



## Intranasal administration of NECA can induce both anti-inflammatory and pro-inflammatory effects in BALB/c mice: Evidence for A<sub>2A</sub> receptor sub-type mediation of NECA-induced anti-inflammatory effects<sup>☆</sup>

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### ABSTRACT

The role of adenosine in allergic inflammation is unclear. This study investigated the effects of the non-selective adenosine receptor agonist, 5-*N*-ethylcarboxamido-adenosine (NECA), on immunized only and immunized and airway challenged mice. The adenosine receptor sub-type(s) mediating the NECA effects and the A<sub>2A</sub> receptor mRNA expression were also investigated.

In mice that were only immunized, intranasal NECA (1 mM) administration caused a significant increase in bronchoalveolar lavage total cell count (TCC), neutrophils and eosinophils (>1.5-, >6 and >60-fold, respectively). Two and four intranasal ovalbumin (OVA) challenges induced a significant ( $P < 0.05$ ) increase in TCC (>2.1- and >4-fold, respectively) and eosinophils (>350- and >1700-fold, respectively). Real-time PCR analysis showed that the A<sub>2A</sub> receptor sub-type mRNA was significantly increased ( $P < 0.05$ ) in the lung tissue of immunized mice following both two and four OVA challenges. NECA (0.3 mM) treatment caused a significant reduction in the increase induced by the two and four OVA challenges in the TCC by 46.1% and 56.6%, respectively, eosinophils by 70.1% and 75.6%, respectively, and in the A<sub>2A</sub> receptor sub-type mRNA by 43.2% and 41.0%, respectively. Treatment with the A<sub>2A</sub> receptor antagonist, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, SCH-58261, completely reversed both the NECA-mediated reduction in TCC and eosinophilia. Moreover, OVA challenge of immunized mice, over 2 consecutive days, resulted in a significant ( $P < 0.05$ ) increase in TCC (4.5-fold) and eosinophils (>2000-fold) that was detected 72 h later. NECA (0.3 mM) treatment, at 24 and 48 h post OVA challenge, significantly reduced the increase in both TCC and eosinophils by 45.0% and 74.8%, respectively. Our data show that in immunized, but not OVA-challenged mice, high dose of NECA (1 mM) induces an inflammatory airway response. In contrast, in models of inflammation, NECA, at mainly 0.3 mM, induces a significant anti-inflammatory effect when administered prior to the induction of airway inflammation or therapeutically following its establishment. The data also indicate that the anti-inflammatory action of NECA seems to be mediated via the A<sub>2A</sub> receptor sub-type and hence the use of selective A<sub>2A</sub> receptor agonists as potential therapeutic agents in the treatment of inflammatory diseases such as asthma should be investigated further.

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### 1. Introduction

Adenosine is a ubiquitous nucleoside which serves mainly as an extracellular messenger [1] whose normally low cellular

concentration is significantly increased during hypoxia or metabolic stress [1]. A role in inflammatory diseases such as asthma has been suggested for over two decades and is based on several lines of evidence including elevated levels of adenosine in bronchoalveolar lavage (BAL) fluid of asthmatic patients [2] and in the blood of asthmatics following provocation with allergen [3]. Moreover, provocation with adenosine or adenosine monophosphate results in bronchoconstriction in asthmatic patients but not in normal subjects [4] and in allergic but not normal animals

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[5–7]. In this regard, the mast cell and mast cell derived mediators may play a role in mediating the adenosine-induced bronchoconstriction [8,9].

Adenosine-induced effects are mainly mediated via the activation of G protein coupled purinergic receptors of the P1 type namely, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>. Although all receptors bind adenosine, they differ in their binding affinities, cellular expression and the effector molecules to which they are coupled [10]. In addition to its accepted role in bronchoconstriction, much attention over the last decade has focused towards elucidating the effects of adenosine on pro-inflammatory cells within the context of asthma [11]. Recently, it has been shown that exposure of asthmatics to adenosine causes eosinophil cell influx into the airways [12]. An earlier study using mice deficient in adenosine deaminase (ADA) showed that they had an increased level of endogenous adenosine in their lung tissue and this was associated with severe pulmonary inflammation [13]. Adenosine has also been shown to enhance ragweed-induced airway inflammation which was also associated with an increase in the levels of several inflammatory mediators [14].

Conversely, several studies have shown that adenosine can also mediate anti-inflammatory effects. For example in a rat model of asthma, the A<sub>2A</sub> agonist, CGS 21680, induced anti-inflammatory effects similar in magnitude to those induced by budesonide [15]. In a murine model of inflammation, mice deficient in the A<sub>2A</sub> receptor sub-type had a significantly higher degree of tissue inflammation compared with normal animals [16]. Furthermore, adenosine, via the A<sub>1</sub> receptor, mediates anti-inflammatory effects in mice such as decreased IL-4 and IL-13 release and reduces goblet cell metaplasia [17]. However, other studies have reported that the A<sub>1</sub> receptor sub-type has significant pro-inflammatory effects [18,19].

Thus, neither the role of adenosine nor its specific receptor sub-types in inflammation are well resolved. In this study, we investigated 1) the airways cellular response of immunized, but not challenged mice, to intranasal administration of the non-selective adenosine receptor agonist, NECA, 2) the effect of NECA administration on the induction of airway inflammation in immunized and challenged mice, 3) the adenosine receptor sub-type(s) involved in mediating the NECA-induced effects, 4) the effect of therapeutic NECA administration on established OVA-induced airway inflammation, and 5) the effect of OVA challenge and NECA treatment on the expression of the A<sub>2A</sub> receptor sub-type mRNA.

## 2. Materials and methods

### 2.1. Animals

Male BALB/c mice approximately 6–8 weeks old, maintained on OVA-free diet, were used throughout this study. All experimental protocols were approved by the Animal Welfare Committee and complied with Kuwait University Health Sciences Centre requirements.

### 2.2. Reagents

5-N-Ethylcarboxamidoadenosine, phosphate buffered saline, ovalbumin (grade V), dexamethasone 21-phosphate disodium salt, halothane, 8-cyclopentyl-1,3-dipropylxanthine, 3-propylxanthine, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH-58261), 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate (MRS 1523) (Sigma–Aldrich, USA), absolute ethanol (Merck KGaA, Germany), formaldehyde (Surechem Products Ltd, UK), Alu-Gel-S (SERVA Electrophoresis GmbH, Germany), Isotone II diluent solution (Beckman Coulter Inc., USA), Zap-OGLOBIN (Coulter Electronics Ltd, UK), Diff-Quick (Baxter Dade AG, Dudingon, Switzerland),

dimethyl sulphoxide (BHD Laboratory Supplies, Poole, England), propylene glycol (Merck Schuchardt, Hohenbrunn, Germany).

### 2.3. Sensitization

BALB/c mice were immunized once intraperitoneally (i.p.) with 10 µg OVA in 0.2 ml of Alu-Gel-S on day 0. All other OVA challenges and drug treatments were given intranasally as specified in the protocols below.

### 2.4. Bronchoalveolar lavage cell counts and differentiation

At the specified time points (see protocols), after intranasal OVA or PBS challenge, BAL was performed as described previously [20]. Results are expressed as total cells or the number of macrophage, lymphocytes, neutrophils and eosinophils per ml of BALF.

