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Adenosine induces airway hyperresponsiveness through activation of A_3 receptors on mast cells

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Abstract

Background—The mechanisms responsible for the development of airway hyperresponsiveness in asthma are poorly understood. Adenosine levels are high in the lungs of patients with asthma, but a role for adenosine in the development of this cardinal feature of asthma has not been previously reported.

Objective—To determine the capacity of adenosine to induce airway hyperresponsiveness, and to investigate the mechanisms behind these effects of adenosine on airway physiology.

Methods—Wild-type C57BL/6 mice were exposed to aerosolized adenosine analog adenosine-5′ N-ethylcarboxamide (NECA), and subsequent hyperresponsiveness to methacholine was investigated by measuring airway mechanics after anesthesia and tracheostomy. Similar experiments were conducted with A_1-deficient, A_3-deficient, and mast cell–deficient mice, as well as with mast cell–deficient mice engrafted with wild-type (wt) or $A_3^{-/-}$ mast cells. The effect of NECA on methacholine-induced tension development in ex vivo tracheal rings was also examined.

Results—Exposure of wt mice to NECA resulted in the robust induction of airway hyperresponsiveness. NECA failed to induce hyperresponsiveness to methacholine in tracheal ring preps ex vivo, and NECA-induced airway hyperresponsiveness in vivo was not affected by the genetic inactivation of the A_1 adenosine receptor. In contrast, NECA-induced airway hyperresponsiveness was abolished in A_3 adenosine receptor-deficient mice and in mice deficient

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in mast cells. Reconstitution of mast cell–deficient mice with wt mast cells restored hyperresponsiveness, whereas reconstitution with A_3 receptor–deficient mast cells did not.

Conclusion—Adenosine induces airway hyperresponsiveness indirectly by activating A_3 receptors on mast cells.

Keywords

Airway hyperresponsiveness; adenosine; mast cell; asthma

Asthma is a major public health problem in developed countries, and it has become the most common chronic illness of children in the United States.[1] Asthma is characterized by 3 major features: (1) reversible airflow obstruction, (2) airway inflammation, and (3) airway hyperresponsiveness (AHR). The mechanisms by which these distinct features of asthma develop have been extensively investigated. The pathogenesis of AHR, however, has remained most elusive.

Airway hyperresponsiveness is characterized by the immediate and excessive airway contraction that occurs after exposure to nonspecific stimuli (eg, cold air, perfumes). Bronchoconstriction after exposure to these normally innocuous stimuli is a major cause of morbidity for patients with this disease. A number of inflammatory cytokines and mediators, airway remodeling, neural reflexes, and secondary dysfunction of airway smooth muscle (ASM) all have been postulated to contribute to the development of AHR in patients with asthma.[2–7] Because of the vast array of inflammatory mediators present simultaneously in the asthmatic airway, the pathophysiological mechanisms responsible for AHR development have been exceedingly difficult to study in human beings.

Adenosine is a metabolic by-product of ATP, present in high levels in exhaled breath condensates and bronchoalveolar lavage fluid from patients with asthma.[8,9] By activating 4 G-protein–coupled adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) on immunocytes, neurons, goblet cells, and ASM, adenosine is believed to contribute to asthma pathogenesis.[8–20] However, a role for adenosine in the development of AHR has not been previously investigated. In smooth muscle cells, Gerwins and Fredholm[21] demonstrated that adenosine could stimulate Ca^{2+} mobilization and enhance the contractile response to activation of $G_{q/11}$-coupled receptors. In human ASM cells, adenosine mobilizes intracellular calcium through an inositol 1,4,5-triphosphate–mediated pathway.[22] Because AHR is a hyperresponsive state of ASM and calcium mobilization is essential for smooth muscle contraction, these studies have suggested to us that adenosine may be capable of inducing AHR. To test this hypothesis, we conducted a series of *in vivo* and *ex vivo* experiments examining the capacity of the nonselective adenosine analog NECA to induce AHR in mice.

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. Female C57BL/6 mice and WBB6F1/J-$Kit^{W/W\text{-}v}$ mast cell–deficient mice were purchased from the Jackson

Laboratory and bred in our animal facility. Female $A_1^{-/-}$ and $A_3^{-/-}$ mice were generated and genotyped as previously described, and backcrossed 12 generations to the C57BL/6 background.[16,23,24] Female C57BL/6 $Kit^{W\text{-}sh/W\text{-}sh}$ mast cell–deficient mice were bred in our animal facility. All mice were housed under pathogen-free conditions with 12-hour day and night switch.

Exposure of mice to aerosolized adenosine analog NECA

The non selective adenosine analogue NECA was used in place of adenosine in all studies. Similar to adenosine, NECA is approximately equipotent on A_1, A_{2A}, and A_3 receptors, but has the advantage over the natural parent compound that its duration in tissues is much longer than just a few seconds.[25] Mice were placed in individual plexiglass chambers and exposed to aerosolized NECA (3 mg/mL in 25% dimethyl sulfoxide) or vehicle (25% dimethyl sulfoxide) for 10 minutes. Ten minutes later, mice were anesthetized and tracheostomized for measurement of airway mechanics.

Measurement of lung mechanics in anesthetized mice

Lung mechanics including lung resistance (R_L), dynamic compliance (C_{dyn}), airway resistance (R_{aw}), and tissue damping (G_{tissue}) were measured in anesthetized mice as previously described.[16] After the determination of basal mechanics (at 10-second intervals for 1 minute), mice were serially challenged with aerosolized methacholine (20 mg/mL, 40 mg/mL, and 80 mg/mL) for 20 seconds, and the R_L, C_{dyn}, R_{aw}, and G_{tissue} after each challenge were recorded every 10 seconds for 2 minutes.

Tension development in tracheal ring preparations

Tracheal ring preparations and *ex vivo* tension measurements were performed by using a modified protocol similar to that described previously with some modification.[16] After the preparation of tracheal rings, the preload tension was set to 0.5 g. The rings were treated with 10 μmol/L methacholine for 5 minutes, then washed and reset to 0.5 g resting tension, which was maintained throughout the 45-minute equilibration period. After a 10-minute pretreatment with vehicle, the rings were challenged with increasing concentrations (1 nmol/L to 10 μmol/L) of methacholine to establish dose-response curves. To assess the effect of NECA on methacholine-induced contraction, after stimulation with the highest tested concentration (50 μmol/L) of methacholine, rings were washed thoroughly and allowed to recover for 30 minutes, adjusted to the original resting tension, then treated for 10 minutes with either vehicle or 50 μmol/L NECA, and the dose-dependent response to methacholine was assessed again. At the conclusion of each experiment, tracheal segments were blotted on a gauze pad and weighed. Force generation was calculated as milligrams tension per milligrams tracheal ring weight.

