Gβγ-coupled adenosine receptors differentially limit antigen-induced mast cell activation

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Non-standard abbreviations:
ASM- airway smooth muscle
AHR- airway hyperresponsiveness
wt- wild type
BMMCs- bone marrow-derived mast cells
NECA- 5’-N-ethylcarboxamido adenosine
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LTB₄- leukotriene B₄
DNP-HSA- dinitrophenyl-human serum albumin
DMSO- Dimethyl sulfoxide
PBS- phosphate buffered saline
PCA- passive cutaneous anaphylaxis
PSA- passive systemic anaphylaxis
SCF- stem cell factor
BAL- bronchoalveolar lavage
DAMP- danger-associated molecular pattern
HUCBMCs- human umbilical cord-blood derived mast cells
ECP- eosinophil cationic protein
LPS- lipopolysaccharide
cAMP- 3'-5'-cyclic adenosine monophosphate

**Recommended section assignment:** Inflammation, Immunopharmacology, and Asthma
Abstract

Mast cell activation results in the immediate release of pro-inflammatory mediators pre-stored in cytoplasmic granules, and also initiation of lipid mediator production and cytokine synthesis by these resident tissue leukocytes. Allergen-induced mast cell activation is central to the pathogenesis of asthma and other allergic diseases. Presently, most pharmacological agents for the treatment of allergic disease target receptors for inflammatory mediators. Many of these mediators, such as histamine, are released by mast cells. Targeting pathways that limit antigen-induced mast cell activation may have greater therapeutic efficacy by inhibiting the synthesis and release of many pro-inflammatory mediators produced in the mast cell. In vitro studies using cultured human and mouse mast cells, and studies of mice lacking A2B receptors suggest that adenosine receptors, specifically the Gs-coupled A2A and A2B receptors might provide such a target. Here, using a panel of mice lacking various combinations of adenosine receptors, and mast cells derived from these animals, we show that adenosine receptor agonists provide an effective means of inhibition of mast cell degranulation and induction of cytokine production both in vitro and in vivo. We identify A2B as the primary receptor limiting mast cell degranulation, while the combined activity of A2A and A2B is required for the inhibition of cytokine synthesis.
Introduction

Mast cells play a central role in the pathogenesis of allergic rhinitis, asthma, and anaphylaxis. Through degranulation and de novo production of lipid mediators, mast cells are the major cell type responsible for the acute and sometimes life-threatening manifestations of these allergic disorders. Chronic mast cell activation is also believed to contribute to the pathogenesis of asthma (Peachell, 2005; Bradding et al., 2006; Yu et al., 2006). Mast cells are an important source of Th2 cytokines including IL-4, IL-5, and IL-13 (Bradding et al., 1994; Brightling et al., 2003; Galli et al., 2005). Infiltration of mast cells into airway smooth muscle (ASM) bundles in asthmatics has been reported, and this proximity may be important for the development of airway hyperresponsiveness (AHR) (Brightling et al., 2002; James et al., 2012). In a murine model of asthma, mast cells have been implicated in the development of AHR, airway remodeling, and IgE synthesis (Yu et al., 2006). Inhibiting mast cell activation has therapeutic efficacy in asthmatics, further supporting the critical role for this immune cell in disease pathogenesis (Rodrigo et al., 2011). However, drug options for the inhibition of mast cell activation are limited.

Currently, two categories of medications are commercially available to suppress the activity of mast cells: mast cell membrane stabilizers such as cromolyn and nedocromil sodium, and the anti-IgE antibody omalizumab. Sodium cromolyn at very high concentrations inhibits degranulation of human lung mast cells by only 10%, suggesting that these drugs may be inefficient at inhibiting the activity of mast cells in vivo (Church and Hiroi, 1987). The clinical efficacy of omalizumab has been demonstrated in several clinical trials, and it is believed to be a more potent inhibitor of mast cell activation than cromolyn (Boulet et al., 1997; Fahy et al., 1997; Busse et al., 2001; Rodrigo et al., 2011).
While omalizumab inhibits IgE/FεRI-mediated activation by antigen, mast cells can also be activated through FεRI-independent pathways. Chronic mast cell activation via these alternative pathways may contribute to airway remodeling (Peachell, 2005; Okayama et al., 2007; Carter and Bradding, 2011). Therefore, additional means to inhibit mast cell activity are of considerable interest.

