Adenosine A3 receptor-mediated potentiation of mucociliary transport and epithelial ciliary motility

MANAKO Taira, JUN TAMAOKI, KAZUYUKI NISHIMURA, JUNKO NAKATA, MITSUKO KONDO, HISASHI TAKEMURA, and ATSUSHI NAGAI

First Department of Medicine, Tokyo Women’s Medical University
School of Medicine, Tokyo 162-8666, Japan

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To examine the effect of adenosine A3 receptor stimulation on airway mucociliary clearance, we measured transport of Evans blue dye in rabbit trachea in vivo and ciliary motility of epithelium by the photoelectric method in vitro. Mucociliary transport was enhanced dose dependently by the selective A3 agonist N6-(3-iodobenzyl)-5’-N-methylcarbamoyladenosine (IB-MECA) and to a lesser extent by the less-selective N6-2-(4-amino-3-iodophenyl)ethyladenosine, whereas the A1 agonist N6-cyclopentyladenosine (CPA) and the A2 agonist CGS-21680 had no effect. The effect of IB-MECA was abolished by pretreatment with the selective A2 antagonist MRS-1220 but not by the A1 antagonist 1,3-dipropyl-8-cyclopentylxanthine or the A2 antagonist 3,7-dimethyl-L-propargylxanthine. Epithelial ciliary beat frequency was increased by IB-MECA in a concentration-dependent manner, the maximal increase being 33%, and this effect was inhibited by MRS-1220. The IB-MECA-induced ciliary stimulation was not altered by the Rp diastereomer of cAMP but was greatly inhibited by Ca2+-free medium containing BAPTA-AM. Incubation with IB-MECA increased intracellular Ca2+ contents. Therefore, A3 agonist enhances airway mucociliary clearance probably through Ca2+-mediated stimulation of ciliary motility of airway epithelium.

METHODS

Agents. The following drugs were purchased from Research Biochemicals (Natick, MA): N6-2-(4-amino-3-iodophenyl)ethyladenosine (APNEA), N6-cyclopentyladenosine (CPA), 3,7-dimethyl-L-propargylxanthine (DMPX), and MRS-1220. N6-(3-iodobenzyl)-5’-N-methylcarbamoyladenosine (IB-MECA), CGS-21680, and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were purchased from Tocris Cookson (Bristol, UK). The Rp diastereomer of cAMP (Rp-cAMPS) was supplied by BIOLOG.

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Life Science Institute (Bremen, Germany). Evans blue, ionomycin, and EGTA were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM (BAPTA-AM) and fura 2-AM were purchased from Dojin (Kumamoto, Japan).

Stock solutions of all adenosine receptor agonists and antagonists were made in dimethyl sulfoxide (DMSO) to concentrations in the range of 1–4 × 10^{-3} M and then diluted in sterile saline. The final concentration of DMSO present at the highest drug concentrations did not exceed 0.05%, a concentration that had no effect on mucociliary transport rate or ciliary beat frequency in our experimental system.

**Measurement of mucociliary transport.** The experiments were approved by the Committee on Animal Research, Tokyo Women’s Medical University. Specific pathogen-free male Japanese white rabbits (2.0–2.4 kg) were anesthetized with intraperitoneal α-chloralose (50 mg/kg) and urethan (500 mg/kg), and the trachea was explored and covered with a Lucite moist chamber maintained at 37°C. A polyethylene tube was cannulated 2 mm above the carina, and mechanical ventilation (tidal volume 10 ml/kg, respiratory rate 60/min) was performed (model SN-480–7, Shinano, Tokyo, Japan). The cartilage rings of upper trachea were incised transaxially, and the surface of the membranous portion was exposed; 1 μl of 0.5% Evans blue dye in sterile saline warmed at 37°C was gently placed on the membranous portion by a microsyringe 1.5 cm above the carina (Fig. 1). Immediately after this procedure, either the selective A_{1} agonist CPA, the selective A_{2} agonist CGS-21680, or the selective A_{3} agonist IB-MECA (100 μg/kg) was given, and the animals treated with 100 μg/kg IB-MECA. For all sections, Evans blue dye was extracted in 2 ml of formamide, kept in water at 40°C for 24 h, and measured by a spectrophotometer (V-550; Nihon Bunko, Tokyo, Japan) at 620 nm. The Evans blue level in each tracheal section was expressed as a percentage of the total amount of the dye in sections 1–4.