### 2.5. RT-PCR

Crushed frozen lung tissue (200–300 mg) dissected from mice following the BAL procedure was resuspended in Trizol (Invitrogen, USA) and total cellular RNA extracted using the manufacturer's protocol. First strand cDNA synthesis (42 °C for 50 min) was performed with 2 µg RNA in 20 µl containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM dNTP mix, 100 ng random hexamers (Pharmacia Biotech, Uppsala) and 200 U MMLV reverse transcriptase (Invitrogen, USA). PCR amplification was performed in the Roche Light Cycler (LC) using the RT<sup>2</sup> SYBR Green qPCR Master Mix from Superarray (Fredrick, MD, USA). The cDNA (1 µl) was added to 12.5 µl of 2× master reaction mix, 1 µl of RT<sup>2</sup> PCR primer set for A<sub>2A</sub> or GAPDH and water to a final volume of 25 µl. Cycling parameters following a pre-incubation at 95 °C for 10 min were: 95 °C for 1 s, 55 °C for 10 s and 72 °C for 13 s. Relative quantitation was performed using the 4.1 LC software package and data expressed as the ratio of signals obtained for A<sub>2A</sub>/GAPDH. After 45 cycles of amplification products were verified by melting curve analysis as well as subsequent electrophoresis.

### 2.6. Histopathology

Resected lungs were immersed in 5 ml of 10% formaldehyde in PBS, fixed and embedded in paraffin. Sections (4 µm) were examined following staining with hematoxylin and eosin.

### 2.7. Expression of results and statistical analysis

Total and differential cell counts are presented as means ± SEM, and represent the number of cells/ml. The expression of A<sub>2A</sub> mRNA in lung tissue was measured by real-time PCR as described in [Materials and methods](#). The expression observed following treatment with PBS, OVA and OVA-NECA was normalised to the level of a 'house-keeping gene' (GAPDH: glyceraldehyde phosphate dehydrogenase). Results are expressed as means ± SEM. Using a one-way analysis of variance (ANOVA) all the mean differences were compared at once and a post hoc analysis (LSD) was used to determine if there were differences between individual groups. The mean was determined as significant at the 0.05 level because the analysis was post hoc.

### 2.8. Specific experimental protocols

All specific protocols were run twice or three times (depending on the size of the groups within the specific protocol) and at each time all groups were included in each protocol. This ensured that all

groups were subjected to the same conditions. The volume for all intranasal OVA and drug administration was 50  $\mu$ l.

#### 2.8.1. Protocol 1a: to assess the effect of NECA treatment (over two days) on airway cellular influx

Four groups were randomly assigned and all were immunized as described above. Group 1 mice ( $n = 5$ ) were treated with the vehicle for NECA (10% ethanol). Groups 2–4 received 0.1 mM ( $n = 8$ ), 0.3 mM ( $n = 7$ ), 1 mM ( $n = 9$ ) NECA, respectively over 2 days. We chose NECA, as an agonist, in preference to adenosine itself as the latter is extensively metabolized [21] whereas NECA is relatively more stable.

#### 2.8.2. Protocol 1b: to assess the effect of OVA challenge on airway cellular influx

Eight groups were randomly assigned. Groups 1 ( $n = 8$ ) and 2 ( $n = 9$ ) were challenged on day 10 with PBS and OVA (dissolved in PBS), respectively, and BAL performed on day 11. Groups 3 ( $n = 8$ ) and 4 ( $n = 8$ ) were challenged on days 10 and 11 with PBS and OVA, respectively, and BAL performed on day 12. Groups 5 ( $n = 8$ ) and 6 ( $n = 7$ ) were challenged on days 10–12 with PBS and OVA, respectively, and BAL performed on day 13. Groups 7 ( $n = 8$ ) and 8 ( $n = 8$ ) were challenged on days 10–13 with PBS and OVA, respectively, and BAL performed on day 14.

#### 2.8.3. Protocol 1c: to assess the effect of NECA on 2 days' OVA challenge-induced airway cellular influx

Six groups were randomly assigned. Groups 1 ( $n = 7$ ) and 2 ( $n = 7$ ) were challenged with PBS and OVA, respectively, on days 10 and 11 and then treated with vehicle for NECA (10% ethanol) 5–10 min thereafter on both days. Groups 3 ( $n = 10$ ), 4 ( $n = 10$ ) and 5 ( $n = 9$ ) were challenged with OVA on days 10 and 11 and then treated with NECA (0.1, 0.3, and 1.0 mM, respectively) 5–10 min thereafter on both days. Group 6 ( $n = 9$ ) was challenged with OVA on days 10 and 11 and then treated with dexamethasone (1.3 mM) 5–10 min thereafter. BAL was performed on day 12 for all groups. To assess the effect of the 2 days' OVA challenge and the NECA treatment on the expression of  $A_{2A}$  mRNA, lungs were taken from the PBS, OVA challenged and the 0.3 mM NECA treated groups for the RT-PCR experiment.

#### 2.8.4. Protocol 1d: to assess the effect of NECA on 4 days' OVA challenge-induced airway cellular influx (these experiments were conducted to confirm the findings of experiments performed in protocol 1c)

Five groups were randomly assigned. Groups 1 ( $n = 10$ ) and 2 ( $n = 10$ ) were challenged with PBS on days 10–13 and treated with vehicle or 0.3 mM NECA. Groups 3 ( $n = 10$ ), 4 ( $n = 12$ ) and 5 ( $n = 7$ ) were all challenged with OVA and treated with either vehicle, 0.3 mM NECA or 1.3 mM dexamethasone 5–10 min thereafter. BAL was performed on day 14 for all groups. To assess the effect of the 4 days' OVA challenge and the NECA treatment on the expression of  $A_{2A}$  mRNA, lungs were taken from the PBS, OVA challenged and the OVA-NECA treated groups for the RT-PCR analysis.

#### 2.8.5. Protocol 1e: to assess the effect of the $A_1$ , $A_{2A}$ , $A_{2B}$ and $A_3$ receptor antagonists (DPCPX, SCH-58261, enprofylline and MRS 1523, respectively) on NECA-mediated reduction of OVA challenge (2 days)-induced airway cellular influx

Seven groups were randomly assigned. Groups 1 ( $n = 9$ ), 2 ( $n = 10$ ) and 3 ( $n = 11$ ) were all pretreated with the vehicle for the blockers (33.3% DMSO, 33.3% propylene glycol and 33.3% PBS). After 20 min, group 1 was challenged with PBS and groups 2 and 3 were challenged with OVA on both days 10 and 11. Groups 1 and 2

were then treated with vehicle (10% ethanol) and group 3 was treated with NECA (0.3 mM) 5–10 min thereafter on both days. Groups 4 ( $n = 11$ ), 5 ( $n = 9$ ), 6 ( $n = 8$ ) and 7 ( $n = 11$ ) were pretreated with the  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$  receptor antagonists (DPCPX, SCH-58261, enprofylline and MRS 1523, respectively) at 1 mM. As above, 20 min later, groups 4–7 were challenged with OVA and then treated with 0.3 mM NECA 5–10 min thereafter. BAL was performed on day 13 for all groups. An additional control experiment assessing the effects of SCH-58261 (NECA was not included) on baseline OVA-induced inflammation was also performed. Three groups were included, a PBS-challenged group ( $n = 5$ ), an OVA-challenged vehicle pretreated group ( $n = 5$ ) and an OVA-challenged SCH-58261 pretreated group ( $n = 5$ ). The dose of 1 mM was chosen for these antagonists as at a dose ratio (antagonist:NECA) was >3.5-fold. This ensures that the dose of the antagonists was sufficient to effectively antagonize the receptor but not too high to cause non-specific effects. Additionally a 0.1 mM dose, for all antagonists, was tested but no effect was observed. These data are not shown.