Pharmacological agents with cAMP-elevating properties have long been observed to inhibit mast cell function (Torphy, 1998). Cyclic AMP is a ubiquitous intracellular second messenger that is produced from AMP following the activation of adenylyl cyclase. We have previously shown that cAMP can inhibit store-operated calcium channels in mast cells through a PKA-dependent pathway, which subsequently limits antigen-induced mast cell degranulation (Hua et al., 2007). Different strategies can raise intracellular cAMP levels, including activation of Gs-coupled receptors. For example, β-adrenergic agonists can increase intracellular cAMP through activation of Gs-coupled β-adrenergic receptors, which in turn directly relaxes airway smooth muscle. Previous studies have shown that β-adrenergic agonists can also increase cAMP in mast cells, and as a result inhibit mast cell activation (Church and Hiroi, 1987; Weston and Peachell, 1998). However, because β-adrenergic receptors on mast cells tend to internalize and desensitize once bound by agonist, the capacity of β-adrenergic receptor agonists to maintain high cAMP levels in mast cells is limited (Church and Hiroi, 1987; Chong et al., 2003; Scola et al., 2004).
In addition to β-adrenergic receptors, mast cells express other Gs-coupled receptors including the A2 adenosine receptors A2A and A2B (Marquardt et al., 1994; Zhong et al., 2003; Hua et al., 2007). Activation of both A2A and A2B has been shown to increase intracellular cAMP levels in many different cell types including mast cells (Pierce et al., 1992; Feoktistov and Biaggioni, 1995; Olah, 1997; Fredholm et al., 2000). However, whether or not activation of Gs-coupled adenosine receptors in mast cells inhibits mast cell activation is still controversial, especially in vivo. In vitro, adenosine has been shown to elicit both pro- and anti-inflammatory effects on mast cells (Peachell et al., 1991; Suzuki et al., 1998; Zhong et al., 2003; Hua et al., 2007); however, in vivo studies have focused largely on adenosine-induced mast cell activation (Tilley et al., 2000; Zhong et al., 2001; Tilley et al., 2003; Oldenburg and Mustafa, 2005; Hua et al., 2008). Since adenosine is a potent anti-inflammatory mediator after the initial phase of an immune response (Hershfield, 2005), we hypothesized that adenosine might also inhibit mast cell activation in vivo through activation of Gs-coupled A2A and/or A2B receptors. In this study, we used genetic methods to investigate the function of Gs-coupled adenosine receptors on mast cells both in vivo and in vitro to evaluate their potential as therapeutic targets for mast cell-mediated diseases.
Methods

Animals

All studies were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of the University of North Carolina at Chapel Hill. A2A−/−, A2B−/−, and A3−/− mice were generated and genotyped by Southern Blot analysis or PCR as previously described (Ledent et al., 1997; Hua et al., 2007; Salvatore et al., 2000). Each mouse line was backcrossed 12 generations to the C57BL/6 genetic background. The double knockout mice, including A2A−/−A2B−/− and A2B−/−A3−/−, were generated by intercrossing the corresponding double heterozygotes. C57BL/6 wild type (wt) control mice were purchased from the Jackson Laboratory and bred in our animal facility. All mice were housed in a pathogen-free facility with 12 hour day and night switch. For all experiments, mice were greater than 8 weeks old and wild type and receptor deficient animals were matched for age and sex.

BMMC culture

Bone marrow-derived mast cells (BMMCs) were isolated from the femurs of 8-12 week-old mice and grown in RPMI1640 medium containing 10% FCS, 20 ng/ml murine IL-3 and 20 ng/ml SCF as described previously (Salvatore et al., 2000). Cell purity was determined by toluidine blue staining. Cell viability was determined by Trypan blue staining.

Hexosaminidase release, LTB4 and IL-6 measurements
Mast cell degranulation was determined by β-hexosaminidase assay as described (Nguyen et al., 2002). For acute treatment, NECA (or adenosine) was added 20 min before the addition of antigen (DNP-HSA). For chronic treatment, NECA was added 20 hours before the antigen challenge. Hexosaminidase was measured 30 min after stimulation by antigen. IL-6 and leukotriene B₄ (LTB₄) concentrations in supernatants 6 h following mast cell activation were determined by ELISA (Nguyen et al., 2002; Hua et al., 2007). NECA treatment for these experiments was the same as described in the degranulation assay.

**Passive Systemic Anaphylaxis**

Passive systemic anaphylaxis was performed as previously described (Hua et al., 2007). Mice were injected intraperitoneally with NECA in DMSO/PBS (10 μg/kg). They were then given 2 μg murine anti-DNP IgE mAb (Sigma, MO) via tail vein. A second NECA treatment (10 μg/kg) was given 12 hours after IgE sensitization. Twenty-four hours after the first NECA treatment, 100 μg DNP-HSA (Sigma, MO) was injected intravenously to induce mast cell activation. Core body temperature was recorded over time for the indicated period of time using a rectal probe. Investigators were blinded to the treatment given to each mouse during all experiments. The control groups were injected intraperitoneally with vehicle only (DMSO/PBS) at each time point. For histamine measurements, mice were sensitized with anti-DNP IgE systemically as described above. They were then treated with NECA by intraperitoneal injection (400 μg/kg). Twenty four hours later, these animals were injected with DNP-HSA via tail vein to induce systemic
anaphylaxis. Serum was collected immediately following antigen injection and histamine levels were measured by EIA (Cayman, MI).

**Passive cutaneous anaphylaxis**

Mice were injected intraperitoneally with NECA in DMSO/PBS (10 μg/kg). Their right ears were then injected subcutaneously with 2 ng murine anti-DNP IgE mAb (Sigma, MO) in 20 μl PBS. The left ears were injected with 20 μl PBS as negative controls. A booster NECA treatment was given 12 hours after IgE sensitization. Twenty-four hours after IgE sensitization, mice were injected with 0.2 ml 0.9% filtered Evan Blue dye containing 100 μg DNP antigen through the tail vein. One hour after DNP injection the mice were euthanized. Both the left and right ears were collected and extravasated dye was extracted with formamide. The OD values at 610 nm of the pinna extracts were measured.