For the assessment of dose-dependent effects of adenosine receptor agonists, various doses (1–100 μg/kg) of IB-MECA, the less-selective A_{3} agonist APNEA (6), CPA, and CGS-21680 were given, and, 10 min later, the contents of Evans blue in section 4 were measured. To confirm whether the stimulation of mucociliary transport by IB-MECA is mediated by A_{3} receptors, the effects of pretreatment with the following drugs were tested: the selective A_{1} antagonist DPCPX (1 mg/kg), the selective A_{2} antagonist DMX (1 mg/kg), and the selective A_{3} antagonist MRS-1220 (100 μg/kg; see Ref. 11). After 15 min of the intravenous injection of an antagonist, IB-MECA (1–100 μg/kg) was given, and the Evans blue contents in section 4 were determined 10 min later. These adenosine receptor antagonists were determined in pilot studies to have no effect per se on the transport of Evans blue dye.

**Measurement of CBF.** The method for the measurement of CBF of rabbit tracheal epithelium has been described in detail previously (15). Briefly, the mucosa of excised rabbit trachea was cut into small pieces (1–2 mm²) and rinsed several times with Hanks’ balanced salt solution (HBSS).

Next, the tissues were placed on a coverglass (18 × 24 mm) coated with human placental collagen (5.8 μg/cm²; Sigma-Aldrich) in a petri dish and incubated in Ham’s nutrient F-12 medium containing 10 μg/ml insulin, 5 μg/ml transferrin, 25 ng/ml epidermal growth factor, 7.5 μg/ml endothelial cell growth supplement, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamicin at 37°C in a CO_{2} incubator (95% air-5% CO_{2}). On the seventh day of incubation, the cover glass on which the cultured explant was adhered was mounted in a Rose chamber (Sasaki Medical, Tokyo, Japan) that was then placed on the temperature-controlled stage (37°C) of the microscope equipped with a phase-contrast condenser and an on-base type of halogen illuminator (Optiphot-XF; Nikon, Tokyo, Japan). The photometer (NFX-II; Hamamatsu Photonics, Hamamatsu, Japan) with a built-in periplanatic eyepiece, a limiting aperture, and a lateral focusing telescope was attached to the head of the microscope. Because of the beating action of cilia, light from the illuminator passed through the preparation in varying intensities. These variations of light intensity were detected by the photometer and transduced to voltage impulses, which were recorded by a pen recorder (VP-6213A; Panasonic, Osaka, Japan). Measurements of CBF were averaged from clumps of two or more cells with free borders devoid of debris. In our separate experiment, variation of CBF among preparations was <0.6 Hz (<7%), and there were no significant differences in the variations between experimental groups. In addition to CBF, we assessed ciliary coordination by the image of the beating pattern recorded on a video camera (VO-5800; Sony, Tokyo, Japan) with a videocassette recorder capable of freeze-frame replay. Ciliary discoordination was
defined as the loss of a metachronal wave on the free border of the cell clump (26, 33).

Before the measurement of CBF, the preparation was allowed to stabilize for 30 min in Krebs-Henseleit (KH) solution of the following composition (in mM): 118 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, 25.5 NaHCO₃, and 5.6 d-glucose, with pH adjusted to 7.4 and warming at 37°C. After determination of the baseline CBF, medium was drained off the chamber and replaced with KH solution containing CPA, CGS-21680, IB-MECA, or APNEA at a concentration of 10⁻⁸ M, and CBF was continuously recorded for 20 min. To examine the effects of adenosine receptor antagonists, the epithelial cells were incubated for 15 min with DPCPX, DMXP, or MRS-1220 at 10⁻⁷ M, and the maximal response of CBF to the subsequent addition of 10⁻⁸ M IB-MECA was determined. To study the dose-response relationship, IB-MECA (10⁻¹⁰ to 10⁻⁶ M) was applied to the chamber, and the highest recorded value in response to each concentration was determined. In this experiment, only one dose of IB-MECA was given per preparation, because tachyphylaxis is a characteristic of adenosine A₃ receptor activation (21).