#### 2.8.6. Protocol 1f: to assess the effect of therapeutic administration of NECA on established airway cell influx

Six groups were randomly assigned. Groups 1 ( $n = 7$ ) and 2 ( $n = 10$ ) were challenged with PBS or OVA, respectively, on days 10 and 11 and then treated with the vehicle on days 12 and 13. Groups 3 ( $n = 13$ ), 4 ( $n = 12$ ) and 5 ( $n = 12$ ) were challenged with OVA on days 10 and 11 and treated with NECA (0.1, 0.3 and 1 mM, respectively) on days 12 and 13. Group 6 ( $n = 8$ ) was challenged with OVA on days 10 and 11 and then treated with dexamethasone (1.3 mM) on days 12 and 13. BAL was performed on day 14 for all groups.

### 3. Results

#### 3.1. Dose–response curve to NECA

Treatment of immunized, but not intranasally OVA challenged, mice with 1 mM NECA induced a significant (1.8-fold) increase in TCC, but not at the other doses, when compared with vehicle challenged mice (Fig. 1a). Additionally, mice challenged with 1 mM NECA exhibited significant ( $P < 0.05$ ) neutrophilia (>6-fold) and eosinophilia (>60-fold) (Fig. 1b).

#### 3.2. Total and differential cell counts following OVA or PBS challenge

OVA challenge of immunized mice induced a significant (>2-fold) increase in total cell influx after 2 and 3 challenges and >5-fold increase after 4 OVA challenges compared with time matched PBS-challenged mice, but not after a single challenge (Fig. 2a). In addition, the TCC post 4 OVA challenges was significantly greater than all other OVA-challenged groups (Fig. 2a). OVA challenge also induced a significant ( $P < 0.05$ ) increase in the number of lymphocytes (>10-fold), neutrophils (>60-fold) and eosinophils (>350-fold) after 4 challenges compared to PBS-challenged group. In addition, eosinophils increased significantly ( $P < 0.05$ ) after all challenges, even after the first challenge, and was highest after 4 challenges. Neutrophil and lymphocyte numbers showed a significant ( $P < 0.05$ ) increase only after the third OVA challenge (Fig. 2b).

#### 3.3. Effect of NECA on OVA (2 days' challenge)-induced cell influx

OVA challenge induced a significant (>2.3-fold) increase in TCC and eosinophils (>350-fold) compared with PBS-challenged mice

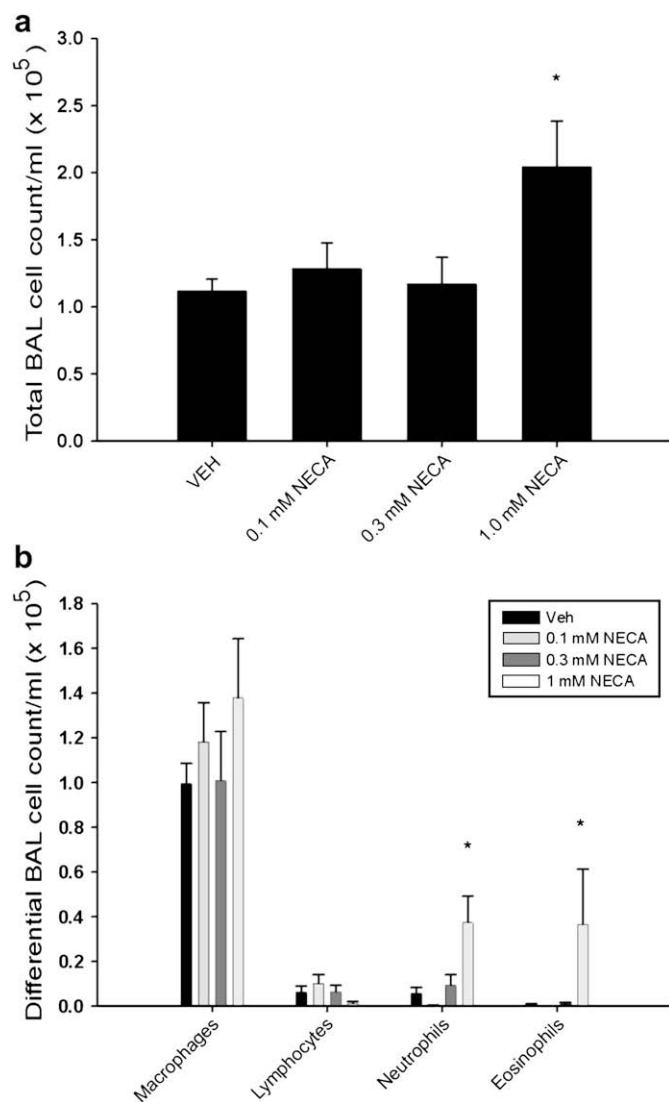


**Fig. 1.** (a and b) Effect of NECA (0.1, 0.3 and 1.0 mM) on total (a) and differential (b) BAL cell count in immunized only mice. Data are presented as means  $\pm$  SEM ( $n = 5-9$ ). \* $P < 0.05$  vs VEH challenged mice.

(Fig. 3a and b). Although OVA challenge increased both lymphocyte and neutrophil numbers, this did not achieve statistical significance ( $P > 0.05$ ). The TCC in both the NECA (0.3 mM) and the dexamethasone (1.3 mM) groups was significantly ( $P < 0.05$ ) reduced relative to the OVA-alone group by 46.1% and 51.5%, respectively (Fig. 3a). Additionally, treatment with NECA, at both 0.3 and 1 mM, and dexamethasone significantly ( $P < 0.05$ ) reduced the increase in eosinophils compared with OVA-challenged mice by 70.1%, 72.2% and 73.4%, respectively (Fig. 3b).

#### 3.4. Effect of NECA on OVA (4 days' challenge)-induced cell influx

OVA challenge induced a significant ( $>4$ -fold) increase in TCC, lymphocyte ( $>8$ -fold), neutrophil ( $>40$ -fold) and eosinophil ( $>1700$ -fold) compared with PBS-challenged mice ( $P < 0.05$ ). The TCC in both the NECA (0.3 mM) and the dexamethasone (1.3 mM) groups was significantly ( $P < 0.05$ ) reduced relative to the OVA-alone group by 56.6% and 75.7%, respectively (Fig. 4a). Treatment with NECA also significantly ( $P < 0.05$ ) reduced the lymphocyte (63.2%), neutrophil (80.0%) and eosinophil (75.6%) cell influx. Dexamethasone also significantly ( $P < 0.05$ ) reduced the OVA-