**Statistical analysis**

All data are presented as mean ± SEM. Paired Student’s t-test was used for comparison before and after treatment in the same subject; two tailed, unpaired student’s t test was used between different groups; repeated measures ANOVA was used to analyze differences between groups over time, from the beginning of DNP-HSA injection through the response period.
Results

Activation of Gs-coupled adenosine receptors inhibits antigen-induced mast cell degranulation in vivo

We employed passive systemic anaphylaxis (PSA) and passive cutaneous anaphylaxis (PCA) models to evaluate mast cell function in vivo. Previous data indicate that murine mast cells, similar to humans, express A2A, A2B, and A3 receptors, but not A1 receptors (Hua et al., 2007). Since adenosine can activate mast cells through A3 receptors in vivo (Salvatore et al., 2000; Tilley et al., 2003), we used mice lacking A3 receptors (A3−/−) to investigate the effect of activation of the Gs-coupled A2A and A2B receptors in PSA and PCA models. For systemic anaphylaxis, A3−/− mice were given murine anti-DNP IgE by intravenous injection. Mice were then treated with NECA intraperitoneally. Twenty four hours later, mast cell degranulation was elicited in vivo by intravenous administration of DNP-HSA. Body temperature drop was selected and recorded as a surrogate marker to evaluate mast cell degranulation, as previously described (Hua et al., 2007). Mice treated with NECA demonstrated less drop in body temperature than mice treated with vehicle (n=7-10 per group, p<0.05 by repeated measures ANOVA), suggesting less mast cell degranulation in the NECA pre-treated group (Figure 1A). Consistent with these findings, PCA experiments also showed that NECA pre-treatment could limit antigen-induced mast cell degranulation in vivo. As shown in Figure 1B, mice treated with NECA prior to antigen challenge demonstrated less plasma extravasation into the ear (n=7 per group, P<0.01 by t test). To further establish that our findings were reflective of mast cell degranulation, we measured the levels of histamine in the serum of a separate cohort of A3−/− mice during PSA. Mice pretreated with NECA exhibited significantly lower
histamine levels in their serum during PSA compared to the vehicle-treated group 
(p<0.01, n=10, 14 and 15 for the controls, DNP and DNP+NECA groups, respectively). Collectively, these experiments demonstrate that activation of Gs-coupled adenosine receptors can inhibit antigen-induced activation of mast cells in vivo.

**Activation of Gs-coupled adenosine receptors inhibits antigen-induced mast cell degranulation in vitro**

In order to further investigate the above in vivo findings, we cultured BMMCs and tested the capacity of NECA to inhibit antigen-induced degranulation. BMMCs from A3−/− mice were cultured in vitro for 5 weeks in media containing murine IL-3 and stem cell factor (SCF). The expression profile of adenosine receptors on A3−/− BMMCs was measured using real time PCR. As expected, these cells only expressed Gs-coupled adenosine receptors A2A and A2B, and expression levels were similar to that of wt BMMCs (data not shown). After passive sensitization with murine anti-DNP IgE (100 ng/ml/million cells for 12 hours), the cultured A3−/− BMMCs were treated with adenosine for 20 min followed by antigen challenge. Mast cell degranulation was quantitated by measuring hexosaminidase release. As shown in Figure 2A, acute activation of Gs coupled adenosine receptors on A3−/− BMMCs failed to modify antigen-induced degranulation of BMMCs.

In our in vivo experiments, the inhibitory effects of NECA on mast cell degranulation were observed approximately 20 hours following the initial treatment; we therefore increased the incubation time prior to antigen challenge in the in vitro experiments from
20 min to 20 h. In order to see whether increased time of incubation with NECA affected cell viability, trypan blue staining was performed. No differences were observed between NECA and vehicle treated cells (data not shown). Antigen-induced hexosaminidase release in A3−/− BMMCs incubated without NECA was 14.9% ± 3%. However, if cells were pretreated with NECA (20 μM) for 20 hours prior to the addition of antigen, the antigen-induced hexosaminidase release from these cells was significantly inhibited (5.8% ± 0.2%, n=3, p<0.01) (Figure 2B). Based on these observations, we conclude that chronic but not acute activation of Gs-coupled adenosine receptors can significantly inhibit antigen-induced mast cell degranulation.

