

Adenosine A₁ and A₃ Receptor Agonists Inhibit Nonadrenergic, Noncholinergic Relaxations in the Guinea Pig Isolated Trachea

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Key Words

Adenosine • Inhibitory nonadrenergic, noncholinergic innervation • Trachea • Guinea pig • Airways

Abstract

Background: Adenosine affects the tone and reactivity of airways by activating specific membrane receptors, named A₁, A_{2a}, A_{2b} and A₃. It affects cellular activities either directly by regulating membrane ion exchanges and polarization, or indirectly by modifying neurotransmitter release. **Objectives:** We assessed the effect of A₁ and A₃ receptor activation on electrically induced nonadrenergic, noncholinergic (NANC) relaxations in the guinea pig isolated trachea and the localization of A₁ and A₃ receptors in tracheal inhibitory neurons. **Methods:** NANC responses at 3 Hz were evaluated in the presence of 2-chloro-N⁶-cyclopentyladenosine (CCPA), a selective A₁ agonist, and 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (CI-IB-MECA), a selective A₃ agonist, before and after the administration of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A₁

antagonist, or 9-chloro-2-(2-furanyl)-5-((phenylacetyl)amino[1,2,4]triazolo[1,5-c]quinazoline (MRS 1220), a selective A₃ antagonist, respectively. For immunohistochemistry, tissues were exposed to antibodies to HuC/D, a general neuronal marker, neuronal nitric oxide synthase (nNOS), and A₁ or A₃ adenosine receptors and processed by indirect immunofluorescence. **Results:** CCPA (10 nM–3 μM) inhibited NANC relaxations. DPCPX (10 nM) failed to antagonize the effect of CCPA, but inhibited per se NANC relaxations (range 0.1–100 nM). CCPA (10 nM–10 μM) contracted unstimulated tracheal preparations, an effect antagonized by 10 nM DPCPX, with a pK_B value of 8.43. CI-IB-MECA (10 nM–3 μM) inhibited NANC relaxations through a mechanism antagonized by MRS 1220 (100 nM). A₁- and A₃-positive neurons containing nNOS were detected in tracheal sections. **Conclusions:** Endogenous adenosine may induce airway hyperresponsiveness by inhibiting NANC relaxations via A₁ and A₃ receptors.

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Introduction

Adenosine is an autacoid that is able to modulate several cellular functions and modify the tone and reactivity of airways. The effects of adenosine are mediated by the activation of specific membrane receptors belonging to the G-protein-coupled receptor family, classified as A₁, A_{2a}, A_{2b} and A₃. Extracellular adenosine affects cellular activities either directly by regulating membrane ion exchanges and membrane polarization, or indirectly by modifying neurotransmitter release [1].

Adenosine, a mediator of several inflammatory processes, is found in bronchoalveolar lavage fluid in response to antigen exposure [2] and in exhaled breath condensate during inflammatory processes [3, 4]; it can affect airway responsiveness by targeting different cell types, in particular mast cells and smooth muscle cells. Indeed, adenosine-induced bronchoconstriction observed in allergic animal models and in asthmatics is thought to be largely due to the release of histamine and leukotrienes from mast cells [5–7]. These cells are both a source and a target of adenosine, which provides a feedback mechanism affecting the release of mast cell mediators [8, 9].

Other potential sources are platelets and neutrophils. Indeed, adenosine has been found to facilitate the expression of cyclooxygenase-2 in human neutrophils, with a consequent increase in PGE₂ production and a reduction in leukotriene B formation [10]. This anti-inflammatory effect of adenosine contrasts with its ability to induce severe asthmatic attacks, an effect which is considered as a specific test to reveal allergic airway inflammation [11]. The bronchoconstrictor action of adenosine in animal models and allergic subjects, together with the absence of bronchoconstriction under physiological conditions, suggests that the type of adenosine response is influenced by the presence of inflammatory processes. On the other hand, inflammatory mechanisms can potentiate the effects of adenosine.

The distribution of adenosine receptors in cells and tissues markedly varies among species [12]. With regard to airway smooth muscle [13], the bronchoconstrictor action of adenosine in the sensitized rabbit is mostly mediated by A₁ receptors [14], which are coupled to G_{i/o} proteins, leading to adenylyl cyclase inhibition [12]. In the same model of allergic asthma [15, 16], A₁ receptor induction has also been shown. A₁-receptor-mediated contraction has also been demonstrated in vascular smooth muscle [17]. The other types of adenosine receptors present in the airways, i.e. A_{2a}, A_{2b} and A₃ receptors, are mostly involved in bronchoconstriction in allergic mice [18]. A_{2b}

receptors are coupled to G_s or G_q proteins whereas A₃ receptors are coupled to G_{i/o} proteins [12].