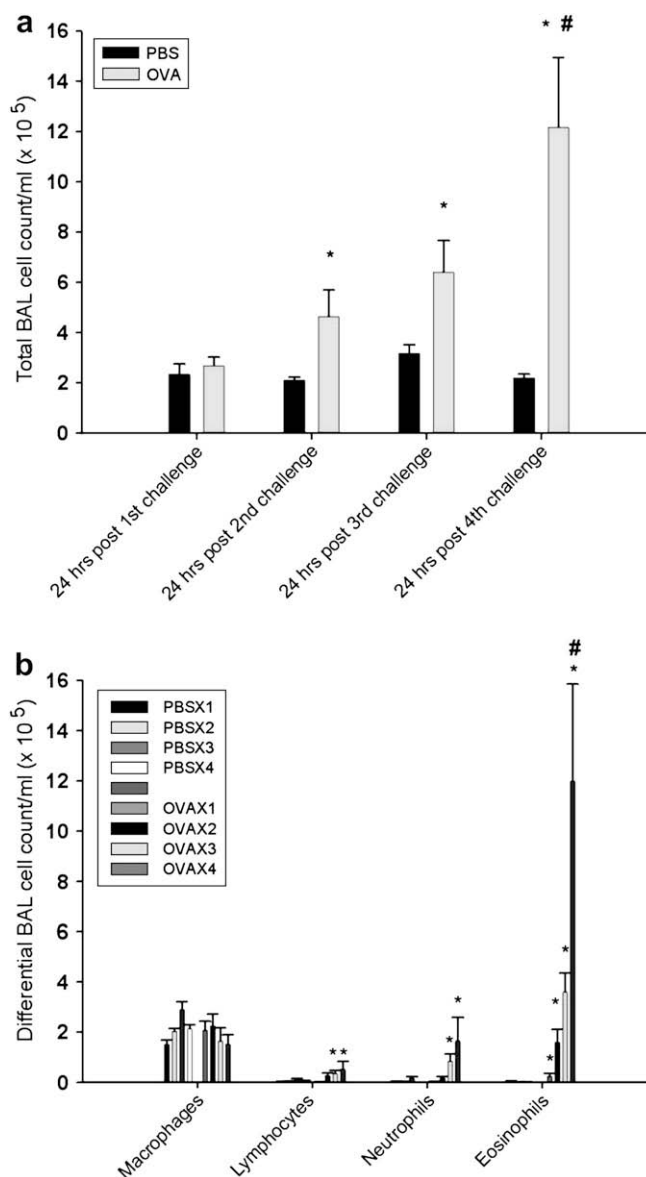


**Fig. 2.** (a and b) Effect of 1st, 2nd, 3rd and 4th OVA challenge on total (a) and differential (b) BAL cell count in immunized mice. Data are presented as mean  $\pm$  SEM ( $n = 7-9$ ). \* $P < 0.05$  vs PBS-challenged mice. # $P < 0.05$  vs OVA-challenged mice.

induced neutrophil and eosinophil influx to a comparable magnitude to the NECA-mediated suppression. However, the dexamethasone-induced lymphocyte suppression was not significant (Fig. 4b). Fig. 5a–d shows H&E stained sections of lung tissue from the 4 treatment groups: (a) OVA-immunized and PBS-challenged: normal lung histology with no apparent peribronchiolar (A) or epithelial (B) inflammation, (b) OVA-immunized and -challenged mice: the peribronchiolar area (A) shows intense inflammatory cell infiltration and the epithelium (B) appears enlarged, (c) OVA-immunized, OVA-challenged and NECA (0.3 mM) treated: the peribronchiolar area (A) shows a much reduced cellular infiltration and the epithelium (B) appears normal compared with OVA-challenged mice, (d) OVA-immunized, OVA-challenged and dexamethasone (1.3 mM) treated mice: the peribronchiolar area (A) also shows a much reduced cellular infiltration and the epithelium (B) appears normal compared with OVA-challenged mice and their appearance is similar to the NECA treated mice.

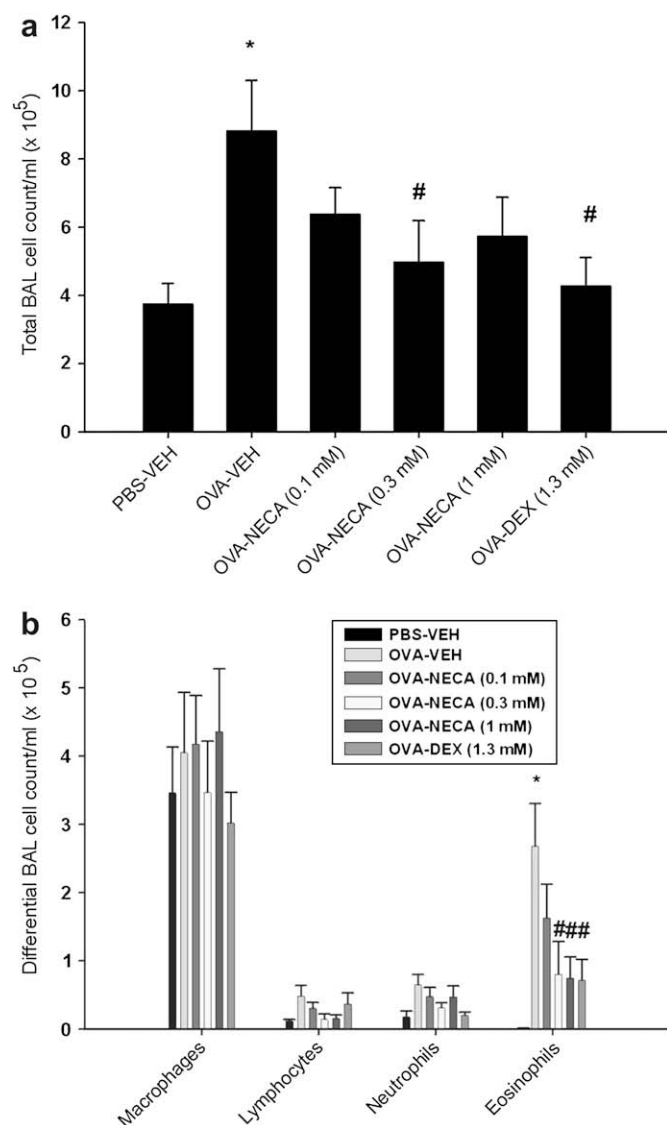


**Fig. 3.** (a and b) Effect of NECA (0.1, 0.3 and 1.0 mM) and dexamethasone (1.3 mM) on OVA (2 days' challenge)-induced increase in total (a) and differential (b) BAL cell count. Data are presented as means  $\pm$  SEM ( $n = 7-10$ ). \* $P < 0.05$  vs PBS-VEH challenged mice. # $P < 0.05$  vs OVA-VEH challenged mice.