**NECA inhibits mast cell degranulation through activation of the A2B receptor**

In order to determine whether the inhibitory effects of NECA on antigen-induced degranulation would still be detectable in cells expressing A3 receptors, we tested the effect of chronic NECA incubation on degranulation of wt BMMCs. IgE-loaded BMMCs were incubated with 20 μM NECA for 20 hours. Antigen-induced mast cell degranulation was then evaluated by measuring hexosaminidase release 30 min after stimulation by DNP antigen. Similar to our findings in the A3−/− BMMCs, chronic treatment with NECA significantly inhibited antigen-induced degranulation of wt mast cells (Figure 3A, 13.8% ± 3.1% in vehicle treated cells vs. 4.7 % ± 1.9 % in chronic NECA treated cells, p<0.001, n=4). In addition, the acute effect of NECA on antigen-induced mast cell degranulation was also evaluated to ensure that functional A3 receptors were expressed on these cells, as observed in our previous studies (Salvatore et al., 2000; Tilley et al., 2003). As expected, NECA added 15-20 min prior to antigen challenge significantly
increased antigen-induced mast cell degranulation (Figure 3A, p<0.05, acute NECA vs. controls). These data indicate that adenosine has a biphasic effect on antigen-induced mast cell degranulation. Acutely, adenosine acts as a pro-inflammatory mediator by potentiating antigen-induced degranulation via A3 receptors, while chronically, adenosine inhibits antigen-induced mast cell degranulation via Gs-coupled receptors regardless of the co-expression of G_{i/o} coupled A3 receptors.

In order to further substantiate this observation and to determine the receptor subtype responsible for the chronic inhibitory effect of NECA on degranulation, both A2A^{-/-} and A2B^{-/-} BMMCs were treated with NECA acutely and chronically and antigen-induced hexosaminidase release measured. As shown in Figure 3B, both the acute potentiation and chronic inhibition of NECA were observed in A2A^{-/-} BMMCs. This finding not only further substantiates the biphasic effect of adenosine on mast cell degranulation, but also indicates that the inhibitory effect of NECA on degranulation is not mediated by A2A receptors. In BMMCs lacking A2B receptors, we first observed an exaggerated mast cell degranulation induced by antigen as compared to the wt controls (26.8% ± 2.4% vs. 13.8% ± 3.1%, p<0.01 by t test), which is consistent with our previous observations (Hua et al., 2007). Second, the acute potentiating effect of NECA was also present in A2B^{-/-} BMMCs, as observed in wt and A2A^{-/-} BMMCs (26.8% ± 2.4% in DMSO vehicle treated A2B^{-/-} cells vs. 33.5% ± 1.6% in NECA acutely treated A2B^{-/-} cells, p<0.05). However, when the A2B^{-/-} BMMCs were chronically treated with NECA, the inhibitory effect on degranulation that was observed in wt, A2A^{-/-} and A3^{-/-} BMMCs was abolished (Figure
3C). These data indicate that the capacity of NECA to inhibit mast cell degranulation is mediated by the A2B receptor.

Next, we conducted experiments to determine whether our *in vitro* observations described above implicating the A2B receptor were also present *in vivo*. Mice lacking the A3 receptor (A3−/−), and mice lacking both A2B and A3 receptors (A2B−/−A3−/−) were sensitized with murine IgE mAb. They were then given an intraperitoneal injection of NECA (400 µg/kg). Antigen-induced mast cell degranulation was evaluated by measuring serum histamine levels the next day immediately following the i.v. administration of DNP antigen. As shown in Figure 4, treatment with DNP antigen resulted in a marked increase in serum histamine level in A3−/− mice. Antigen-induced histamine levels in the serum were significantly attenuated in A3−/− mice, but not A2B−/−A3−/− mice, pretreated with NECA. These data demonstrate that A2B receptors mediate the inhibitory effect of NECA in systemic anaphylaxis.

*A2A receptors do not influence antigen-induced mast cell degranulation*

*A2A* receptors have long been implicated as the major adenosine receptor subtype eliciting inhibitory effects on leukocytes including macrophages and lymphocytes. Surprisingly, based on the above experiments, activation of *A2A* receptors had no modulatory effect on mast cell degranulation. In order to further examine the biological role of *A2A* receptors on mast cells, we investigated the impact of genetic deletion of *A2A* receptors on antigen-induced mast cell degranulation both *in vitro* and *in vivo*. BMMCs from wt and *A2A*−/− mice were cultured for 5 weeks, and dose response studies of antigen-
induced mast cell degranulation were conducted. As shown in Figure 5A, mast cells lacking A$_{2A}$ receptors showed no significant differences in the magnitude of antigen-induced hexosaminidase release compared to wt mast cells. In order to further validate this *in vitro* observation, we conducted PSA and PCA experiments in mice lacking A$_{2A}$ receptors. Ear plasma protein extravasation as the result of PCA (Figure 5B), and core temperature drop as a result of PSA (Figure 5C), was not different between A$_{2A}$-/- and wt mice. Collectively, these data indicate that activation of A$_{2A}$ receptors does not modify antigen-induced mast cell degranulation.