Bone marrow–derived mast cell culture and mast cell reconstitution in C57BL/6 $Kit^{W\text{-}sh/W\text{-}sh}$ mice

Murine bone marrow–derived mast cells (BMMCs) were harvested and cultured from C57BL/6 mice (wild-type [wt] or $A_3^{-/-}$) 8 to 12 weeks old as previously described.[10] After 5 weeks of culture, cells were collected, washed, and injected into C57BL/6$Kit^{W\text{-}sh/W\text{-}sh}$

mice (10 million cells per mouse) via tail vein. Reconstituted mice were housed in pathogen-free circumstance with 12 hours of day-night shift for 7 months. Confirmation of mast cell reconstitution in these recipients was histologically confirmed by toluidine blue stain of tissues from several different organs.

Primary lung mast cell culture and hexosaminidase release measurement

Murine primary lung mast cells were isolated and cultured as described previously.[26] Cells were used after 6 weeks in culture. After loading with murine anti-dinitrophenyl (DNP) IgE (100 ng/mL/million cells) overnight, cells were treated with antigen (DNP-human serum albumin) or adenosine for 20 minutes. Mast cell degranulation was determined by β-hexosaminidase activity assay as described previously.[10]

Statistical analysis

All data are presented as means ± SEMs. Two-tailed, unpaired Student *t* test was used between different groups; repeated-measures ANOVA was used to analyze differences between groups over time, from the beginning of baseline measurements through the response period after each methacholine exposure. Least significant difference was used for multiple comparisons.

RESULTS

NECA robustly induces AHR in C57BL/6 mice

C57BL/6 mice were exposed to NECA (3 mg/mL) for 10 minutes by aerosol. Twenty minutes later, R_L, C_{dyn}, R_{aw}, and G_{tissue} were measured at the basal level and in response to graded methacholine challenge. Control animals were exposed to vehicle rather than NECA. NECA exposure had no effect on basal respiratory mechanics. Methacholine aerosolization at 20, 40, and 80 mg/mL only modestly increased R_L in vehicle-pretreated mice (Fig 1, A). However, the same methacholine dosing resulted in much larger R_L increases in NECA-pretreated animals (Fig 1, A; $P = .004$). The maximum responses to 20, 40, and 80 mg/mL methacholine in NECA pretreated groups versus controls were, respectively, 135% ± 9% versus 131% ± 4%, 171% ± 11% versus 134% ± 10%, and 242% ± 25% versus 152% ± 8% of basal R_L (Fig 1, B; $P = .002$). In addition, methacholine-induced changes in C_{dyn} were influenced by NECA, but to a lesser extent than changes in R_L (see this article's Fig E1, A and B, in the Online Repository at www.jacionline.org; $P < .05$). Because C_{dyn} predominantly reflects the changes in mechanics in the periphery of the lung, these data suggest that NECA acts on proximal airways to mediate the major part of these changes in airway physiology.[27]

To evaluate further the specific region of the tracheobronchial tree involved in adenosine-induced AHR, we used a second method, the forced oscillation technique, to directly measure R_{aw} and G_{tissue} (representing changes in small airways and lung parenchyma[27]). Similar to our findings by measuring R_L and C_{dyn}, NECA pretreatment robustly potentiated methacholine-induced increases in R_{aw} (Fig 1, C; $P = .012$). The maximum R_{aw} after 20, 40, and 80 mg/mL methacholine in the NECA-exposed group versus the vehicle group was 146% ± 6% versus 143%± 6.%, 178% ± 13% versus 146% ± 9%, and 310% ± 45% versus

185% ± 12% of basal R_{aw}, respectively (Fig 1, D; $P = .037$). In addition, NECA potentiated methacholine-induced increases in G_{tissue}, but to a lesser extent than changes in R_{aw}, suggesting that NECA induces AHR throughout the tracheobronchial tree, but with greater effects on the proximal airways ($P = .08$ in Fig E1, C; $P = .046$ in Fig E1, D). Collectively, using 2 different methodologies, these data demonstrate that exposure of murine airways to NECA *in vivo* induces AHR to subsequent cholinergic stimulation, and that this effect on airway physiology is most marked in the proximal airways.

NECA does not enhance methacholine-induced contraction of tracheal rings

To determine whether NECA acts directly on ASM to induce AHR, tracheal rings from C57BL/6 mice were isolated, and the effect of previous NECA exposure on the dose-dependent contractile response to methacholine was assessed *ex vivo*. As shown in this article's Fig E2 in the Online Repository at www.jacionline.org, after an intervening pretreatment with vehicle, the dose-dependent effect of methacholine is similar to that occurring with the initial (dose-dependent) challenge. For those rings instead receiving an intervening pretreatment with NECA, contractile responses to methacholine were also essentially unchanged. These data suggest that NECA-induced AHR cannot be explained by the result of activation of adenosine receptors on ASM.

NECA-induced AHR is mediated by the A_3 adenosine receptor

Although activation of A_1 receptors in smooth muscle cells can increase Ca^{2+} mobilization and enhance responses to $G_{q/11}$-coupled receptors,[21,22] our *ex vivo* data do not support a role for A_1 receptors on ASM cells in adenosine-induced AHR. To determine further whether A_1 adenosine receptors mediate adenosine-induced AHR indirectly (eg, via neural reflex as previously observed for adenosine-induced bronchoconstriction[16]), we examined the capacity of NECA to induce AHR in A_1-deficient mice, as described for wt mice. NECA pretreatment significantly enhanced methacholine-induced R_L increases (Fig 2, A; $P = .002$), C_{dyn} decreases (see this article's Fig E3, A, in the Online Repository at www.jacionline.org; $P = .018$), R_{aw} increases (Fig 2, B; $P = .002$) and G_{tissue} increases (Fig E3, B; P = .046) in $A_1^{-/-}$ mice to a degree similar to that observed in wt animals ($P = .93$ in Fig 2, A; $P = .46$ in Fig 2, B), suggesting that an adenosine receptor other than A_1 mediates the induction of AHR by adenosine. In addition, we observed that the potentiating effects of NECA on methacholine-induced increase in R_L and R_{aw} in $A_1^{-/-}$ mice were more robust than the effects on methacholine-induced changes in C_{dyn} and G_{tissue}, again supporting the development of hyperresponsiveness of the major conducting airways.