### 3.5. Effect of the $A_1$ , $A_{2A}$ , $A_{2B}$ and $A_3$ receptor antagonists (DPCPX, SCH-58261, enprofylline and MRS 1523) on NECA-mediated reduction of OVA (2 days' challenge)-induced cell influx

OVA challenge induced a significant ( $>2$ -fold) increase in TCC compared with PBS-challenged mice ( $P < 0.05$ ; Fig. 6a). Also, eosinophil influx was significantly ( $P < 0.05$ ) increased after OVA challenge ( $>20$ -fold; Fig. 6b). Although neutrophils were increased in the OVA-challenged mice, this did not achieve statistical significance ( $P > 0.05$ ). Treatment with NECA significantly ( $P < 0.05$ ) reduced the OVA-induced increase in total and eosinophil cell influx by 50.0% and 55.3%, respectively. Treatment with the  $A_1$ ,  $A_{2B}$  or the  $A_3$  receptor blockers did not significantly affect the NECA-mediated inhibition of the TCC and eosinophil influx ( $P > 0.05$ ; Fig. 6a and b). In contrast, treatment with SCH-58261 not only completely reversed the NECA-induced reduction in TCC ( $P < 0.05$ ) but indeed significantly ( $P < 0.05$ ) increased the eosinophil influx above that induced by OVA (Fig. 6a and b). In addition the numbers

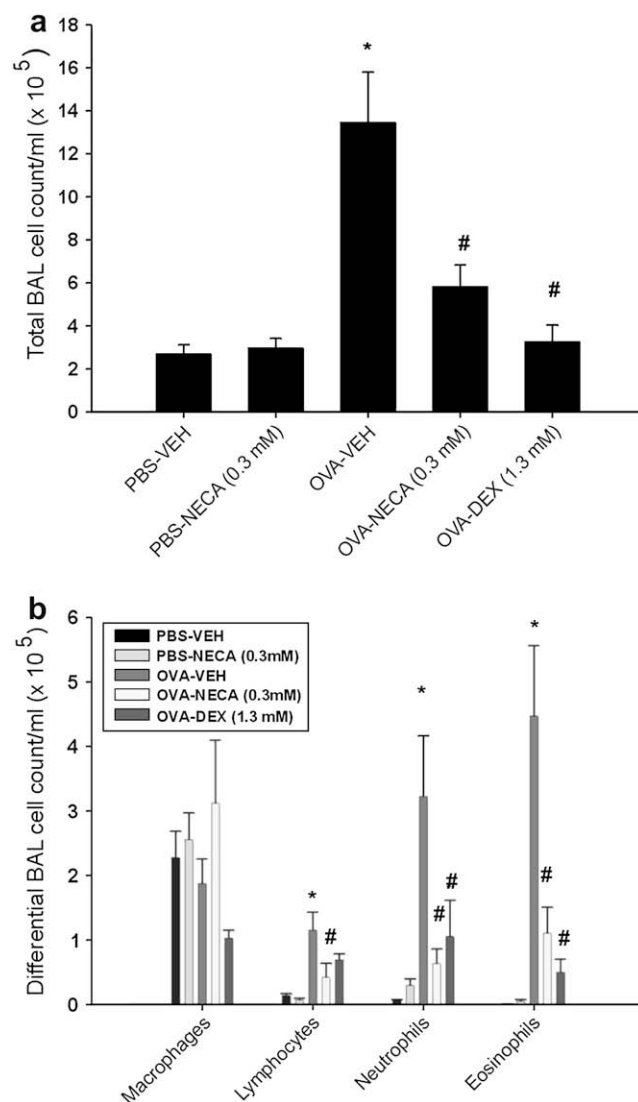


**Fig. 4.** (a and b) Effect of NECA (0.3 mM) and dexamethasone (1.3 mM) on OVA (4 days' challenge)-induced increase in total (a) and differential (b) BAL cell count. Data are presented as means  $\pm$  SEM ( $n = 7-10$ ). \* $P < 0.05$  vs PBS-VEH and PBS-NECA (0.3 mM) challenged mice. # $P < 0.05$  vs OVA-VEH challenged mice.

of neutrophils in the SCH-58261 treated group were significantly ( $P < 0.05$ ; Fig. 6a and b) increased compared to the PBS, the OVA and the OVA-NECA treated groups (Fig. 6b). Moreover, the control experiments with  $A_{2A}$  receptor antagonist, SCH-58261, showed that this compound did not significantly affect either the OVA-induced increase in TCC or eosinophils ( $P > 0.05$ ; Fig. 6c and d).

### 3.6. Effect of therapeutic NECA administration on established OVA-induced cell influx

OVA challenge induced a significant ( $>4.5$ -fold) increase in TCC following challenges on days 10 and 11 as assessed by BAL on day 14 compared with PBS-challenged mice (Fig. 7a). Lymphocyte, neutrophil and eosinophil numbers were also significantly ( $P < 0.05$ ) increased ( $>20$ -,  $15$ - and  $2000$ -fold, respectively) following OVA challenge. The TCC in the NECA (0.1 and 0.3 mM) and the dexamethasone (1.3 mM) groups was reduced relative to the OVA-alone group by 35.1%, 45.3% and 81.5%, respectively (Fig. 7a). This reduction was significant for the 0.3 mM NECA, but not the 0.1

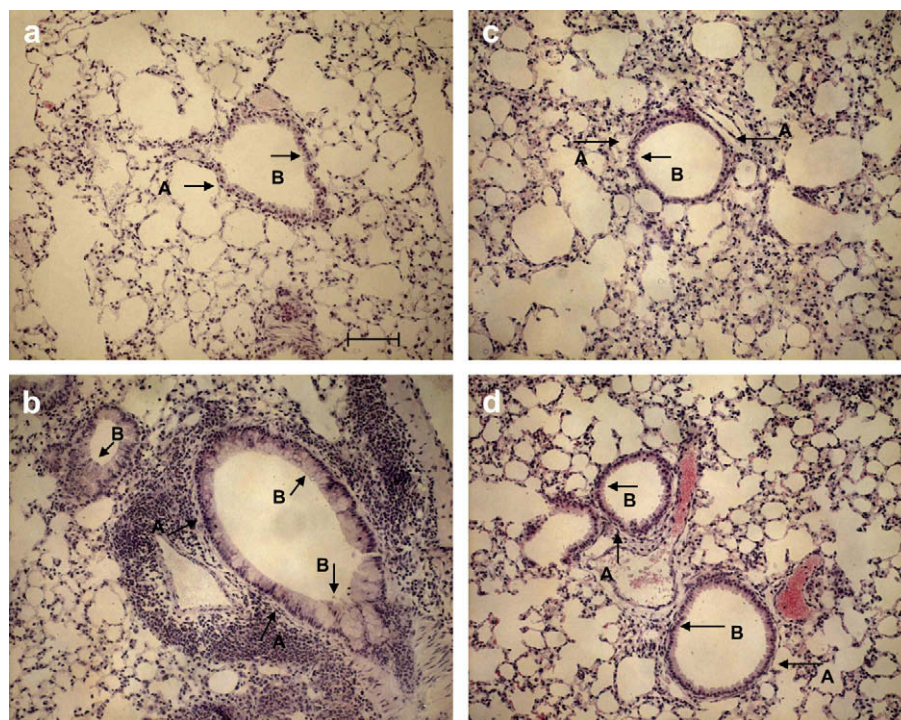


**Fig. 5.** (a–d) Lung histology sections ( $\times 20$  magnification) from the 4 treatment groups: (a) OVA-immunized and PBS-challenged: normal lung histology with no apparent peribronchiolar (A) or epithelial (B) inflammation, (b) OVA-immunized and challenged mice: the peribronchiolar area (A) shows intense inflammatory cell infiltration and the epithelium (B) appears enlarged, (c) OVA-immunized, OVA-challenged and NECA (0.3 mM) treated: the peribronchiolar area (A) shows a much reduced cellular infiltration and the airway epithelium (B) appears normal compared with OVA-challenged mice, (d) OVA-immunized, OVA-challenged and dexamethasone (1.3 mM) treated mice: the peribronchiolar area (A) shows a much reduced cellular infiltration and the epithelium (B) appears normal compared with OVA-challenged mice and their appearance is similar to the NECA treated mice. Bar represents 50  $\mu\text{m}$ .

and 1 mM, and 1.3 mM dexamethasone. Additionally, both NECA (0.3 mM) and dexamethasone (1.3 mM) significantly inhibited eosinophil influx by 79.9% and 87.9%, respectively. Although both lymphocyte and neutrophil numbers were reduced in the dexamethasone treated group, this did not reach statistical significance ( $P > 0.05$ ; Fig. 7b).