*NECA inhibits antigen-induced IL-6 synthesis in BMMCs through A$_{2A}$ and A$_{2B}$ receptors*

In addition to degranulation, activated mast cells can also synthesize pro-inflammatory cytokines. In order to investigate the effect of activation of Gs-coupled adenosine receptors on antigen-induced cytokine synthesis, we treated cultured BMMCs with NECA chronically as described above, and the effects of this treatment on antigen-induced IL-6 synthesis was analyzed. Passively sensitized wt BMMCs were incubated with 20 μM NECA. Twenty hours later, cells were treated with DNP for 6 hours and IL-6 levels in the supernatant were measured. Chronic incubation with NECA did not cause significant production of IL-6. In cells chronically pretreated with NECA (20 h), DNP antigen challenge could still elicit synthesis of IL-6, however, the production of IL-6 in these cells was significantly reduced (27% lower than vehicle/DNP group, n=14-15 per group, p<0.0001) (Figure 6A). These data indicate that chronic treatment with NECA can inhibit antigen-induced cytokine synthesis in BMMCs. In order to determine the
adenosine receptor subtype(s) mediating this inhibitory effect on cytokine synthesis, both 
A2A+/− and A2B+/− BMMCs were cultured and chronically treated with NECA prior to DNP 
antigen challenge, and the capacity of NECA to inhibit antigen-induced IL-6 synthesis 
was examined. Surprisingly, the deletion of neither A2A nor A2B receptors influenced the 
inhibitory effects of chronic NECA treatment on IL-6 synthesis (Figure 6B). These data 
further substantiate the inhibitory effects of chronic NECA treatment on antigen-induced 
cytokine synthesis in BMMCs, and also suggest that the inhibition of IL-6 synthesis by 
NECA is not mediated by either the A2A or A2B receptor. To investigate these 
observations further, we next cultured BMMCs lacking both A2A and A2B receptors, and 
tested the chronic inhibitory effect of NECA on antigen-induced IL-6 synthesis. As 
shown in Figure 5C, the deletion of both A2A and A2B genes completely abolished the 
chronic inhibitory effect of NECA on IL-6 synthesis in these mast cells. Collectively, 
these data indicate that activation of Gs-coupled adenosine receptors can also inhibit 
antigen-induced cytokine synthesis in BMMCs. In contrast to mast cell degranulation 
where the inhibitory effect of chronic NECA was only mediated by the A2B receptor, 
NECA-induced inhibition on cytokine synthesis was co-operatively mediated by both 
A2A and A2B adenosine receptors.

Effect of NECA on antigen-induced lipid mediator release from BMMCs

In addition to degranulation and cytokine synthesis, activated mast cells can also release 
arachidonic acid-derived lipid mediators. In order to determine whether activation of Gs- 
coupled adenosine receptors could modify antigen-induced lipid mediator release from 
mast cells, we measured LTB4 levels in the supernatants of immunologically activated
mast cells that were incubated acutely (20 min) or chronically (20 h) with NECA prior to DNP-antigen challenge. Unlike the potentiating effect of acute NECA on antigen-induced mast cell degranulation, antigen-induced lipid mediator release was not affected when DNP-stimulated cells were pre-treated acutely with NECA (Figure 7A). Also in contrast to the effects of chronic NECA on degranulation and cytokine synthesis, DNP-antigen-induced LTB₄ production was no different between mast cells incubated chronically (20 h) with vehicle or NECA. However, we did observe a 50% decrease DNP-triggered LTB₄ release by mast cells incubated for 20 h with DMSO vehicle (Figure 7A Vehicle, ~100,000 pg/ml) compared to mast cells incubated for 20 min with DMSO vehicle (Figure 7B Vehicle, ~200,000 pg/ml). Thus, we cannot rule out the possibility that chronic NECA might inhibit DNP-induced LTB₄ release and that such an inhibitory effect could not be observed due to potential effects of the DMSO vehicle.
Discussion

Previous in vitro studies have shown biphasic effects of adenosine on mast cell activity; however, the receptor subtypes that mediate the inhibitory effects of adenosine are still controversial (Peachell et al., 1991; Yip et al., 2009). Mast cells express two distinct Gs-coupled adenosine receptors; their biological roles have not been comprehensively defined, especially in vivo. Since activation of Gs-coupled adenosine receptors increases intracellular cAMP, we hypothesized that the inhibitory effects of adenosine on mast cells are mediated by the Gs-coupled adenosine receptors. In this study, we used both genetically modified animal models and mast cell cultures to comprehensively investigate the role of Gs-coupled adenosine receptors on mast cells both in vitro and in vivo. First, our data demonstrates a potent inhibitory effect of the non-hydrolysable adenosine analogue NECA on IgE-induced mast cell degranulation; this inhibitory effect of NECA was abolished by the genetic deletion of the A2B but not the A2A receptor. Second, while A2A receptors also couple to Gs, we were unable to demonstrate a role for this receptor subtype in antigen-induced mast cell degranulation in vitro or in vivo. Third, in addition to mast cell degranulation, activation of Gs-coupled adenosine receptors significantly inhibited antigen induced synthesis of IL-6 in mast cells; this inhibitory effect was mediated by both A2A and A2B receptors. Lastly, our in vitro studies revealed a time-dependent biphasic effect of adenosine on mast cell activation. Acutely, adenosine enhances mast cell degranulation but chronically, it limits mast cell activation.