Because both intracellular cAMP and Ca²⁺ play a major role in the regulation of airway epithelial ciliary motility (15, 24), we assessed the contributions of these to the action of IB-MECA. To do so, the cells were treated for 15 min with the cAMP antagonist Rp-cAMPS (10⁻⁴ M) or with Ca²⁺-free KH solution containing both EGTA (5×10⁻³ M) and the intracellular Ca²⁺-chelating agent BAPTA-AM (5×10⁻⁶ M), and the concentration-response curves for IB-MECA were generated. In our preliminary experiment, Rp-cAMPS (10⁻⁴ M) per se had no effect on the baseline value of CBF but abolished the increase in CBF produced by 8-bromo-cAMP (10⁻⁶ M). In addition, Ca²⁺-free medium containing EGTA and BAPTA-AM decreased baseline CBF by only 7.3 ± 1.1% (P < 0.05, n = 9).

Measurement of intracellular Ca²⁺. To confirm whether the effects of adenosine receptor agonists on ciliary motility were associated with Ca²⁺ mobilization, we measured intracellular levels of Ca²⁺ ([Ca²⁺]i) in response to 10⁻⁸ M CPA, CGS-21680, and IB-MECA. We also determined the effect of pretreatment for 15 min with MRS-1220 (10⁻⁷ M) on IB-MECA-induced [Ca²⁺]i responses. The cells grown on a cover glass were washed with HBSS that contained 10 mM HEPES (pH 7.4) and loaded with fura 2-AM for 20 min at 37°C. The cover glass was then washed again and held with a rigid holder in a continuously stirred cuvette containing HEPES-buffered HBSS maintained at 37°C, and the fluorescence intensity was measured with a spectrophotometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). For excitation of fura 2 fluorescence, ultraviolet lights of 340- and 380-nm wave lengths were automatically exchanged at a rate of 50 Hz, the emitted light from cells [fluorescence at 340 (F₃₄₀) and 380 nm (F₃₈₀)] was detected with a photomultiplier tube through a 510 ± 10-nm band-pass filter, and the fluorescence intensity ratio, F₃₄₀/F₃₈₀, was automatically calculated. Maximal and minimal values for the ratio were determined in the presence of ionomycin (10⁻⁵ M) and EGTA (5×10⁻³ M), respectively, and [Ca²⁺]i was calculated using the external calibration standards and formula previously described (9).

Statistics. All values were expressed as means ± SE. The drug concentrations producing a half-maximal response of CBF (EC₅₀ values) were calculated using the concentration-effect curves by nonlinear regression analysis. Statistical analysis was performed by ANOVA using Scheffé’s F-test (Unistat 3.0 statistical software; Megalon, Novato, CA), and a P value <0.05 was considered statistically significant.

RESULTS

Mucociliary transport. Spontaneous mucociliary transport in the rabbit tracheal mucosa is shown in Fig. 1. The contents of Evans blue dye in section 2, where the dye had been placed at time 0 on the mucosal surface, gradually decreased. During the first 10 min, the decrease in Evans blue contents in section 2 was accompanied by corresponding increases in the dye contents in sections 3 and 4. Next, the contents in section 3 decreased, whereas those in section 4 increased. These findings indicate that Evans blue was transported from the lower trachea toward the larynx in the control condition.