### 3.7. Adenosine $2A$ receptor mRNA levels in lungs of immunized mice challenged with OVA with or without NECA treatment

Adenosine receptor mRNA levels were quantified in total RNA preparations isolated from whole lungs. The 2 days' OVA challenge induced a significant increase in the  $A_{2A}$  mRNA ( $>2.3$ -fold increase over the PBS-challenged mice). NECA (0.3 mM) treatment resulted in a significant (43.2%) inhibition of OVA enhanced expression ( $P < 0.05$ ; Fig. 8a). The 4 days' OVA challenge also significantly increased ( $>1.6$ -fold) the  $A_{2A}$  receptor mRNA ( $P < 0.05$ ; Fig. 8b). However, mRNA levels were not significantly higher following 4 challenges compared to the 2 challenges. Again treatment with NECA resulted in a significant (41.0%) inhibition of OVA enhanced  $A_{2A}$  transcript expression ( $P < 0.05$ ; Fig. 8b).

## 4. Discussion

Our data show that in immunized, but not OVA-challenged mice, high dose of NECA (1 mM) induces an inflammatory airway response. In contrast, in models of inflammation, NECA, at mainly 0.3 mM, induces a significant anti-inflammatory effect when administered either prior to the induction of airway inflammation or therapeutically following the establishment of airway inflammation. The data also show that the  $A_{2A}$  receptor antagonist, SCH-

58261, completely reversed NECA-mediated anti-inflammatory effects, suggesting that adenosine receptor activation can induce both pro- and anti-inflammatory effects and that the anti-inflammatory action is mediated mainly via the  $A_{2A}$  receptor sub-type.

Administration of NECA to immunized, but not challenged, mice induces an inflammatory response that is characterized by neutrophilia and eosinophilia and was seen only at the highest dose of NECA. This effect may well be mediated via the  $A_{2B}$  receptor sub-type, activation of which was reported to result in pro-inflammatory effects [22,23]. The  $A_{2B}$  is a low affinity receptor activated only at high levels of adenosine and hence 1 mM may be the threshold level for activation. Pro-inflammatory actions of adenosine, in immunized but not allergen challenged animals, have also been previously noted in guinea pigs [24] where eosinophils were significantly increased following AMP provocation, albeit after a shorter period of time. The mechanism by which NECA induces this pro-inflammatory response remains to be elucidated, but strong evidence suggests involvement of mast cell derived mediators. Mast cell tryptase and chymase are potent stimuli of eosinophil accumulation following their injection into the skin of guinea pigs [25]. Also, adenosine has been shown to augment allergen-induced histamine release [26].

The experiments conducted to establish the optimal time point to study the effects of NECA on the OVA-induced inflammation showed that TCC was significantly increased in all OVA-challenged groups except in animals where OVA was given only once and eosinophils were increased even following a single challenge. On the other hand, lymphocytes and neutrophils were only significantly increased after the third OVA challenge. On the basis of these findings, the two OVA challenge model was selected to assess the effects of NECA as it was important to use a response model where

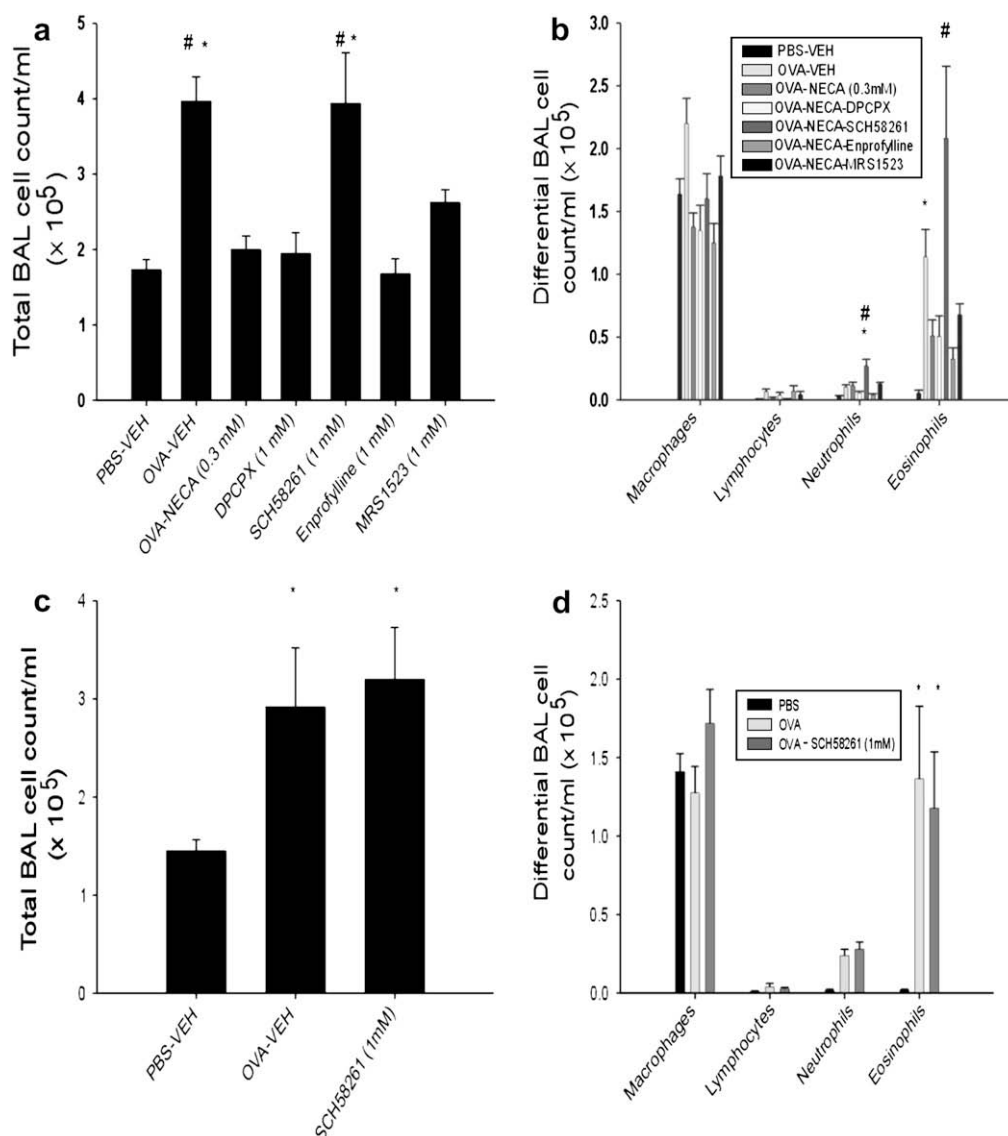


**Fig. 6.** (a and b) Effect of 1 mM of DPCPX, SCH-58261, enprofylline and MRS 1523 on NECA (0.3 mM)-mediated reduction of OVA-induced increase in total (a) and differential (b) BAL cell count ( $n = 9-11$ ). Data are presented as means  $\pm$  SEM. <sup>\*</sup> $P < 0.05$  vs PBS-VEH challenged mice. <sup>#</sup> $P < 0.05$  vs OVA-NECA challenged mice. (c and d) Effect of SCH-58261 (1 mM) on OVA-induced increase in total (a) and differential (b) BAL cell count ( $n = 5$ ). Data are presented as means  $\pm$  SEM. <sup>\*</sup> $P < 0.05$  vs PBS-VEH challenged mice.

both potential inhibitory and enhancement effects of NECA on OVA-induced inflammation could be assessed.