Adenosine levels are elevated in BAL fluid from asthmatics (Driver et al., 1993). Adenosine inhalation causes immediate bronchoconstriction in asthmatics but not in
normal subjects, an effect that can be inhibited by antagonizing adenosine-induced mast cell activation (Cushley et al., 1983; Phillips et al., 1989). Based on these observations, it has been posited that adenosine activates mast cells and contributes to asthma pathogenesis, and that antagonizing the pro-inflammatory adenosine receptor subtype may benefit asthmatics (Polosa and Blackburn, 2009). The adenosine receptor subtypes that potentially mediate these pro-inflammatory effects on mast cells include A₁, A₂B and A₃ receptors as suggested by previous studies (Ali et al., 1994; Feoktistov and Biaggioni, 1995; Salvatore et al., 2000; Feoktistov et al., 2001; Feoktistov et al., 2003; Tilley et al., 2003; Zhong et al., 2004; Chen et al., 2006; Young et al., 2006; Yip et al., 2009; Hua et al., 2011). However, clinical trials have failed to demonstrate efficacy of antagonizing these three adenosine receptors in asthmatics, suggesting that the biological roles of adenosine in asthmatic lungs needs to be re-evaluated (Ball et al., 2003; Pascoe, 2007).

In this study, we observed a time dependent, biphasic effect of adenosine on mast cell activation. Acutely, adenosine enhanced mast cell degranulation, but chronically adenosine limited mast cell activation. Adenosine is produced by the metabolism of ATP released by cells during hypoxia and other pathophysiological conditions resulting in cellular stress. ATP and its metabolites adenosine and uric acid are major danger associated molecular patterns (DAMPs); the biological role of which is to help maintain and restore in vivo homeostasis following cellular stress (Willart and Lambrecht, 2009). The time dependent, biphasic effects that we and others have observed with adenosine may confer this molecule a unique role in maintaining homeostasis following tissue injury. Acutely, adenosine functions as a DAMP, signaling the in vivo “alarm system” by
up-regulating the inflammatory response. Chronically, adenosine through preconditioning and inhibition of the inflammatory response, may act to prevent excessive tissue injury and to help eventually restore homeostasis (Fredholm, 2007). Human studies from our center have shown increased adenosine levels in the induced sputum from asthmatics with mild, moderate, and severe persistent asthma. These subjects were stable, and not in the midst of an asthma exacerbation, suggesting that adenosine levels are chronically elevated in the lungs of asthmatics, particularly those with more severe disease. Therefore, in combination with previous studies, this report suggests that the chronically accumulated adenosine in asthmatic lungs may be anti-inflammatory rather than pro-inflammatory.

Our observations of the inhibitory effects of adenosine on antigen-induced mast cell degranulation in vitro are consistent with several previous reports on human lung fragments, dispersed human lung mast cells and human umbilical cord blood-derived mast cells (HUCBMCs) (Hughes et al., 1984; Peachell et al., 1988; Peachell et al., 1991; Suzuki et al., 1998). At least three independent studies have shown that adenosine can inhibit antigen-induced histamine release from human lung fragments and dispersed human lung mast cells (Hughes et al., 1984; Peachell et al., 1988; Peachell et al., 1991). While these investigators showed that the inhibitory effects of adenosine could be blocked by inhibiting cell-surface adenosine receptors, the tools to identify and isolate specific receptors were not available at the time of these investigations. Peachell et al. found that low concentrations of adenosine potentiated (<1 μM), and high concentrations inhibited (100 μM) antigen-induced degranulation of human lung mast cells (Peachell et
al., 1988). Given the low affinity of the A2B receptor for adenosine, these observations suggest that the A2B receptor might mediate these inhibitory effects on human lung mast cells. Yip et al also observed a similar biphasic effect of adenosine on anti-IgE induced human mast cell activation (Yip et al., 2011). By using pharmacological antagonism, this group reported that the potentiating and inhibitory effect of adenosine was mediated by A1 and A2B receptors respectively. In contrast, using in vitro cultured HUCBMCs, Suzuki et al. found that IgE crosslink-induced mast cell degranulation could be inhibited by the A2A receptor agonist CGS-21680, and, that this inhibition could be effectively prevented by ZM241385, a potent A2A receptor antagonist, suggesting a role for the A2A receptor in adenosine-mediated inhibition of degranulation (Suzuki et al., 1998). In addition, Rork et al have found that activation of A2A receptors can limit mast cell degranulation in the murine heart, decreasing reperfusion injury (Rork et al., 2008). A study using cheek pouch of the golden hamster also revealed an inhibitory effect of activation of A2A receptors on inosine-induced periarteriolar mast cell degranulation (Fenster et al., 2000). In contrast, our mouse studies both in vitro and in vivo do not support a role for A2A receptors in antigen-induced mast cell degranulation.