Besides mast cells and smooth muscle cells, other cell types are involved in the ability of adenosine to modify airway responsiveness. In particular, a role for neuronal pathways has been hypothesized [7]. Regarding the neuronal effects of adenosine, it has been shown that the stimulation of A₁ receptors can inhibit the release of acetylcholine from autonomic neuroeffector junctions in the airways, intestine and motor endplates [19–23] whereas a facilitatory effect, probably mediated by A₂ receptors, has been demonstrated in rabbit and rat airways and other organs [24–26]. Adenosine also increases the activity of pulmonary sensory nerves via A₁ receptors in rats and humans [27].

The neurogenic control of airway tone depends on the balance of bronchoconstrictor (mainly dependent on excitatory pathways) and bronchodilator output, essentially provided by the nonadrenergic, noncholinergic (NANC) inhibitory system, which has nitric oxide (NO) and carbon monoxide (CO) as prominent neurotransmitters [28]. As a consequence, the effect of adenosine in the airways may also depend on its influence on the NANC inhibitory innervation. This study was aimed at evaluating the modulation of the NANC inhibitory responses by the adenosine system in the guinea pig trachea, in particular by means of A₁ and A₃ receptors. In addition, the expression of A₁ and A₃ receptors was evaluated in neuronal NO synthase (nNOS)-containing tracheal neurons.

Materials and Methods

Animals

Male albino guinea pigs weighing 500–600 g (Bettinardi, Momo, Italy) were used. Guinea pigs were housed in standard animal facilities, providing constant temperature (21 ± 1°C), relative humidity (50–55%), and alternating 12-hour light and 12-hour dark cycles. The animals were provided with food and water *ad libitum*. Care and handling of the animals were in accordance with the European Union Directive 86/609 for the human use of animals and with the *NIH Guide for the Care and Use of Laboratory Animals*. All experimental procedures were reviewed and approved by the Animal Use Committee of the University of Pavia, Italy, where the experiments were performed. The animals were killed by cervical dislocation and rapid exsanguination. The number of animals sacrificed was kept to the minimum necessary for a meaningful interpretation of the data.

Functional Studies

The trachea was excised and transferred to a Petri dish containing oxygenated (95% O₂ and 5% CO₂) standard Tyrode's solution. A 3-cm-long whole tracheal segment was prepared. Then, mucosa was removed gently [29] to avoid the electrical stimulation of epi-

thelial cells, which may release substantial amounts of prostanoids, adenosine triphosphate and NO, which may distort the nerve-mediated response to electrical-field stimulation (EFS) [30, 31]. Since this technique enables a histologically proved total mucosa ablation, the use of prostanoid synthesis inhibitors (e.g. indomethacin) was no longer needed, as can also be deduced from several studies on the regulation of airway tone [32–34]. This was confirmed in a series of pilot experiments in which indomethacin (1–10 μM) neither affected the basal tone of the tracheal tube nor the amplitude of EFS-induced NANC relaxations. Then, the tracheal tube was cannulated at each extremity using two polyvinyl chloride tubes (outer diameter, 2.0 mm; inner diameter, 1.35 mm), and was set up horizontally in a 10-ml organ bath containing standard Tyrode's solution, maintained at 37°C and bubbled with a mixture of 95% O₂ and 5% CO₂ according to the method described by Tanihata and Uchiyama [35] and validated by other groups [36–38], including ours [28]. The preparation was flushed intraluminally with a peristaltic pump delivering Tyrode's solution at 0.4 ml · min⁻¹ for 30 min. Then one end of the preparation was occluded and the other was connected to a pressure transducer for intraluminal pressure recording. Signals were recorded using a PowerLab data acquisition system (ADInstruments Ltd., Crowborough, UK), and analyzed using PowerLab Chart v4.1.1 software.