Our data showed that NECA administration, in the 2 days' OVA challenge model, clearly had an inhibitory effect on the OVA-induced increase in both the total and the eosinophil cell count. Although all the doses of NECA reduced the OVA-induced increase in TCC, this was only significant with the 0.3 mM dose, but not at 0.1 or 1 mM dose. The lack of dose dependency, on TCC, may be due to the fact that both macrophages and neutrophils were higher in the 1 mM dose group possibly due to activation of pro-inflammatory adenosine receptors on these cells.

Due to the opposing findings in the effects of NECA in the immunized, but not challenged mice, where administration of NECA (1 mM) was found to have pro-inflammatory effects and the anti-inflammatory effects in the 2 days' OVA challenge model, NECA's anti-inflammatory effect was re-investigated using the 0.3 mM dose, on the 4 days' OVA challenge model, a time point at which the highest inflammatory response had previously been noted. Our findings with the 4 days' OVA challenge model clearly

confirmed the results of the 2 days' OVA challenge model. Indeed, NECA (0.3 mM) significantly reduced the OVA-induced increase in total, lymphocyte, neutrophil and eosinophil cell infiltration in the 4 days' OVA challenge model and was comparable to the effects of dexamethasone.

Although our data clearly showed that administration of the non-selective adenosine receptor agonist NECA, at 0.3 mM, concurrently with an inflammatory stimulus, induces an anti-inflammatory response, several other studies have suggested a pro-inflammatory role for adenosine. A recent report [13] has shown that mice deficient in adenosine deaminase exhibit a significant increase in numbers of eosinophils and activated macrophages in both the lungs as well as BAL. Van den Berge et al. [12] have also shown that challenge with AMP induces a significant increase in sputum eosinophils in asthmatic subjects. This discrepancy between our findings and the studies showing that adenosine may be pro-inflammatory could be explained by differences in the concentration of adenosine or adenosine analogues used. In the studies demonstrating a pro-inflammatory effect of adenosine, the

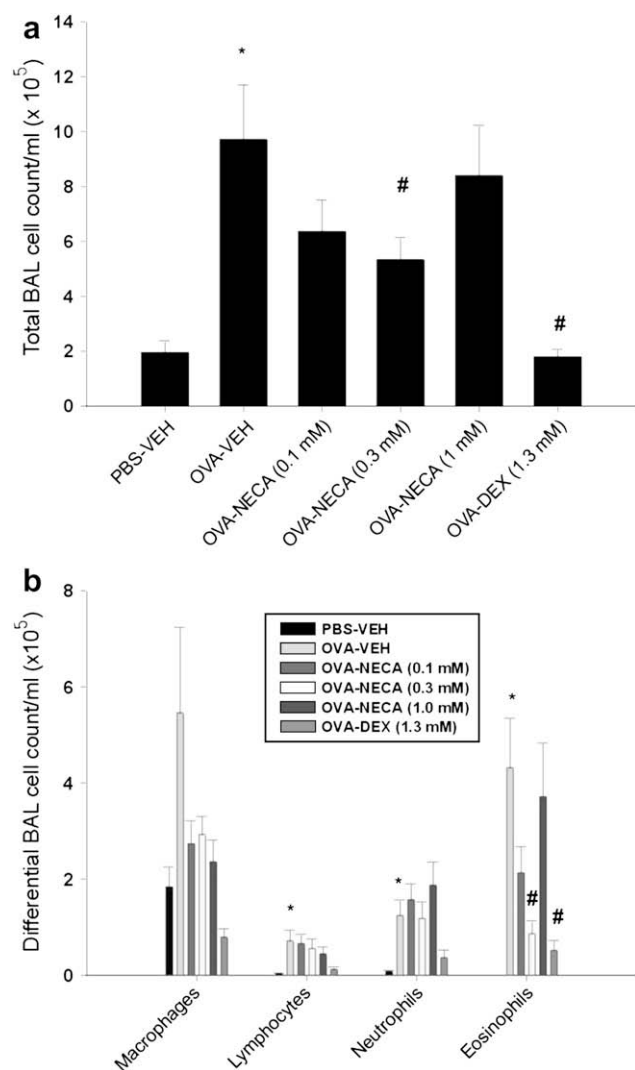


**Fig. 7.** (a and b) Effect of NECA (0.1, 0.3 and 1.0 mM) and dexamethasone (1.3 mM) on established increase in total (a) and differential (b) BAL cell count ( $n = 7-12$ ). Data are presented as means  $\pm$  SEM. \* $P < 0.05$  vs PBS-VEH challenged mice. # $P < 0.05$  vs OVA-VEH challenged mice.

levels of adenosine achieved in the lungs are significantly high and although all adenosine receptors are activated, the predominant effect is a pro-inflammatory response.

NECA is a relatively non-selective agonist ( $pK_A A_1 -7.8$ ;  $pK_A A_{2A} -7.7$ ;  $pK_A A_{2B} -6.5$  and  $pK_A A_3 -8.2$ ) [1] and its anti-inflammatory effects could potentially be mediated via any of the receptor sub-types. Our results with the adenosine receptor blockers would appear to favour the  $A_{2A}$  receptor as antagonizing this receptor with the SCH-58261 compound completely reversed the inhibitory effects of NECA. Additionally, both the eosinophil and the neutrophil influx were significantly increased in the SCH-58261 pretreated group compared with the OVA group. The lack of effect of SCH-58261 on baseline OVA-induced increase in TCC and eosinophils suggests these effects are not due to non-specific effects of SCH-58261 or to the masking of underlying anti-inflammatory effect of  $A_{2A}$  to dampen down the OVA-induced inflammation such as eosinophil influx. The anti-inflammatory effect of the  $A_{2A}$  receptor sub-type is consistent with the literature. A recent study showed that mice deficient in  $A_{2A}$  receptors had increased I $\kappa$ B- $\alpha$  expression and nitrooxidative stress in the lung suggesting a possible involvement of  $A_{2A}$  receptor sub-type in down-