Next, we examined the capacity of NECA to induce AHR in A_3-deficient mice. As shown in Fig 3, A and B, and this article's Fig E4, A and B, in the Online Repository at www.jacionline.org, NECA-induced changes in R_L, C_{dyn}, R_{aw}, and G_{tissue} were abolished in mice lacking the A_3 receptor ($P > .2$). As a positive control, wt animals were examined concurrently with A_3-deficient mice, and robust AHR, similar to our findings in Fig 1 and Fig E1, was observed ($P < .0001$). These results demonstrate that NECA-induced AHR is mediated by activation of A_3 adenosine receptors.

NECA-induced AHR is abolished in mast cell–deficient mice

We and others have previously shown that adenosine can activate mast cells through the A_3 receptor;[12,26] therefore, NECA-induced AHR was examined in mast cell–deficient mice (WBB6F1/J-Kit^{W}/Kit$^{W\text{-}v}$). As shown in Fig 4, A and B, and this article's Fig E5, A and B, in the Online Repository at http://www. jacionline.org, NECA-induced AHR was abolished in mast cell–deficient animals, similar to our findings in A_3-deficient mice. These data suggest that NECA induced AHR is the result of activation of A_3 receptors on mast cells.

NECA-induced AHR is repaired in wt but not $A_3^{-/-}$ mast cell–reconstituted mice

To establish that NECA-induced AHR occurs indirectly as a result of activation A_3 adenosine receptors on mast cells, we repeated the described *in vivo* experiments in mast cell–deficient mice reconstituted with wt and $A_3^{-/-}$ BMMCs. For these studies, we used a second mast cell–deficient strain that is inbred on the C57BL/6 background (C57BL/6Kit$^{W\text{-}sh}$/Kit$^{W\text{-}sh}$ mice) to facilitate the reconstitution of mast cells derived from C57BL/6-$A_3^{-/-}$ and C57BL/6-wt mice. Successful reconstitution of mast cells in these mice was determined by toluidine blue staining of the lungs (see this article's Fig E6, A–F, in the Online Repository at http://www.jacionline.org), and mast cell numbers in the lungs did not differ between mice reconstituted with wt or $A_3^{-/-}$BMMCs (Fig E6, G). As shown in Fig 5, A and B, NECA pretreatment robustly potentiated methacholine-induced increases in R_{aw} in C57BL/6Kit$^{W\text{-}sh}$/Kit$^{W\text{-}sh}$ mice reconstituted with wt but not $A_3^{-/-}$ BMMCs (P = .026). These data establish that that NECA-induced AHR is the result of activation of A_3 adenosine receptors on mast cells. We failed to observe a significant effect of NECA on methacholine-induced G_{tissue} increase in wt and $A_3^{-/-}$ mast cell–reconstituted mice (see this article's Fig E7 in the Online Repository at http://www.jacionline.org), further suggesting that mast cell activation by adenosine acts predominantly on proximal airways to produce AHR in mice.

The potency of adenosine to elicit lung mast cell degranulation

Our results show that aerosolized NECA can induce robust AHR through a mast cell–dependent mechanism, but cannot produce bronchoconstriction directly in anesthetized mice. In contrast, we and others have found that antigen-induced mast cell degranulation can produce bronchoconstriction in naive mice under anesthesia (data not shown). To determine whether differences in the magnitude of mast cell degranulation in response to each stimulus might be responsible for these differential effects on airway physiology, we examined the capacity of adenosine and antigen to degranulate murine lung mast cells acutely *in vitro*. As shown in Fig 6, adenosine induced modest degranulation of lung mast cells. In contrast, the magnitude of degranulation was much greater after stimulation with antigen. These findings support the hypothesis that the magnitude of adenosine-induced degranulation is insufficient to produce direct bronchoconstriction, but sufficient to prime the airway to become hyperresponsive to subsequent stimulation.

DISCUSSION

Airway hyperresponsiveness is a cardinal feature of asthma, characterized by bronchoconstriction after exposure to numerous nonantigenic stimuli, including cold air,

perfumes, and exercise. In this report, we describe a previously unrecognized role for adenosine as an inducer of AHR. Because it is well established that adenosine levels are elevated in the asthmatic lung,[8,9] there is a strong implication that adenosine may contribute to the development of AHR in patients with asthma.

Modulatory effects of adenosine on airway physiology have long been observed.[28] Previous studies have focused on the pathways mediating exogenous adenosine-induced bronchoconstriction.[29,30] However, it remains unclear whether endogenous adenosine produces direct bronchoconstriction in patients with asthma. Adenosine inhalation–induced bronchoconstriction in patients with asthma can be largely (80%) relieved by pretreatment with either mast cell membrane stabilizers or antihistamines.[31,32] However, these interventions are largely ineffective for the control of airflow obstruction during asthma attacks or status asthmaticus, in which endogenous levels of adenosine should be markedly increased. In addition, although A_1 receptors have also been shown to partially mediate adenosine-induced bronchoconstriction through a mast cell–independent pathway, targeting the A_1 receptor gene in human beings with asthma has been largely ineffective.[16,19,33,34] These observations suggest that endogenous adenosine may affect airway pathophysiology in ways other than eliciting bronchoconstriction. In this study, we have discovered that adensosine produces AHR in mice. On the basis of these findings, we propose that the pathophysiological role of adenosine in the asthmatic lung may be to prime the airway and produce AHR through activation of mast cells, rather than by serving as a direct bronchospastic mediator.

We used 2 different methodologies, the single compartment model (R_L and C_{dyn}) and the constant phase model (R_{aw} and G_{tissue}) of lung mechanics, to evaluate adenosine-induced AHR *in vivo* in mice. Both methods have demonstrated that acute NECA exposure renders the airways hyperresponsive, significantly increasing the response to methacholine challenge 20 minutes later. One limitation of our study is that we do not know how long these effects of NECA on airway physiology persist. Although the natural cognant ligand adenosine is subject to rapid metabolism *in vivo*, it is elevated in the bronchoalveolar lavage fluid from patients with asthma, suggesting that continuous generation of adenosine in the human lung, likely a function of elevated metabolism associated with inflammation, provides a persistent stimulus that could possibly contribute to a sustained effect on AHR. In addition, we observed that the changes in R_L and R_{aw} were greater than those in C_{dyn} and G_{tissue}. Because increases in R_{aw} reflect narrowing of the proximal airways, these data initially suggested that NECA may be acting on ASM *in vivo* to produce AHR.