After 1 h of equilibration, the tracheal tube was stimulated via two platinum electrodes placed in parallel, 1 cm apart, and connected to an electrical stimulator (MARB ST 87). Trains of rectangular pulses (0.5 ms duration, 1-, 3- and 10-Hz frequency at 60 V) were delivered for 5 s at 10-min intervals. EFS-induced NANC relaxations were measured as reduction in the intratracheal pressure. They consisted of an initial fast response (peak response) followed by a late, slow recovery of the tone to the basal value. To evaluate the overall inhibitory response (i.e. both the peak and duration of the relaxant response), the area under the curve (AUC) was calculated as the integral from baseline for each response (Pa · s). All the experiments were carried out in the presence of hyoscine (1 μM) to block muscarinic acetylcholine receptors, and piperoxan (1 μM) and propranolol (1 μM) to block α - and β -adrenoceptors. Since EFS at the frequencies employed did not elicit a tachykinergic component of response (as shown by the abolition of any contraction by hyoscine alone), we did not need to use tachykinin antagonists. NANC relaxations to EFS were elicited before (control) and after the administration of the following drugs: 2-chloro-N⁶-cyclopentyladenosine (CCPA), a selective A₁ agonist at concentrations ranging from 10 nM to 3 μM , 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A₁ antagonist, 10 nM, 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (Cl-IB-MECA), a selective A₃ agonist, 10 nM–3 μM , 9-chloro-2-(2-furanyl)-5-((phenylacetyl)amino-[1,2,4]triazolo[1,5-c]quinazoline (MRS 1220), a selective A₃ antagonist, 0.1 μM . Noncumulative concentration-response curves to CCPA and Cl-IB-MECA were constructed in separate tissues in the absence or in the presence of the antagonists at A₁ or A₃ receptors. DPCPX and MRS 1220 were left in contact for 45 min before agonist administration, whereas agonists were left in contact for 3 min before EFS.

CCPA-induced contractions were measured as peak pressure minus baseline. In a set of experiments, CCPA and Cl-IB-MECA were given together, each at 100 nM, to assess their combined effect. Tetrodotoxin (1 μM) was given in a number of experiments, to ensure that EFS-evoked inhibitory responses were neurogenic and CCPA-induced contractions were not.

Data and Statistical Analysis

Data are given as raw data or as the mean $\pm$ SEM of the percent residual response after pharmacological treatment compared to control response (100%). Apparent affinity estimates (pK_B) from single antagonist concentrations were calculated by the Gaddum (1957) equation using agonist concentration ratios determined at the EC₅₀ levels (which were evaluated by nonlinear curve fitting) in control and test curves. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by means of analysis of variance followed by Bonferroni's test for multiple comparisons. A *p* value <0.05 was considered statistically significant.

Drugs

Hyoscine, isoprenaline, MRS 1220, propranolol, tetrodotoxin (all purchased from Sigma-Aldrich, St. Louis, Mo., USA) and piperoxan (Rhône-Poulenc, Courbevoie, France) were dissolved in distilled water. CCPA, Cl-IB-MECA and DPCPX (Sigma-Aldrich) were prepared by first dissolving them in dimethylsulfoxide and then diluted in distilled water. Indomethacin (Sigma-Aldrich) was dissolved in ethanol and then diluted in distilled water.

Immunohistochemistry

The trachea was quickly dissected and fixed in 4% paraformaldehyde in 0.1 M, phosphate buffer (PB; Fisher Scientific, Fair Lawn, N.J., USA), pH 7.4 for 12 h at 4°C and then stored in 30% sucrose/PB for cryoprotection at 4°C overnight. Specimens were embedded in OCT compound (Tissue-Tek Sakura, Torrance, Calif., USA), frozen in liquid nitrogen and cut into 12- to 14- μm -thick sections using a cryostat. Sections were then processed for double-labeling indirect immunofluorescence as described previously [39, 40]. Briefly, sections were washed in PB, incubated in 10% normal goat serum for 1 h at room temperature and then incubated overnight at 4°C with the following mixtures containing: rabbit polyclonal anti-A₁ adenosine receptor (1:100; Sigma-Aldrich Chemie, Steinheim, Germany) and either mouse monoclonal anti-HuCD (1:200; Molecular Probes Inc., Eugene, Oreg., USA) or anti-nNOS (1:200, Santa Cruz Biotechnology, Santa Cruz, Calif., USA), a general neuronal marker and a major inhibitory neurotransmitter, respectively. Other double-labeling experiments included sections incubated with the rabbit polyclonal anti-A₃ adenosine receptor antiserum (1:100; Sigma-Aldrich Chemie) and either anti-HuCD or anti-nNOS used at the same dilutions as indicated above. Following washing, sections were incubated for 1 h at room temperature with a mixture of affinity-purified goat anti-rabbit IgG coupled with fluorescein isothiocyanate (FITC; 1:400; Calbiochem, La Jolla, Calif., USA) and goat anti-mouse IgG conjugated with Alexa Fluor 495 (1:500; Molecular Probes Inc., Eugene, Oreg., USA), washed again and attached to coverslips with glycerol in PB (9:1). Fluorescent images were acquired with a Zeiss Axioplan (Zeiss, Thornwood, N.Y., USA) microscope equipped with the appropriate filter cubes to discriminate between FITC and Alexa 495. Images were recorded with a Polaroid DMC digital photocopier (Polaroid, Cambridge, Mass., USA) and DMCV 2 software. Images were further processed using Corel Photo Paint and Corel Draw software programs (Corel, Milan, Italy). Control experiments to assess the specificity of A₁ and A₃ receptor antibodies were performed by omitting the primary antibodies or replacing them with a nonimmune rabbit serum.