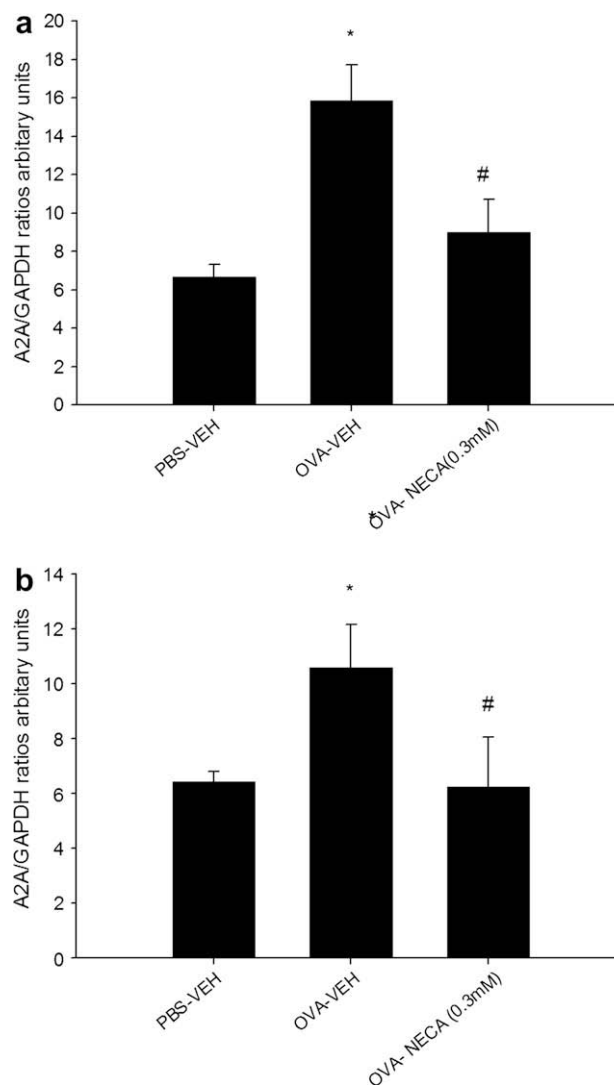


**Fig. 8.** (a and b) Effect of 2 days' OVA (a) and 4 days' OVA (b) challenges and treatment with NECA (0.3 mM) on the transcription level of the  $A_{2A}$  receptors. Histograms represent the mean ratio ( $\pm$ SEM) of  $A_{2A}$  to GAPDH ( $n = 5-7$ ) following the treatments indicated. \* $P < 0.05$  vs PBS-VEH challenged mice. # $P < 0.05$  vs OVA-VEH challenged mice.

regulating allergic inflammation in this model [27]. In addition, others have shown that the  $A_{2A}$  agonist, CGS 21680, has potent anti-inflammatory effects in an allergic rat model of inflammation [15]. The anti-inflammatory effects of the  $A_{2A}$  receptor have been reported in several other types of inflammation such as colitis and peritonitis [28,29]. It is interesting that in a recent clinical trial the  $A_{2A}$  agonist, GW328267x, did not improve therapeutic outcomes for asthmatic patients [30], although it should be noted that a low dose of the drug was used. Moreover, GW328267x may also antagonize the  $A_3$  receptor [30] which others have reported to mediate the anti-inflammatory effects of adenosine [31,32]. Blockade of the  $A_1$ ,  $A_{2B}$  or the  $A_3$  receptors had no significant effect on the NECA-mediated reduction of OVA-induced airway inflammation. The lack of involvement of both the  $A_1$  and the  $A_{2B}$  in the NECA-mediated anti-inflammatory action was not surprising as several studies have shown that both these receptors have a pro-inflammatory role [33–37]. Equally, these receptors may also mediate anti-inflammatory effects [17,38]. We found that although treatment with the  $A_3$  receptor blocker increased both the TCC and

the eosinophils numbers, compared with the OVA-NECA group, this was not statistically significant. This finding was somewhat surprising since it has been previously reported that activation of the A<sub>3</sub> receptor sub-type, in both eosinophils and neutrophils, inhibits their degranulation and superoxide anion release [31,32].

In view of the previous experiment highlighting the important role for the A<sub>2A</sub> receptor, we examined the expression of this receptor sub-type in our model. We found that both the two and four OVA challenges induced a significant increase in the A<sub>2A</sub> receptor mRNA level compared to the PBS-challenged mice. Lack of complete association between both challenges and numbers of inflammatory cells would suggest that this higher A<sub>2A</sub> mRNA probably reflects a real increase in A<sub>2A</sub> gene expression in the lung tissue, with the caveat being that without more precise information on the location and relative density of the receptors the confounding effect of inflammatory cell influx cannot be definitively ruled out. More recently however, several studies have shown that A<sub>2A</sub> receptor gene expression can be potently up-regulated by inflammatory mediators such as TNF $\alpha$  and IL-1 $\alpha$  [39]. Furthermore, this up-regulation seems to be dependent upon NF- $\kappa$ B activation [40]. In our study, the fact that NECA treatment, in both the two and the four OVA challenges, decreased the A<sub>2A</sub> receptor mRNA over-expression was not totally surprising as the increased expression of this receptor mRNA is secondary to inflammation. Therefore the down-regulation of this mRNA is more than likely due to the general anti-inflammatory effects of NECA (0.3 mM). Of interest is the finding of a recent study showing that inflammatory mediators like TNF $\alpha$  can increase the receptor functionality via reversal of A<sub>2A</sub> desensitization [41].

The recognition that asthma is a chronic disease makes it incumbent for any novel therapy to demonstrate effectiveness when given therapeutically in an ongoing disease. With this in mind, we investigated whether NECA treatment following the establishment of airway inflammation would also reduce this inflammatory response. Our data clearly show that when NECA is given therapeutically it decreases both the TCC and the eosinophil count at 0.3 mM. It is of interest to note that in this therapeutic approach, unlike the prophylactic approach, the 1 mM NECA dose had no effect on eosinophil recruitment. This suggests that, within the context of established inflammation, the adenosine receptor(s) mediating the pro-inflammatory effects may be primed and their effects, at high doses of NECA, are more prominent than when NECA is given prior to the induction of inflammation. We believe that this is the first demonstration of anti-inflammatory effects of NECA when given therapeutically in an animal model. Interestingly, the studies by Fan and Mustafa [14] where adenosine was given to mice 24 h following ragweed allergen challenge showed significant enhancement of the ragweed-induced inflammatory cell influx. Again, the explanation for this could be in the dose. In these studies, a higher dose of adenosine (0.02 M) was used compared with our study and therefore it is very likely that this high dose of adenosine enhanced the ragweed-induced inflammation as we noted with the 1 mM concentration, in the immunized only mice.

## 5. Conclusion

Our data show that in immunized, but not OVA-challenged mice, a high dose of NECA (1.0 mM) induces an inflammatory airway response. In contrast, in models of inflammation, NECA, at mainly 0.3 mM, induced significant anti-inflammatory effects when given prior to the induction of inflammation or following the establishment of overt airway inflammation. The anti-inflammatory effects of NECA seem to be mediated predominantly via the A<sub>2A</sub> receptor sub-type and therefore the potential use of A<sub>2A</sub>

receptor agonists as therapeutic agents in inflammatory diseases such as asthma merits further investigation.

## 6. Contribution of the work

This study shows that activation of adenosine receptors can result in both anti-inflammatory and pro-inflammatory actions. The anti-inflammatory action can be seen when a non-selective adenosine agonist, NECA, is administered prior to the induction of airway inflammation or more importantly when it is administered therapeutically, following the induction of overt inflammation. The study also shows that the A<sub>2A</sub> is the main receptor sub-type that mediates the anti-inflammatory action of NECA. Additionally, the study shows that the mRNA for the A<sub>2A</sub> receptor is increased following allergen challenge and is down-regulated with NECA treatment.

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