Previous studies have shown that adenosine can elicit calcium mobilization through activation of A_1 adenosine receptors in cultured human ASM cells, suggesting that adenosine acts through A_1 receptors to modulate the contractility of ASM.[21,22] However, our studies with *ex vivo* isolated tracheal rings showed that pretreatment with NECA failed to change methacholine-induced tension development. Furthermore, our *in vivo* studies showed that mice deficient in A_1 receptors developed AHR of similar magnitude as wt animals in response to NECA. Collectively, these experiments demonstrate that adenosine-induced AHR is not the result of activation of A_1 adenosine receptors on ASM, or A_1 receptors on any other cell type.

Our study has identified a critical role for mast cells in the development of adenosine-induced AHR. Adenosine-induced AHR was abolished by genetic deletion of mast cells in mice and was restored by the reconstitution of mast cells in mast cell–deficient mice, indicating that mast cells are critical intermediaries in the induction of AHR by adenosine. The potential role of mast cells in AHR development has been proposed in several previous studies. Mast cell infiltration in ASM bundles has been reported to be the major difference between asthma and eosinophilic bronchitis (a disease that has asthmalike airway inflammation but lacks AHR), where the ASM is devoid of mast cells.[35] In patients with asthma, anti–TNF-α therapy can simultaneously reduce AHR and sputum histamine levels but has no effect on airway inflammation, suggesting that there may be a special link between AHR and mast cells.[36] In mice, mast cells can promote the formation of multiple features of chronic asthma including AHR.[37] However, it remains unclear how mast cells are activated in the asthmatic lung and subsequently contribute to AHR development.

It has long been held that mast cells either exist at resting state or become transiently activated to induce mediator release via degranulation and *de novo* synthesis of lipids and cytokines. Mast cell degranulation, which is usually triggered by antigen-induced cross-linking of IgE/FcεRI receptors, is responsible for the acute and sometimes life-threatening manifestations of asthma and other allergic disorders. Recently, however, mounting evidence has revealed that mast cells can be modestly and differentially activated by other stimuli, which differs from classic IgE/antigen-induced " anaphylactic degranulation," both in kinetics and in amounts and/or spectrum of secreted mediators.[38–42] These nonanaphylactoid activation pathways, including so-called *piecemeal degranulation*, may not trigger the acute clinical manifestations of an anaphylactic response, but nevertheless contribute to the formation of chronic features of asthma such as AHR.[37,43] In the mouse, Martin et al[44] demonstrated that degranulation of mast cells at modest magnitude by antigen primed the airway to be more responsive to methacholine, rather than directly producing bronchoconstriciton. Our data show that although adenosine can degranulate mast cells, it causes only modest degranulation compared with that associated with antigen/IgE, a finding consistent with previous observations.[26] In addition, anti-IgE therapy improves airway inflammation but not AHR in patients with asthma, suggesting that alternative activation pathways, such as adenosine-induced degranulation, may perhaps play a more important role in AHR development.[45]

Extensive mast cell degranulation results in the release of numerous mediators capable of stimulating ASM contraction. These mediators include histamine, serotonin, tryptase, prostaglandin D_2, leukotriene C_4/leukotriene D_4, tryptase, major basic protein, thromboxane A_2, platelet-activating factor, and angiotensin II. Our data suggest that levels of mast cell mediators achieved by A_3 activation *in vivo* are insufficient to produce bronchoconstriction by themselves but render the airway hyperresponsive to methacholine. One possible mechanism includes alteration of smooth muscle dynamics. Although an attractive hypothesis, preliminary studies from our laboratory have been unable to replicate this phenomenon convincingly *ex vivo*, because pretreatment of murine tracheal rings with subthreshold concentrations of serotonin or leukotriene D_4 produced only modest increases in methacholine-induced contraction (data not shown). Other potential mechanisms include

changes in airway geometry or luminal patency as the result of mucosal edema or mucus secretion. Because the naive mouse airway has few goblet cells, this latter mechanism, that NECA pretreatment causes changes in airway geometry via acting on goblet cells, seems less likely. We failed to detect any effects of NECA on lung mechanics before methacholine challenge, suggesting that major changes in luminal diameter are not occurring. We cannot exclude the possibility that AHR results from subtle changes in luminal diameter that are insufficient to alter airway resistance, or mucosal edema that does not change luminal diameter.

Airway hyperresponsiveness is a defining feature of asthma, but the mechanisms underlying its origins remain poorly understood. For many years, adenosine has been suspected to contribute to the pathogenesis of asthma, but a role for this ubiquitous biological mediator in AHR development has not been previously described. Our data have revealed a new role for adenosine as an inducer of AHR, through its capacity to degranulate mast cells by binding to the A_3 adenosine receptors. Limiting adenosine-induced mast cell degranulation may represent a novel means for controlling this important feature of asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
BMMC	Bone marrow–derived mast cell
C_{dyn}	Dynamic compliance
G_{tissue}	Tissue damping
R_{aw}	Airway resistance
R_L	Lung resistance
wt	Wild-type

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Key messages

- Adenosine can induce AHR in mice indirectly via activation of A_3 adenosine receptors on mast cells.
- Because adenosine levels are elevated in the lungs of patients with asthma, these studies suggest that endogenous adenosine may contribute to the development of AHR in asthma.