The effects of intravenous administration of adenosine A₁, A₂, and A₃ receptor agonists (30 μg/kg) on mucociliary transport are demonstrated in Fig. 2. When CPA was given, Evans blue contents at 10 min tended to be greater in section 2 and were significantly smaller in section 4 compared with controls (P < 0.05, n = 10), suggesting that mucociliary transport was retarded. Administration of CGS-21680 was without effect, and IB-MECA caused a decrease in Evans blue contents in section 2 (P < 0.01, n = 10) and increases in the dye contents in sections 3 (P < 0.05, n = 10) and 4 (P < 0.01, n = 10), indicating a potentiation of mucociliary transport. As shown in Fig. 3, IB-MECA enhanced tracheal mucociliary transport in a dose-dependent manner, where the maximal increase in Evans blue contents in section 4 was observed at a dose of 30 μg/kg (9.7 ± 0.9 to 24.0 ± 1.5%, P < 0.01, n = 8) and the effect of a higher dose (100 μg/kg) was less potent. Administration of APNEA likewise caused a dose-dependent but weaker stimulation of Evans blue transport, CGS-21680 had no significant effect, and CPA at 30 and 100 μg/kg caused an inhibition of the transport (P < 0.05 for each dose). The IB-MECA-induced increase in Evans blue transport was not altered by pretreatment with DPCPX but was almost completely abolished by MRS-1220 (Fig. 4). Pretreatment with DPCPX only enhanced the response to 100 μg/kg IB-MECA (20.9 ± 1.0 to 29.8 ± 1.3%, P < 0.05, n = 8).

Ciliary motility. Addition of IB-MECA (10⁻⁸ M) to the chamber elicited a rapid increase in CBF of rabbit tracheal epithelium from the baseline value of 11.2 ± 0.3 to 14.4 ± 0.6 Hz (P < 0.01, n = 11) within 1 min, which was followed by a small decrease and the subsequent stable response (Fig. 5). The CBF value 20 min after the addition was still significantly greater than the baseline value (P < 0.01). Addition of APNEA produced a smaller increase in CBF at 1 and 3 min, CGS-21680 was without effect, and CPA caused a small but significant decrease in CBF at 3 and 5 min (maximal decrease: 11.0 ± 0.3 to 9.5 ± 0.3 Hz, P < 0.05, n = 11). The increase in CBF produced by IB-MECA was not altered by DPCPX or DMXP but was abolished by MRS-1220 (P < 0.01, n = 10; Fig. 6). Discoordination of ciliary beating was not observed in the recorded video film throughout the experiments.
As shown in Fig. 7, IB-MECA (10^{-10} to 10^{-7} M) increased CBF in a concentration-dependent fashion: the maximal increase from the baseline value was 33.2 ± 4.6% (P < 0.01, n = 8), and the EC_{50} value was 3.1 ± 0.5 \times 10^{-9} M (n = 8). The CBF value declined at the higher concentration of 10^{-6} M IB-MECA. Incubation of cells with Ca^{2+}-free medium containing EGTA and BAPTA-AM greatly attenuated the IB-MECA-induced increase in CBF, but Rp-cAMPS did not alter the effect of IB-MECA.

**Intracellular Ca^{2+} levels.** The baseline [Ca^{2+}]_i in the rabbit tracheal epithelium was 147 ± 11 nM (n = 16). Exposure of cells to CPA or CGS-21680 at 10^{-8} M had no effect on [Ca^{2+}]_i. However, IB-MECA (10^{-8} M) caused a rapid increase in F_{340/F380} (Fig. 8). This [Ca^{2+}]_i response was biphasic, consisting of an initial

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**Fig. 2.** Effects of adenosine receptor agonists on mucociliary transport in the rabbit trachea. EB contents in the tracheal sections were determined 10 min after iv administration of N-cyclopentyladenosine (CPA), CGS-21680, or N^6-(3-iodobenzyl)-5'-N-methylcarbamoyladenosine (IB-MECA; 30 \mu g/kg for each). In the control experiment, no drug was given. The EB level in each tracheal section was expressed as a percentage of the total amount of dye in sections 1–4. Values are means ± SE; n = 10 for each column. *P < 0.05 and **P < 0.01, significantly different from control values.

