reduced basal levels of cAMP and to produce an excessive influx of extracellular calcium through store-operated calcium channels following antigen activation. The functional role of A_{2B} ARs was further demonstrated by the observed increased sensitivity to IgE-mediated anaphylaxis in mice lacking these receptors. The authors thus conclude that the response of mast cells towards antigen stimulation was limited by modulation of the IgE receptor-induced signalling pathways by A_{2B} AR. A more recent study in a murine model of endotoxin-induced lung injury has further confirmed a general protective effect of A_{2B} AR against pulmonary inflammation (Schingnitz *et al.*, 2010). Pharmacological suppression of A_{2B} ARs by the specific A_{2B} AR antagonist MRS 1754 or genetic deletion of the A_{2B} AR in functional studies of LPS-induced lung injury resulted in dramatic increases in lung inflammation and histological tissue injury. Furthermore, pretreatment of mice with a specific A_{2B} AR agonist (BAY 60-6583) significantly attenuated lung inflammation and pulmonary oedema in wild-type, but not in A_{2B} AR-deficient mice. During an inflammatory response the local concentration of adenosine in tissues has been reported to exceed micromolar concentrations (Driver *et al.*, 1993), and this would allow the relatively insensitive A_{2B} AR to be activated with the consequence of turning on the associated anti-inflammatory and tissue protective signalling in various acute inflammatory conditions.

In conclusion, our studies using HCMC have confirmed the original observation in HLMCs that low concentrations of adenosine potentiate, while high concentrations of this nucleotide suppress IgE receptor-mediated histamine release from human mast cells (Peachell *et al.*, 1991). In addition, we have provided the first direct evidence that the A_1 and A_{2B} ARs are responsible for the potentiating and suppressing actions of adenosine, respectively. Such effects are in accordance with the respective coupling of A_1 and A_{2B} ARs to $G\alpha_i$ and $G\alpha_s$ proteins. We also confirmed that adenosine does not stimulate normal, non-activated mast cells, and our results do not support a facilitating role of A_{2B} ARs in human mast cell activation as proposed by studies employing HMC-1 cells. With reference to the propagation of allergen-induced inflammatory reactions, the enhancement of IgE-mediated mast cell activation by low physiological concentrations of adenosine through the high affinity A_1 AR would be important in amplifying the immediate immunological response against an invading allergen, whereas the suppression of mast cell activation induced by a high level of adenosine through A_{2B} ARs would serve to limit the extent of inflammation and prevent excessive tissue damage. Although current research suggests that A_{2B} AR antagonists are potentially useful in the control of asthma (Brown *et al.*, 2008b), inhibition of mast cell activation, as indicated by our results, does not seem to be a part of their mechanism of action. Alternatively, unjustified use of A_{2B} antagonists in non-asthmatic subjects may jeopardize the protective effect of A_{2B} ARs against endotoxin-induced pulmonary inflammation. Our findings further suggest that the development of A_1 AR antagonists as anti-asthmatic agents may reduce the contribution of mast cells to allergen-induced asthma. Finally, one cannot rule out the possibility that airway mast cells in asthmatic patients display different profiles of adenosine-mediated responses, but further studies are required to provide direct evidence.

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Conflicts of interest

None.

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