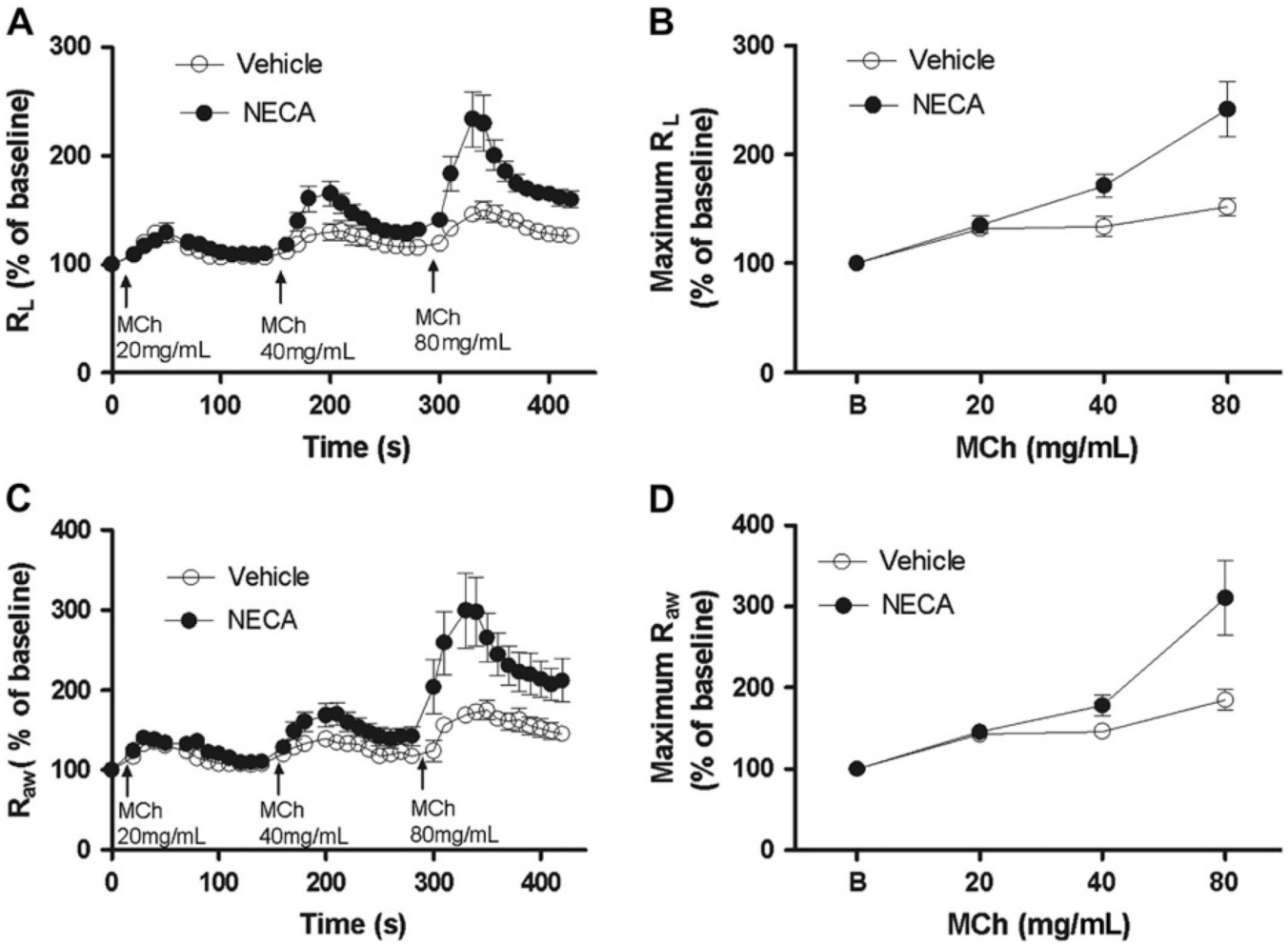

FIG 1.

Adenosine analogue NECA induces AHR in mice. Methacholine (*MCh*)–induced changes in resistance of lung (R_L) and resistance of airway (R_{aw}) were measured in mice pretreated with aerosolized NECA (3 mg/mL; n = 13) or vehicle (n = 11). Data are expressed as percent of baseline ± SEMs. **A** and **C**, respective changes in R_L and R_{aw} over time; **B** and **D**, peak R_L and R_{aw} after each MCh exposure. *P* <.01 in *A* and *B*; *P* <.05 in *C* and *D*; NECA vs vehicle groups by repeated-measures ANOVA.