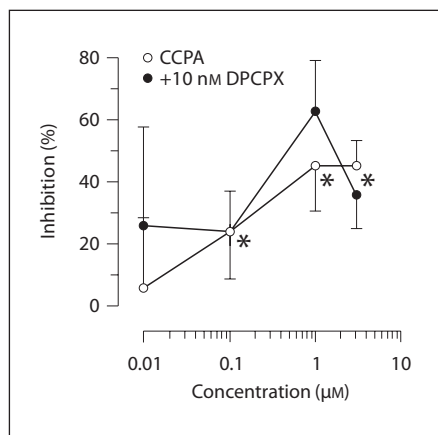


Fig. 1. The A_1 agonist CCPA (0.01–3 μ M) concentration-dependently reduces NANC relaxations induced by EFS at 3 Hz in the guinea pig trachea. The A_1 antagonist DPCPX (10 nM) fails to affect the response to CCPA. $n = 5$. * $p < 0.05$ as compared to control relaxations.

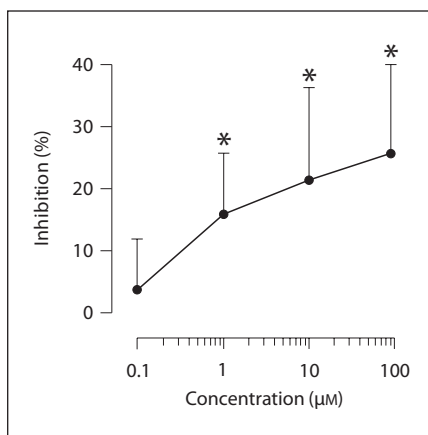


Fig. 2. The A_1 antagonist DPCPX (0.1–100 nM) reduces tracheal NANC relaxations in a concentration-dependent fashion. $n = 9$ –27. * $p < 0.05$ as compared to control relaxations. This suggests that DPCPX behaves as a (partial) agonist in the guinea pig trachea.

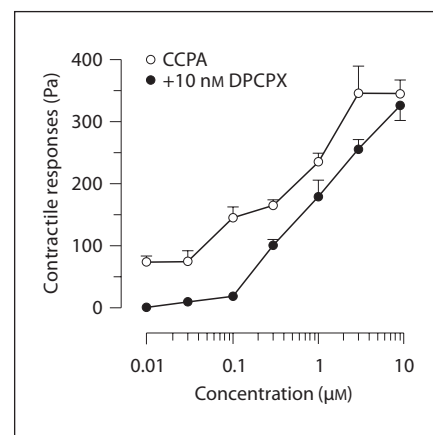


Fig. 3. The A_1 agonist CCPA induces concentration-dependent tracheal contractions ($n = 9$ –20) evaluated as intratracheal pressure increases. The A_1 antagonist DPCPX (10 nM) causes a parallel shift to the right of the concentration-response curve to CCPA ($n = 4$, $p < 0.05$).

Results

Under NANC conditions, EFS at 1, 3 and 10 Hz induced biphasic relaxant responses consisting of an initial fast response followed by a late slow response up to recovery of the basal tone; the responses were reproducible over a 7-hour time interval and were abolished by 1 μ M tetrodotoxin ($n = 4$, data not shown). EFS-induced relaxations were frequency dependent and submaximal compared with the response evoked by 10 μ M isoprenaline (19, 45 and 67%, respectively, $n = 4$). We chose to evaluate the effect of adenosine receptor agonists and antagonists on the relaxations induced at a 3-Hz frequency stimulation.