Mast cells are a highly heterogeneous cell type. They originate in bone marrow but mature in peripheral organs where they acquire different phenotypic properties following differentiation in the local milieu, which may differ between tissues. It is possible that the environment in which the mast cell resides influences adenosine receptor expression and function, accounting for the differences in receptor effects observed between these studies. While several studies have suggested that A2A receptors mediate anti-
inflammatory effects (Antonioli et al.,; Fenster et al., 2000; Harada et al., 2000; Kreckler et al., 2006; Rork et al., 2008; Trevethick et al., 2008), a clinical trial failed to demonstrate efficacy of an A2A agonist in attenuating allergen-induced early and late reactions, sputum total cell counts and EG2+ cells numbers, ECP (eosinophil cationic protein) levels, or inflammatory cytokine production in asthmatics (Luijk et al., 2008).

Investigations on the biological role of the Gs-coupled A2B receptor have yielded conflicting results. Studies on HMC-1 cells have revealed a pro-inflammatory role for A2B receptors (Feoktistov and Biaggioni, 1995; Meade et al., 2002; Ryzhov et al., 2004; Ryzhov et al., 2006). Similarly, our study using HUCBMCs showed that IgE-crosslinking-induced mast cell activation was augmented by A2B receptor activation, further suggesting a pro-inflammatory role for the A2B receptor on mast cells in vitro (Hua et al., 2011). However, these in vitro observations have not held up in vivo. For example, in a placebo controlled, double blinded, randomized, two way crossover Phase Ib clinical trial, the compound QAF 805 which antagonizes both A2B and A3 receptors failed to demonstrate efficacy in attenuating AMP-induced bronchial hyper-responsiveness in asthmatics (Pascoe, 2007).

Previous studies by our group and others have ascribed an anti-inflammatory phenotype to the A2B receptor. We have previously reported that murine mast cells lacking A2B receptors exhibited an exaggerated response to antigen (Hua et al., 2007), suggesting an anti-inflammatory role for A2B receptors in mice. In addition, activation of A2B receptors have been reported to reduce the severity of inflammation in LPS-induced murine
macrophage activation, murine sepsis, and a mouse model of ventilation-induced lung injury (Kreckler et al., 2006; Yang et al., 2006; Eckle et al., 2008; Csoka et al.). In this study, we also found that the chronic inhibitory effect of adenosine on mast cell activation was abolished by genetic deletion of the A2B receptors both \textit{in vivo} and \textit{in vitro}, further supporting an anti-inflammatory role for A2B receptors on mast cells.

In addition to inhibiting degranulation, we also found that chronic stimulation of Gs-coupled adenosine receptors by NECA could inhibit antigen-induced cytokine synthesis. Surprisingly, we were unable to attribute this inhibition to a single adenosine receptor sub-type. Mast cells lacking either A2A or A2B receptors responded similarly to wt cells, suggesting that the presence of an A2A-Gs or A2B-Gs signal was sufficient to mediate the inhibitory effects of NECA. Interestingly, activation of both Gs-coupled receptors on wt mast cells was no more effective than activation of either receptor in isolation on mast cells from A2A- or A2B-deficient mice. When mast cells devoid of both Gs-coupled receptors were examined, the inhibitory effect of NECA on antigen-induced IL-6 synthesis was abolished.

The capacity of adenosine to potently regulate inflammation has made the G-protein-coupled adenosine receptors attractive pharmacological targets. Our findings of an inhibitory role for the A2B receptor, both constitutively (Hua et al., 2007) and when chronically stimulated, may have implications for A2B antagonists under development for the treatment of asthma. A more detailed understanding of the mechanisms by which adenosine regulates the immune response in a bi-phasic fashion will be critical to the
successful introduction of adenosine receptor ligands for the treatment of asthma and other inflammatory diseases.

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Authorship Contributions

Participated in research design: Hua, Tilley

Conducted experiments: Hua, Chason, Jania, Acosta, Tilley

Contributed new reagents or analytic tools: Ledent

Performed data analysis: Hua, Chason, Jania, Tilley

Wrote or contributed to the writing of the manuscript: Hua, Tilley
References


hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor.

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Footnotes

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Legends for Figures

Figure 1. Effect of NECA on antigen-induced mast cell degranulation in vivo.

A. Passive systemic anaphylaxis (PSA). Mice were injected intraperitoneally with NECA (10 μg/kg) (filled square, n=7) or DMSO/PBS vehicle (open square, n=10), and 2 μg murine anti-DNP IgE mAb (Sigma, MO) was administered via tail vein. A second NECA/vehicle treatment was given 12 hours after IgE sensitization. Twenty-four hours after the first NECA treatment, 100 μg DNP-HSA antigen was injected intravenously. Data represent the mean body temperature ± SEM every 10 min post-antigen administration. * P<0.01 by repeated measures ANOVA. B. Passive cutaneous anaphylaxis. Mice were injected intraperitoneally with NECA or vehicle as described for PSA. Right ears were then injected subcutaneously with 2 ng murine anti-DNP IgE mAb in 20 μl PBS. Left ears were injected with 20 μl PBS. Twenty-four hours after IgE sensitization, mice were injected with 0.2 ml 0.9% filtered Evan Blue dye containing 100 μg DNP antigen by tail vein. One hour after DNP injection, ears were collected and extravasated dye was extracted with formamide. Data represent the mean OD values at 610 nm (Right ear-left ear) of the pinna extracts ± SEM. * P <0.05 by student t test between NECA (filled bar, n=7) vs. vehicle (open bar, n=7) groups. C. Serum histamine levels during PSA. Mice were subjected to PSA as described in A, except 400 μg/kg of NECA was used. Blood was obtained immediately following antigen injection for measurement of serum histamine levels by ELISA. Data represent mean histamine level ± SEM. ** P<0.001 by student t test between NECA treated mice (n=14) vs. vehicle
treated mice (n=15). ## P<0.001 between both NECA and vehicle treated mice vs. naïve controls (n=10).