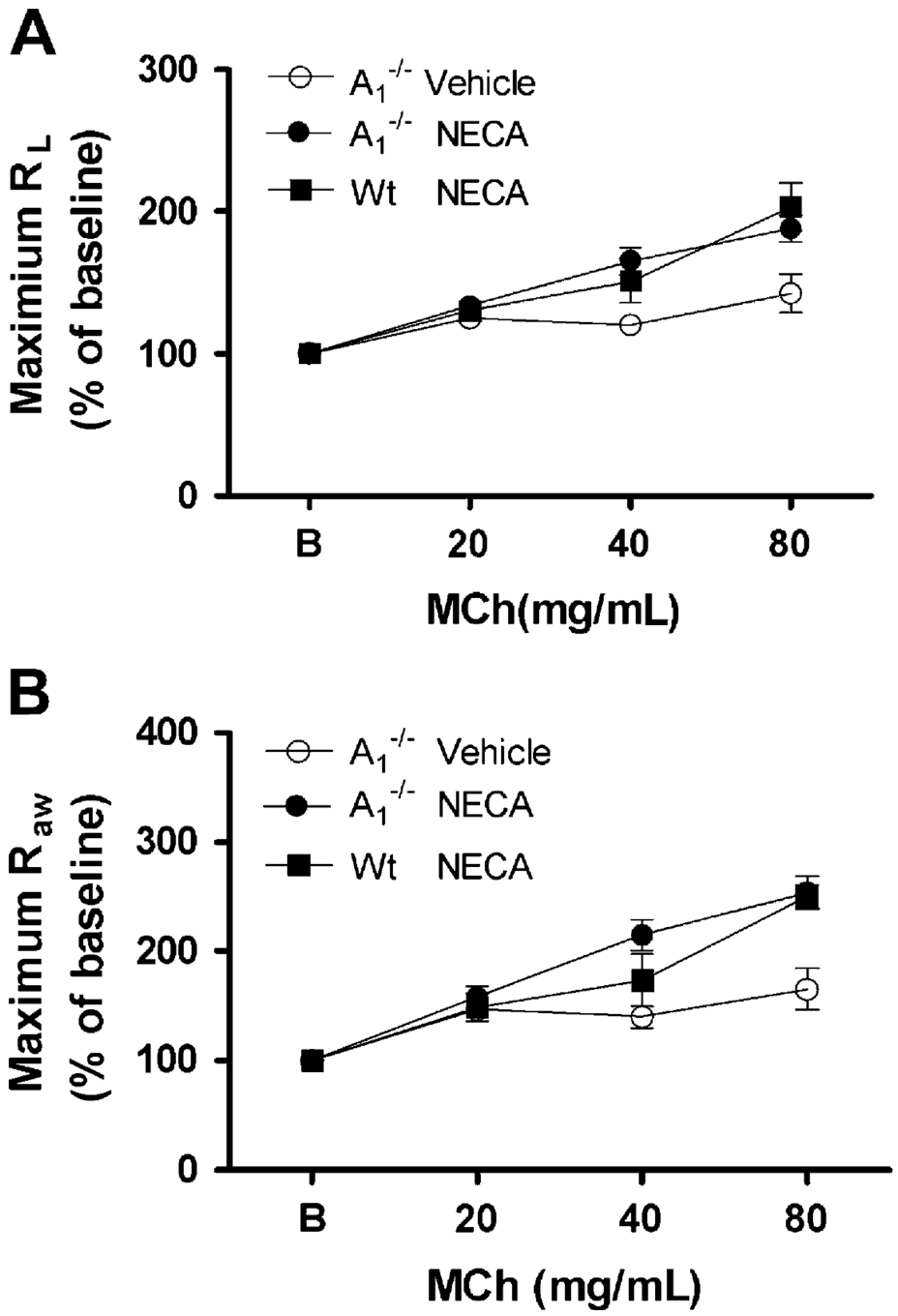

FIG 2.

NECA-induced AHR in $A_1^{-/-}$ mice. Data represent the peak R_L (**A**) and R_{aw} (**B**) after methacholine (*MCh*) exposure and are expressed as percent of baseline ± SEMs. $P < .01$ in *A* and *B*, for $A_1^{-/-}$ NECA (n = 17) vs $A_1^{-/-}$ vehicle (n = 11) groups; $P = .92$ in *A*, 0.46 in *B* for wt NECA (n = 5) vs $A_1^{-/-}$ NECA groups by repeated-measures ANOVA.

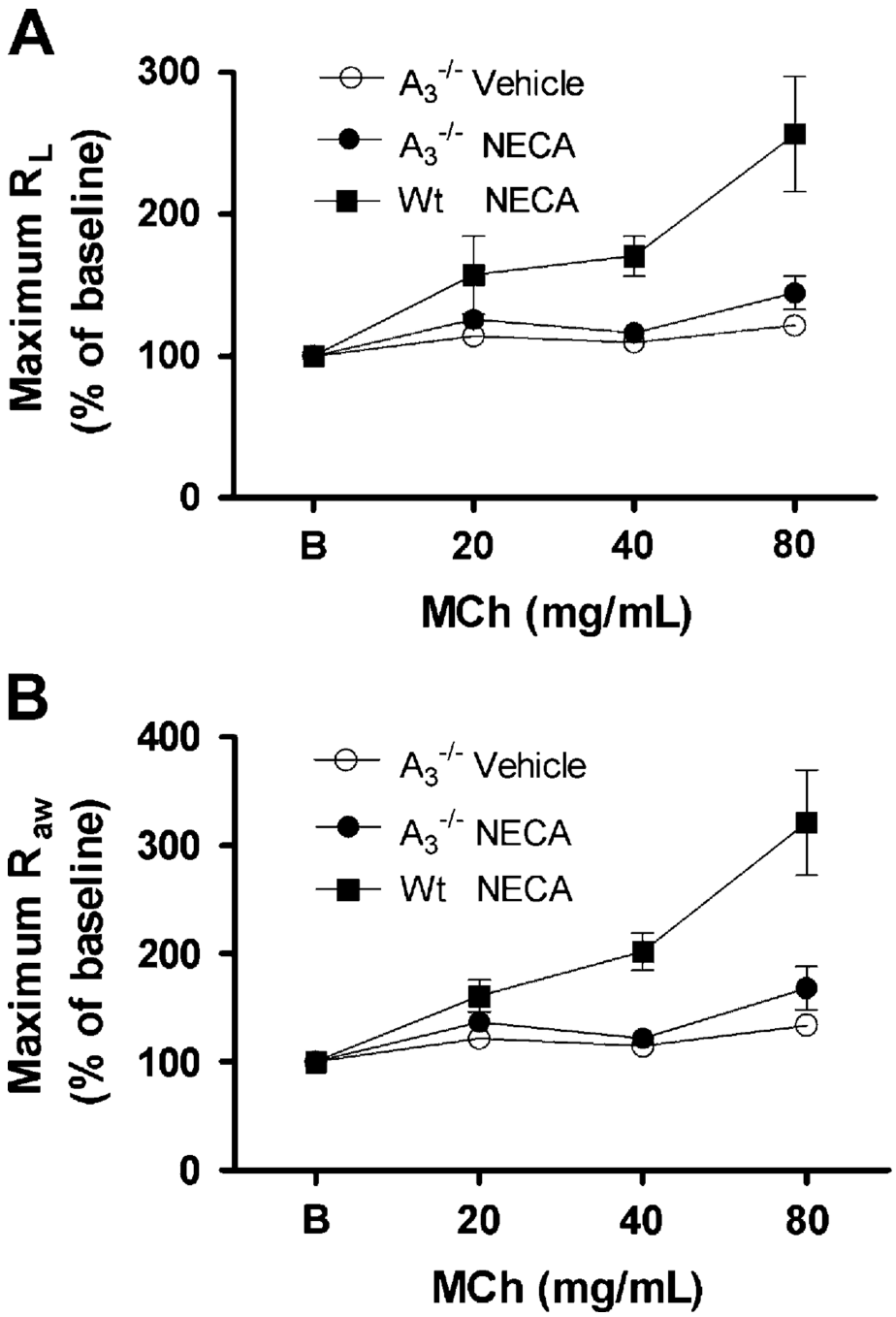

FIG 3.

NECA-induced AHR is A_3 receptor-dependent. Data represent the peak R_L (**A**) and R_{aw} (**B**) after each methacholine (*MCh*) exposure and are expressed as percent of baseline ± SEMs. $P < .01$ in both *A* and *B*, wt NECA (n = 4) vs both $A_3^{-/-}$ vehicle (n = 6) and $A_3^{-/-}$ NECA (n = 7); $P > .2$ in *A* and *B*, $A_3^{-/-}$ NECA vs $A_3^{-/-}$ vehicle, by repeated-measures ANOVA and least significant difference test.