EFS-induced relaxations were significantly reduced in a concentration-dependent fashion after treatment with the A_1 agonist CCPA (0.01–3 μ M) (fig. 1). In particular, NANC relaxations were maximally reduced by 45% at 3 μ M, with an EC_{50} of 76 nM. The effect of CCPA (1 and 3 μ M) was totally reversible by prolonged (30 min) washout. However, the A_1 antagonist DPCPX alone (0.1–100 nM) reduced EFS-induced relaxations in a concentration-dependent fashion up to 25% (fig. 2) and did not antagonize the effect of CCPA at 10 nM, the standard dose recommended in the literature [41–43] (fig. 1).

In unstimulated preparations, CCPA (0.01–10 μ M) induced tracheal contractions in a concentration-dependent fashion (fig. 3), an effect that was not modified by

tetrodotoxin, indicating a direct action on smooth muscle. Contractions were maximal at 3 μ M (342 Pa pressure increase), and the EC_{50} was 221 nM. Conversely, this effect was antagonized by DPCPX 10 nM, which raised the EC_{50} of CCPA to 811 nM, with a pK_B value of 8.43.

To ensure that the reduction in NANC relaxations caused by CCPA was a specific effect via activation of postjunctional A_1 receptors, and not related to the CCPA-induced enhancement of muscular tone, we contracted tracheal preparations with histamine. The contraction induced by 0.1 μ M histamine was similar to that induced by 0.1 μ M CCPA. In the presence of the contraction caused by 0.1 μ M histamine, the EFS-induced NANC relaxations were not reduced (123.1% as compared to baseline).

EFS-induced relaxations were also reduced, though to a lesser extent (maximal inhibition 29%), by the A_3 agonist Cl-IB-MECA (0.01–3 μ M; fig. 4) with an EC_{50} of 127 nM. This inhibitory effect was totally reversible by prolonged washout. In contrast with CCPA, Cl-IB-MECA did not directly affect tracheal tone. The A_3 antagonist MRS 1220 (100 nM) caused a shift to the right of the concentration-response curve to Cl-IB-MECA, which raised the EC_{50} of the agonist to 341 nM, with a pK_B of 7.22 (fig. 4). MRS 1220 alone did not affect EFS-induced relaxations.

When CCPA and Cl-IB-MECA were given concomitantly (fig. 5), each at 100 nM, EFS-induced relaxations were reduced by 46.5%, a value that corresponds to the

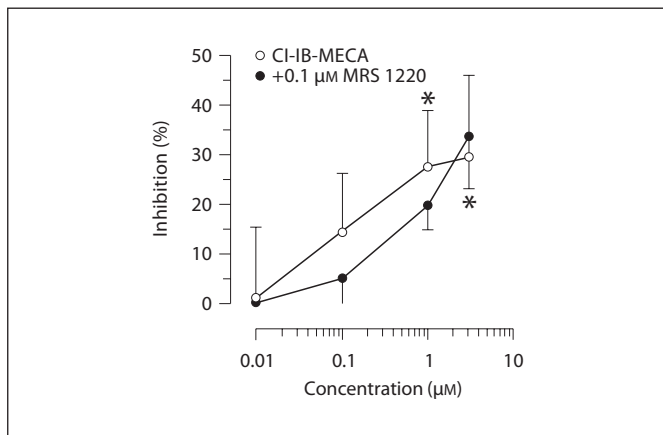


Fig. 4. The A_3 agonist CI-IB-MECA (0.01–3 μM) reduces tracheal NANC relaxations in a concentration-dependent fashion. The A_3 antagonist MRS 1220 (0.1 μM) causes a parallel shift to the right of the concentration-response curve to CI-IB-MECA. $n = 6$. * $p < 0.05$ as compared to control relaxations.

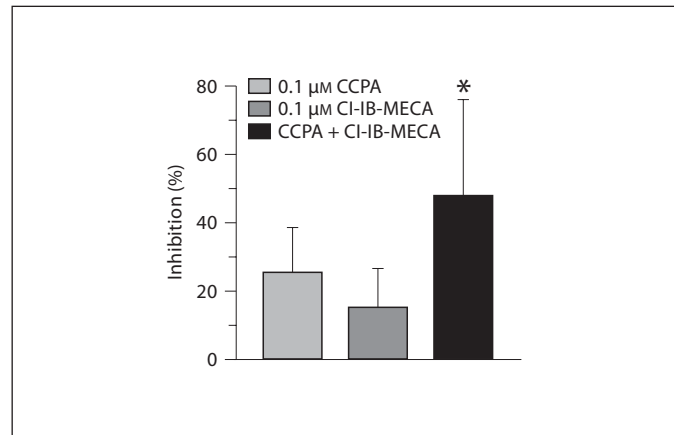


Fig. 5. The figure illustrates the inhibitory effect of CCPA (0.1 μM) and CI-IB-MECA (0.1 μM), administered singularly (values taken from fig. 1 and fig. 4, respectively) or concomitantly, on NANC relaxations. When administered concomitantly, these agonists show an additive effect, suggesting an action on two separate receptors. $n = 8$. * $p < 0.05$ as compared to each individual agonist.

sum of the inhibitory effects produced by each individual compound.