**Fig. 3.** Dose-dependent effect of adenosine receptor agonists on mucociliary transport. EB contents in tracheal section 4 were determined 10 min after iv administration of CPA, CGS-21680, N^6-2-(4-amino-3-iodophenyl)ethyladenosine (APNEA), or IB-MECA (1–100 \mu g/kg). In the control experiment (C), no drug was given. The EB level was expressed as a percentage of the total amount of dye in sections 1–4. Values are means ± SE; n = 8 for each point. *P < 0.05 and **P < 0.01, significantly different from control values.

**Fig. 4.** Effects of adenosine receptor antagonists on IB-MECA-induced stimulation of mucociliary transport. Rabbits received iv 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 1 mg/kg), 3,7-dimethyl-1-propargylyxanthine (DMFX; 1 mg/kg), or MRS-1122 (100 \mu g/kg), and 15 min later IB-MECA (1–100 \mu g/kg). EB contents in tracheal section 4 were determined 10 min after administration of IB-MECA. The EB level was expressed as a percentage of the total amount of dye in sections 1–4. Values are means ± SE; n = 8 for each point. *P < 0.05 and **P < 0.01, significantly different from values for IB-MECA alone.

**Fig. 5.** Time course of effects of adenosine receptor agonists on ciliary beat frequency (CBF) of rabbit tracheal epithelium. IB-MECA, APNEA, CGS-21680, or CPA at a concentration of 10^{-8} M was added to the chamber at time 0 (arrow). Values are means ± SE; n = 11 for each point. *P < 0.05 and **P < 0.01, significantly different from baseline values.
transient rise that peaked within 15 s and a following sustained response. In this phase, periodic increases in [Ca\textsuperscript{2+}]\textsubscript{i} (Ca\textsuperscript{2+} oscillations) were not observed. The peak value of [Ca\textsuperscript{2+}]\textsubscript{i} was 566 ± 39 nM, indicating an increase in [Ca\textsuperscript{2+}]\textsubscript{i} by 419 ± 32 nM (P < 0.001, n = 16). Pretreatment with MRS-1220 (10^{-7} M) did not affect the baseline [Ca\textsuperscript{2+}]\textsubscript{i} levels but reduced the subsequent IB-MECA-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise by 84 ± 9% (P < 0.001, n = 16).

**DISCUSSION**

In the present experiment, we developed an in vivo method to evaluate airway mucociliary clearance by determining the transport rate of Evans blue dye that had been placed on the tracheal mucosal surface above the carina in rabbits and demonstrated for the first time that stimulation of adenosine A\textsubscript{3} receptors enhances mucociliary transport. This conclusion is based on the following findings. First, the rate of propulsion of the dye toward the larynx was increased by intravenous administration of IB-MECA, a potent and selective A\textsubscript{3} receptor agonist (1), and to a lesser extent by the relatively less selective A\textsubscript{3} agonist APNEA (6), but not with the selective A\textsubscript{1} and A\textsubscript{2} agonists CPA and CGS-21680, respectively. Second, pretreatment with the selective A\textsubscript{3} receptor antagonist MRS-1220 (11) blocked the IB-MECA-induced stimulation of Evans blue transport, whereas DPCPX and DMPX, selective A\textsubscript{1} and A\textsubscript{2} antagonists, respectively, did not.
It has been generally accepted that mucociliary transport is governed by ciliary activity and the depth and rheological properties of periciliary fluid (27, 30), and we hypothesized that the A3 receptor-mediated transport of Evans blue dye may be accounted for by the stimulation of ciliary activity. Furthermore, because adenosine A3 receptors are present on mast cells and the receptor activation results in the release of a variety of mediators (22), the observed effect of IB-MECA could be attributed to mast cell-derived mediators. To test these possibilities, we conducted in vitro experiments using cultured tracheal epithelium, and the results were consistent with our in vivo findings. Addition of IB-MECA rapidly increased CBF, indicating a direct action on airway epithelial cells. The ciliary stimulatory effect is likely mediated by the A3 receptor, since IB-MECA was effective at nanomolar concentrations, consistent with A3 selectivity (12), and since it was specifically blocked by MRS-1220. To date, A3 receptors have been cloned from rats (17), rabbits (10), and humans (25), and mRNA for these receptors are expressed in particularly high abundance within the human lung (25), but localization of the receptors to airway epithelial cells warrants further studies. Additionally, the effectiveness of ciliary action in transporting Evans blue dye may depend on several characteristics of ciliary beating, of which the CBF is but one. The coordination of the beating pattern, for instance, also plays a role in ciliary performance (27). In the present study, no ciliary discoordination was noted among adjacent cilia on the same cell or several bordering cells in association with the increased CBF in response to IB-MECA. Thus it seems reasonable to speculate that the observed increase in CBF can be translated into the enhanced mucociliary transport, as predicted by theoretical models of mucociliary pumping (23). However, although there is no evidence that selective stimulation of adenosine A3 receptors causes bronchoconstriction, possible influence of the contraction of trachealis musculature on the IB-MECA-induced mucociliary transport cannot be ruled out.