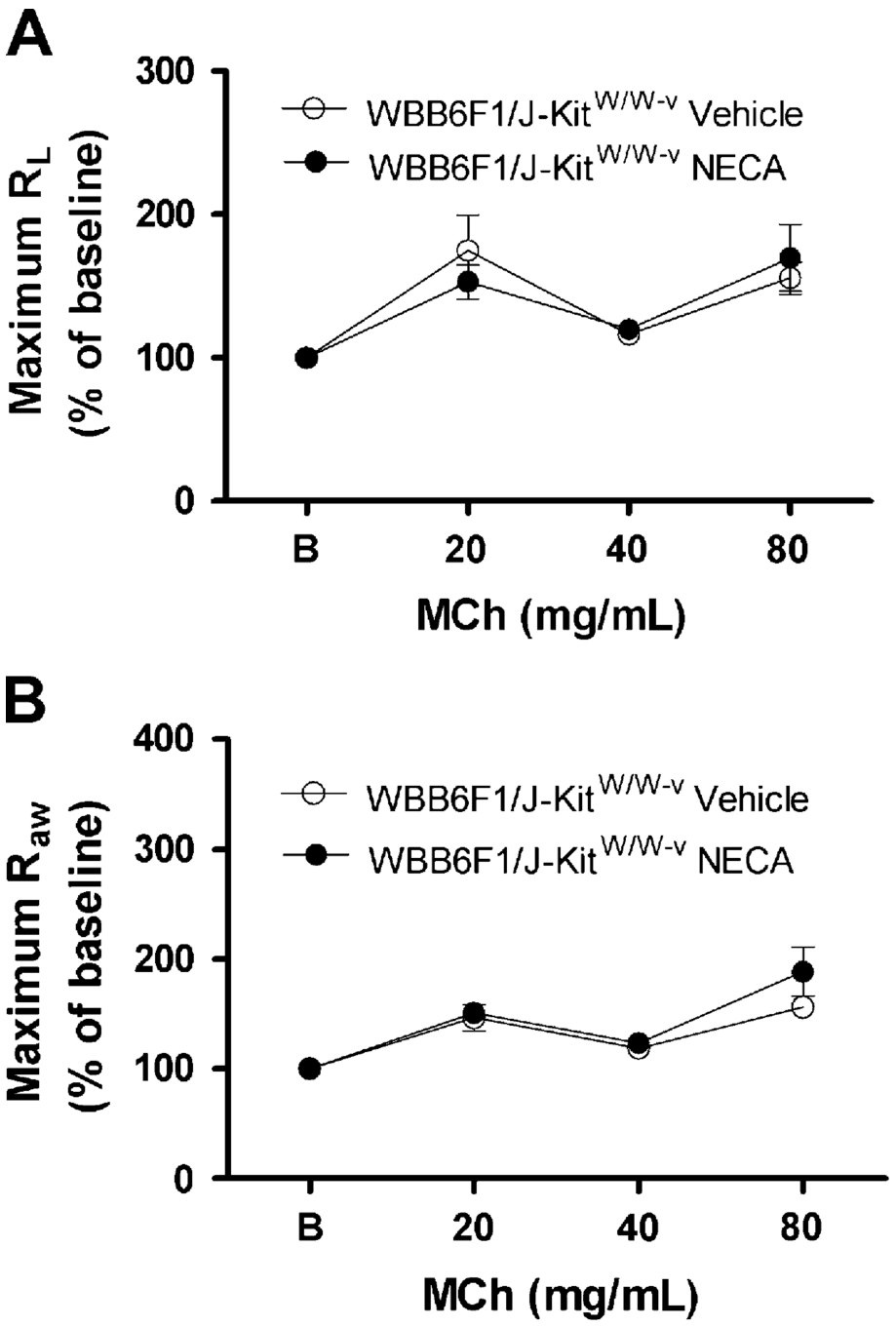

FIG 4.

NECA-induced AHR is mast cell–dependent. Data represent the peak R_L (**A**) and R_{aw} (**B**) after each methacholine (*MCh*) exposure and are expressed as percent of baseline ± SEMs. $P > .18$ in *A* and *B*, NECA (n = 8) vs vehicle (n = 7) groups by repeated-measures ANOVA.