Cells positive for A_1 and A_3 immunoreactivities were identified in the guinea pig trachea either as single elements or in small clusters. Double-labeling immunohistochemistry showed that all A_1 - and A_3 -labeled cells co-expressed HuC/D immunoreactivity, a well-known pan-neuronal marker, indicating that both A_1 and A_3 were expressed by neurons. A subset of A_1 - and A_3 -positive neurons contained nNOS, indicating the presence of these receptors in NO-containing inhibitory neurons (fig. 6, 7). Interestingly, some nNOS-positive neuronal cells did not display A_3 immunoreactivity (fig. 7). Specificity of A_1 and A_3 immunoreactivities was demonstrated by the absence of labeling in sections in which the primary antibodies were omitted or replaced with nonimmune serum.

Discussion

The present study provides the first evidence that adenosine agonists at A_1 and A_3 receptors reduce the NANC inhibitory control in the guinea pig isolated trachea. This evidence leads to the hypothesis that a reduction in NANC inhibitory responses may contribute to airway hyperresponsiveness under conditions of extracellular adenosine accumulation such as during inflammatory processes.

NANC relaxations were reduced by the A_1 agonist CCPA in a concentration-dependent fashion, suggesting a reduction in neurotransmitter release. In our hands, the A_1 antagonist DPCPX, which was found to antagonize A_1 -mediated responses in transfected Chinese hamster ovary cells [42], rat brain [44] and guinea pig myenteric excitatory neuronal cells [43], failed to counteract the effect of CCPA on NANC relaxations. Furthermore, DPCPX inhibited per se NANC relaxations at concentrations of 1–100 nM, indicating that, in the guinea pig trachea, this molecule exerts partial agonist properties. Similarly, DPCPX has been found to behave as a partial agonist in the descending relaxation (a NANC inhibitory response) of rat ileum [45].

As reported in the literature, CCPA caused a direct excitation of smooth muscle cells, which is considered the main effect of A_1 receptor stimulation [13–17], leading to a concentration-dependent increase in tracheal tone. The contractile response induced by CCPA was shifted to the right by DPCPX (10 nM) with a pK_B value of 8.43, which is not far from the pK_i value of 9.5 originally reported in rat brain [12, 44] or from the pK_D values of 8.79–9.07 reported in rat smooth muscle preparations from different organs [46]. These observations suggest that adenosine A_1 receptors located prejunctionally on intrinsic NANC neurons are not ‘identical’ to those located postjunctionally, or they may be coupled to different signal transduction mechanisms. Indeed, the presence of unidentified