**Figure 2.** Effect of acute vs. chronic adenosine receptor stimulation on antigen-induced degranulation in vitro. A. BMMCs from A_3^{+/−} mice were sensitized with murine anti-DNP IgE antibody and incubated with adenosine (100 μM) or vehicle for 20 min. Mast cell degranulation was triggered by addition of DNP-HSA antigen. Data represent % of hexosaminidase release of vehicle treated cells (controls) ± SEM. P>0.05 by student t test. n=3 cell lines. B. Anti-DNP IgE sensitized A_3^{+/−}BMMCs were treated with 20 μM NECA or vehicle for 20 hand hexosaminidase measured 30 min after stimulation with DNP-HSA antigen.** P <0.01 by student t test between NECA vs. vehicle (n=3 cell lines per group).

**Figure 3.** Effect of acute and chronic NECA on antigen-induced degranulation of wt, A_2A^{−/−} and A_2B^{−/−} mast cells. Cultured wt (A), A_2A^{−/−} (B) and A_2B^{−/−} (C) mast cells were passively sensitized with murine IgE. The cells were then incubated with NECA acutely (20 min) or chronically (20 h) prior to the addition of antigen. Controls were treated with aliquot vehicle. Hexosaminidase release was then measured to evaluate mast cell degranulation. * P<0.05 vs. vehicle. Data are from 3-4 different sets of cells, and presented as mean % hexosaminidase release ± SEM.

**Figure 4.** Effect of NECA on antigen-induced mast cell degranulation in mice lacking both A_3 and A_2B receptors in vivo. A_3^{−/−} and A_2B^{−/−}A_3^{−/−} mice were sensitized with
murine IgE. These mice were then given NECA i.p. (400μg/kg) as described previously in this study. Blood was obtained immediately following antigen injection the next day for the measurements of serum histamine levels by ELISA. Controls include two groups of A3<sup>-/-</sup> mice treated with IgE/Veh/PBS and IgE/Veh/ DNP. Data represent mean histamine level ± SEM. N=5 in each group. * P<0.05 by student t test between NECA treated A3<sup>-/-</sup> vs. both vehicle treated A3<sup>-/-</sup> and NECA treated A2B<sup>-/-</sup>A3<sup>-/-</sup> mice.

**Figure 5. In vivo and in vitro evaluation of antigen-induced degranulation in A2A<sup>-/-</sup> mice.**

**A.** Dose response curve of hexosaminidase release with DNP-HSA antigen in wt and A2A<sup>-/-</sup> BMMCs. P>0.05 between wt. and A2A<sup>-/-</sup> cells by repeated ANOVA. Data are from BMMCs from 3 mice of each genotype, and presented as mean % hexosaminidase release ± SEM. **B.** Passive cutaneous anaphylaxis in wt and A2A<sup>-/-</sup> mice. n=5-6 per group; p>0.05 by student t test. **C:** Systemic anaphylaxis in wt and A2A<sup>-/-</sup> mice. Data represent mean temperature drop 20 min post DNP antigen ± SEM. n=5-7 per group, p>0.05 by student t test.

**Figure 6. Effect of NECA on antigen-induced IL-6 synthesis.** Cultured wt (A), A2A<sup>-/-</sup>, A2B<sup>-/-</sup> (B) and A2A<sup>-/-</sup>A2B<sup>-/-</sup> (C) mast cells were passively sensitized with murine IgE. The cells were then incubated with NECA (20 μM) chronically (20 h) prior to the addition of antigen. Controls were treated with aliquot vehicle. The IL-6 levels in the supernatants from these cells were then measured 6 h after the addition of antigen. *P<0.05 between NECA/DNP and vehicle/DNP treated groups by student t test. Data represent 15 different
lines of cells in A; 3-4 different lines of cells in both B and C; and are presented as mean % of the IL-6 levels in antigen (DNP) treated cells ± SEM.

Figure 7. Effect of NECA on antigen-induced LTB₄ synthesis by BMMCs.

Mast cells were sensitized with murine anti-DNP IgE and incubated chronically (20h) (A) or acutely (20 min) (B) with 20 μM NECA, and LTB₄ synthesis induced with DNP-HSA antigen. LTB₄ release in supernatants was measured 6 h later by ELISA. Data for both A and B were from 6 different lines of cells, and presented as mean LTB₄ levels ± SEM.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

(A) NECA/DNP:
- 0/0
- 20/0
- 0/50
- 20/50

(B) IL-6

(C) WT vs A2A−/− and A2B−/−

**Significant difference
## Very significant difference
Figure 7