In both in vivo and in vitro experiments, the A1 agonist CPA caused small but significant decreases in Evans blue transport and CBF. This is consistent with our previous finding that the A1 agonist CPA inhibited rabbit tracheal ciliary motility (34). In addition, we noted that although IB-MECA dose dependently increased mucociliary transport and ciliary motility at relatively low doses (up to 30 μg/kg and 10^{-7} M, respectively), the responses to high doses (100 μg/kg and 10^{-6} M) tended to decline. The reason for this is uncertain, but desensitization of A3 receptors is unlikely because only one dose of IB-MECA was given for each experiment. Another possible reason would be that the high dose of IB-MECA is not A3 receptor selective. In fact, it has been shown that IB-MECA bind to cloned adenosine A3 receptors at concentrations of as low as 10 nM (8, 10) and also bind to cloned rabbit adenosine A1 receptors at high concentrations (10). This hypothesis seems likely, because the decline in Evans blue transport in response to 100 μg/kg IB-MECA was reversed by the selective A1 receptor antagonist DPCPX.

Ciliary motility of airway epithelium is regulated mainly by cAMP and Ca^{2+} (15, 24). Intracellular cAMP activates glycogenolysis and subsequently stimulates the production of ATP, an energy source of ciliary beating, via the tricarboxylic acid cycle, and the mobilization of intracellular Ca^{2+} apparently acts on the ciliary axoneme via formation of Ca^{2+}-calmodulin complexes. In our experiment, the increase in CBF produced by IB-MECA was not altered by pretreatment of cells with the cAMP antagonist Rp-cAMPS, but it was greatly attenuated by Ca^{2+}-free solution containing the intracellular Ca^{2+}-chelating agent BAPTA-AM, which inhibits both Ca^{2+} influx and Ca^{2+} release from intracellular stores. These results suggest that the ciliary stimulatory action of IB-MECA may be mediated by Ca^{2+} mobilization. We confirmed this by measuring [Ca^{2+}], and found that IB-MECA but not CPA or CGS-21680 increased [Ca^{2+}], and that the IB-MECA-induced response was greatly attenuated in the presence of MRS-1220. In support of this, recent studies have shown that stimulation of A3 receptors elevate intracellular Ca^{2+} in human nonpigmented ciliary epithelial cells (18), rat cardiomyocytes (29), and the rat mast cell line (28) probably via generation of inositol phosphate through phosphoinositide breakdown. It has been demonstrated that stimulation of nucleotide receptors with ATP and UTP produces an initial transient increase in [Ca^{2+}], and the following Ca^{2+} oscillations in airway epithelium (3). In contrast, such Ca^{2+} oscillations were not noted in the response to IB-MECA. The reason for the difference is uncertain, but this could be because of the difference in receptors and/or experimental condition.

In conclusion, our present studies show that stimulation of adenosine A3 receptors increases ciliary motility of airway epithelium and mucociliary transport in the respiratory tract. Therefore, selective A3 receptor agonists might be useful in the treatment of impaired mucociliary clearance.

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