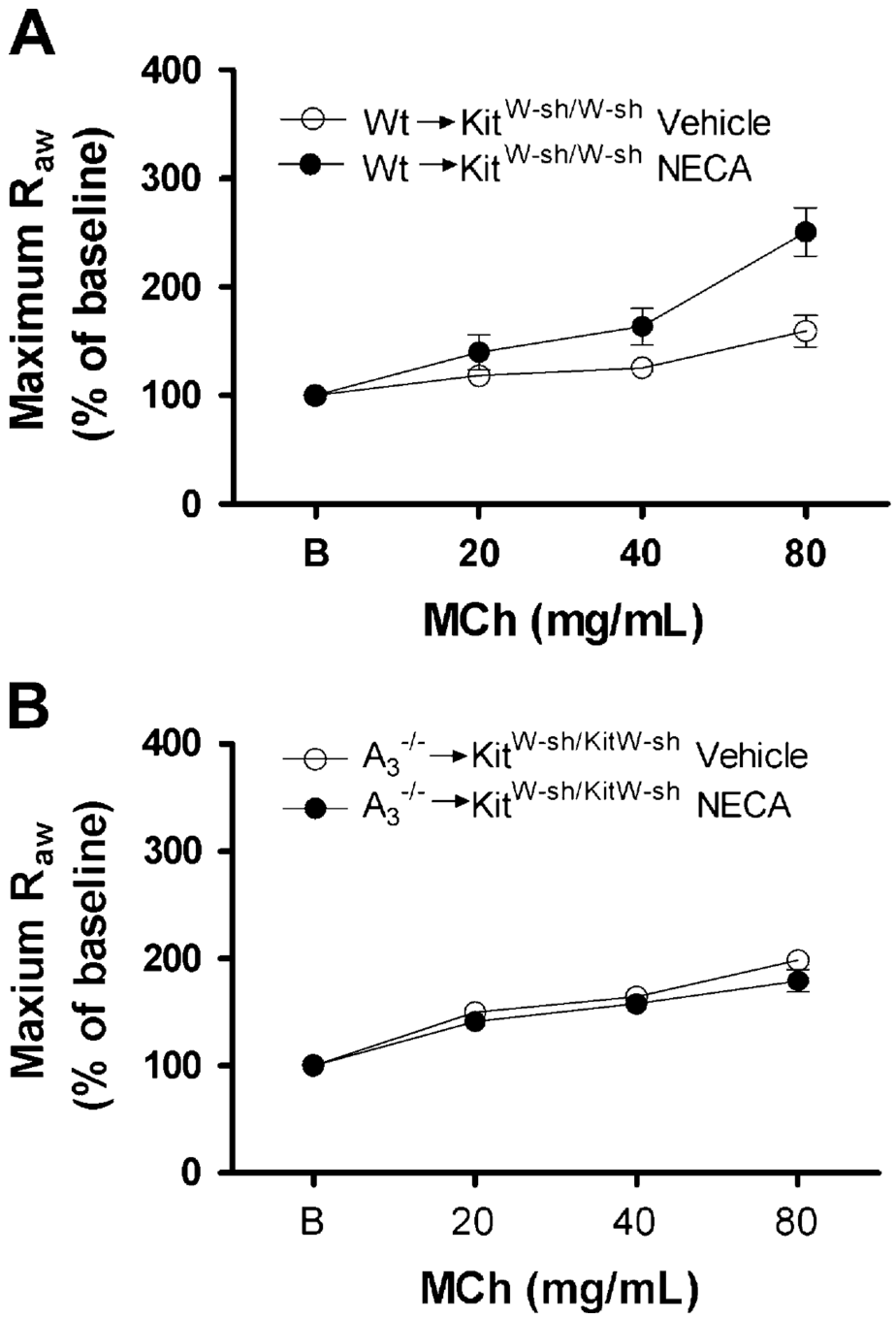

FIG 5.

NECA-induced AHR in mast cell–reconstituted mice. Mast cell–deficient mice were reconstituted (→) with wild-type (**A**) or $A_3^{-/-}$ (**B**) mast cells. Data represent the peak R_{aw} after each methacholine (*MCh*) exposure and are expressed as percent change from baseline ± SEMs. *P* = .026 in *A* (n = 8/group) and .187 in *B* (n = 4–5/group); NECA vs vehicle groups by repeated-measures ANOVA.

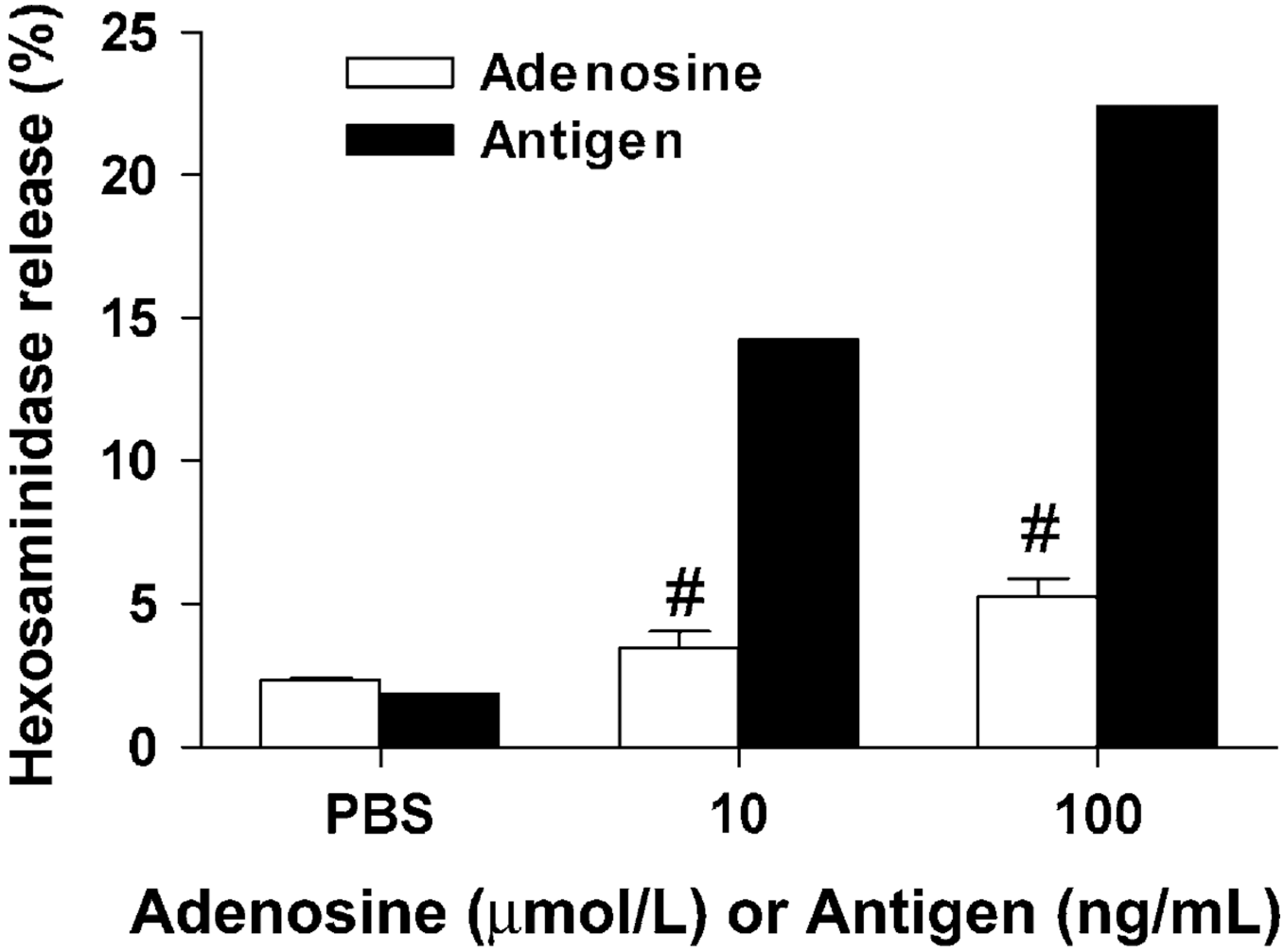

FIG 6.

Adenosine-induced degranulation of primary lung mast cells. Murine primary lung mast cells were cultured *in vitro* in the presence of stem cell factor and IL-3 for 5 weeks. Cells then were loaded with IgE for 12 hours. Antigen (DNP-HSA) and adenosine-induced mast cell degranulation was determined by measuring hexosaminidase release. #*P* <.05 by *t* test vs PBS-treated cells.