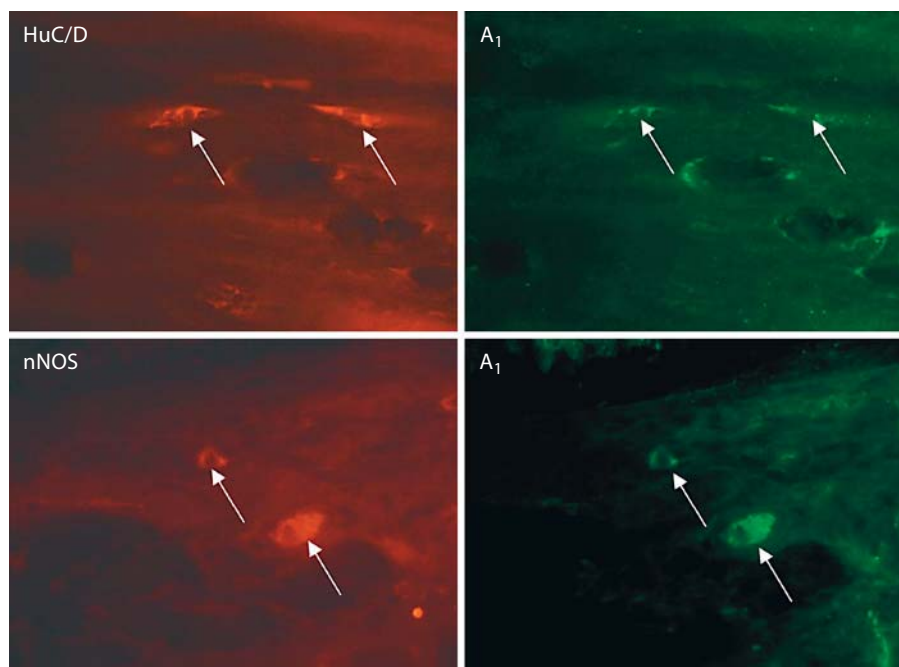


Fig. 6. Representative photomicrographs of neurons of a guinea pig trachea showing A₁ (FITC, green fluorescence), HuC/D and nNOS (Alexa 495, red fluorescence) immunoreactivities in the cytoplasm of neuronal cell bodies in tracheal ganglion cells. A₁/HuC/D and A₁/nNOS show colocalization of A₁ with HuC/D or nNOS immunoreactivity, respectively. Arrows indicate neurons that contain A₁ and HuC/D or nNOS immunoreactivity. Original magnification for each image: $\times 400$.

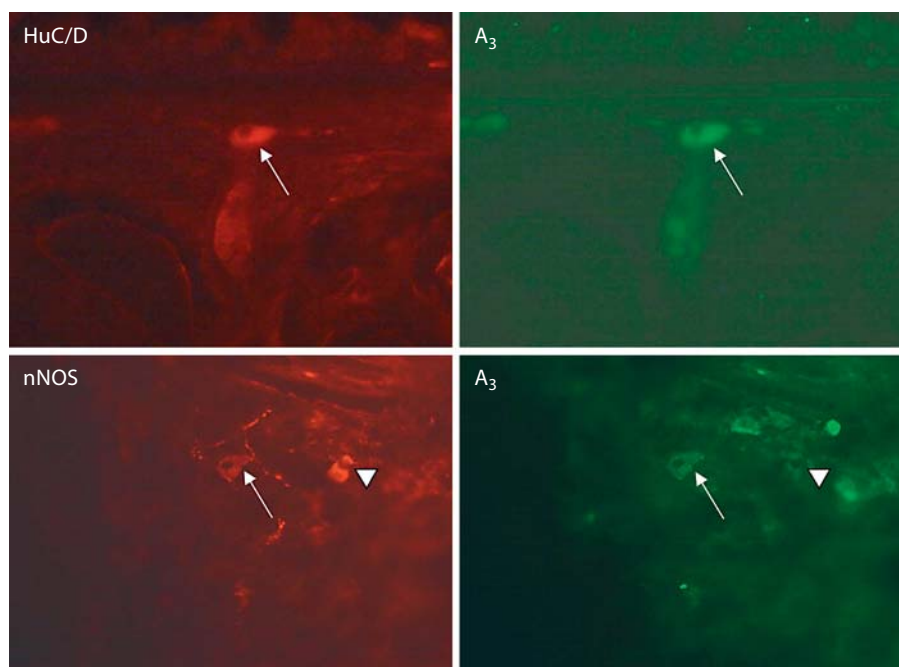


Fig. 7. Representative photomicrographs of neurons of a guinea pig trachea showing A₃ (FITC, green fluorescence), HuC/D and nNOS (Alexa 495, red fluorescence) immunoreactivities in the cytoplasm of neuronal cell bodies in tracheal ganglion cells. A₃/HuC/D and A₃/nNOS show colocalization of A₃ with HuC/D or nNOS immunoreactivity, respectively. Arrows indicate neurons that contain A₃ and HuC/D or nNOS immunoreactivity. Occasionally, some nNOS-positive/A₃-negative cells were observed (arrowheads). Original magnification for each image: $\times 400$.

G_i-coupled adenosine receptor subtypes in intrinsic neurons has been hypothesized to explain the failure of DPCPX to inhibit the adenylyl cyclase activation induced by the A₁ agonist cyclopentyladenosine or 5'-N-ethylcarboxamidoadenosine (a nonspecific adenosine receptor agonist) in human smooth muscle cell cultures [13]. The failure of DPCPX to antagonize the effect of CCPA on

NANC relaxations, in spite of its ability to antagonize the CCPA-induced muscular contraction, rules out the possibility of a merely functional antagonism by CCPA, related to the increase in smooth muscle tone. This is also confirmed by the failure of histamine-induced contraction to inhibit NANC relaxations.

Although the majority of studies have attributed the bronchoconstrictor effect of adenosine to a secondary release of histamine and leukotrienes from inflammatory cells, A₁ receptors have been identified as an additional target of adenosine in rabbit airway smooth muscle [14] and A₁ receptor induction has been shown in the same allergic-rabbit model of asthma [15, 16] and, recently, in human asthmatic subjects [47]. Adenosine has been found to moderately contract healthy guinea pig and human airways through a direct mechanism on smooth muscle cells or indirectly via release of prostaglandins and thromboxanes [48, 49]. In human bronchiolar segments, adenosine contraction was about 20% of the maximum contraction induced by carbachol. A₁-receptor-mediated contraction has also been demonstrated in vascular smooth muscle. This effect is due to the coupling of A₁ receptors to G_i proteins that leads to adenylyl cyclase inhibition [17].

In the central nervous system, A₁-receptor-mediated effects include a reduction in neurotransmitter release via activation of presynaptic receptors located on excitatory, but not inhibitory nerve endings [50]. Our observations suggest that, in the periphery, A₁ receptor activation may reduce not only the cholinergic excitatory neurotransmission [41], but also the release of inhibitory neurotransmitters from intrinsic NANC nerves. Conversely, A₂ receptors have been found to facilitate cholinergic transmission by acting prejunctionally in rat isolated [25] airways.

With regard to its effects on the noncholinergic (i.e. tachykinergic) excitatory system, adenosine has been found to reduce the EFS-induced substance-P-mediated contraction in the guinea pig airways through the activation of prejunctional A₁ and A₂ receptors [41]. By contrast, another study questioned any involvement of adenosine in the modulation of the noncholinergic excitatory system in the airways of the same species [51].

The A₃ agonist Cl-IB-MECA reduced EFS-induced relaxations, although to a lesser extent as compared to equimolar concentrations of CCPA. By contrast, Cl-IB-MECA did not affect smooth muscle tone, indicating the absence of A₃ receptors on effector cells. Therefore, prejunctional A₃ receptors, similarly to A₁ receptors, negatively regulate the NANC inhibitory system in the guinea pig trachea. The A₃ receptor antagonist MRS 1220, employed at 100 nM [13], antagonized the effect of Cl-IB-MECA with an apparent receptor affinity value (pK_B) of 7.22. This value is lower than reported in the literature. Indeed, the pK_B of MRS 1220 ranged from 8.77 to 9.46, depending on the type of assay employed [52–56]. In addition, species differences in the affinity of MRS 1220 at human (where

MRS 1220 shows high affinity) and rodent A₃ receptors [12, 57] could partly explain a lower pK_B value, as observed in our experiments.

CCPA and Cl-IB-MECA, when given concomitantly at a concentration of 100 nM, reduced NANC relaxations by approximately 46%, a value that corresponds to the sum of the inhibitory effects produced by each individual compound, thus suggesting an additive effect due to the participation of two distinct adenosine receptor subtypes.

Our immunohistochemical findings revealed that A₁ and A₃ receptors are colocalized with nNOS in the cytoplasm of neuronal cell bodies in tracheal ganglion cells. This supports the involvement of these adenosine receptors in the modulation of tracheal NANC inhibitory innervation. As observed in functional experiments, in which the A₃ receptor stimulation caused a weaker inhibition of NANC relaxations, compared to the A₁ receptor, the overlapping of A₃ receptors with nNOS-positive neurons seems to be lower than that observed for A₁ receptors.

In conclusion, this study shows that prejunctional adenosine A₁ and A₃ receptors exert an inhibitory control on the NANC inhibitory innervation in the guinea pig trachea. In agreement with the literature data, postjunctional A₁ receptors located on the effector cells enhance airway smooth muscle tone. Therefore, based on our findings, adenosine may enhance smooth muscle tone through several mechanisms. They include (1) the reduction of the NANC inhibitory control via prejunctional A₁ and A₃ receptors, (2) the direct activation of postjunctional excitatory A₁ receptors, and possibly (3) an enhanced acetylcholine release from cholinergic nerve endings due to activation of prejunctional A₂ receptors [24, 25, 52]. On the other hand, adenosine is known to exert a potent bronchoconstrictor action when administered in the presence of inflammatory processes of the airways [6, 11, 15, 18]. This may be due, at least in part, to the suppression of the NANC inhibitory innervation, which exerts the main inhibitory control on airway smooth muscle. This may be regarded as a novel pathophysiological explanation of the bronchoconstrictor effect of adenosine in allergic inflammation.

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