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Original Article

Adrenomedullin stimulates cyclic AMP production in the airway epithelial cells of guinea-pigs and in the human epithelial cell line

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Abstract

This study was designed to examine the effects of adrenomedullin (AM) on airway epithelial cells. Primary cultures of guinea-pig tracheal epithelial cells and the human bronchiolar epithelial cell line NCI-H441 were used. Intracellular cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), prostaglandin E_2 (PGE_2), and stable end-products of nitric oxide were assayed. Adrenomedullin (10^{-6} mol/L) stimulated cAMP production in guinea-pig epithelial cells. Indomethacin (10^{-5} mol/L) significantly decreased the basal level of intracellular cAMP in guinea-pig epithelial cells, but not in NCI-H441 cells. However, AM did not stimulate production of PGE_2, a major product that can increase cAMP formation. In the case of NCI-H441 cells, AM (10^{-8} – 10^{-6} mol/L) did not significantly affect intracellular cGMP levels or nitrite content in conditioned medium. Adrenomedullin and calcitonin gene-related peptide (CGRP) each stimulated cAMP production in NCI-H441 cells, but AM-stimulated cAMP production was antagonized by the CGRP fragment $CGRP_{8-37}$. These findings suggest that AM stimulates cAMP production and functionally competes with CGRP for binding sites in airway epithelial cells, at least in human epithelial cells, but that it does not stimulate the release of PGE_2 and nitric oxide. Though cyclooxygenase products contribute to some extent to cAMP formation in guinea-pigs, AM independently stimulates intracellular cAMP formation in airway epithelial cells.

Key words: adrenomedullin, airway, cAMP, epithelial cell, guinea-pig, human.

Introduction

Adrenomedullin (AM) is a hypotensive peptide that was discovered in human pheochromocytoma in 1992.[1] Human AM consists of 52 amino acids, includes one intramolecular disulfide bond, and exhibits slight homology with calcitonin gene-related peptide (CGRP).

Immunoreactive AM and AM mRNA are present not only in normal adrenal medulla but also in the lungs and kidneys.[2] In a normal human lung, immunohistochemical studies have shown that AM is expressed in the airway in the columnar epithelium, in some glands, in neurons of the parasympathetic nervous system, in endothelial cells, chondrocytes, alveolar macrophages and smooth muscle cells.[3] Additionally, recent studies suggest that the lung is rich in AM receptors.[4,5] The presence of immunoreactive AM and its binding sites in the airway suggest that this peptide has an important role in the regulation of airway functions. In fact, several studies have revealed the physiological effects of AM on the airway. In the guinea-pig airway, inhalation of AM has potent bronchodilatory effects.[6,7] In rat alveolar macrophages, AM suppressed LPS-stimulated cytokine-induced neutrophil chemoattractant (CINC) production.[8]

These findings suggest that AM may play a broad role in the regulation of airway functions. However, to our knowledge, very few studies of AM in airway epithelial

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cells have been performed, although it is well known that the airway epithelium modulates bronchial tonus and plays an important role in airway inflammation. Augmentation of intracellular cyclic nucleotides in the airway seems important, especially in airway inflammation. For example, bronchodilatory agents for bronchial asthma, such as theophyllines, are now focused on its anti-inflammatory effects.

This study was designed to investigate the effect of AM on guinea-pig airway epithelial cells and a human airway epithelial cell line.

Materials and methods

Materials

Human AM, CGRP and $CGRP_{8-37}$ were purchased from the Peptide Institute Inc. (Osaka, Japan). Forskolin, protease type XXIV, 3-isobutyl-1-methylxanthine (IBMX) and indomethacin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Kits for the determination of cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and prostaglandin E_2 (PGE_2), were obtained from Amersham (Bucks, UK). Dulbecco's modified Eagle's medium (DMEM)/F12, RPMI1640 culture medium and penicillin/streptomycin were purchased from Gibco BRL (Rockville, MD, USA). The Bio-Rad protein assay system was used for the determination of protein content of cultured cells. Other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan).

Cell culture

Guinea-pig airway epithelial cell culture

Primary cultures of the guinea-pig tracheal epithelial cells were performed using a modified method of Wu and Smith.[9] Briefly, guinea-pigs (Hartley, 300–400 g, both sexes) were killed by cervical dislocation. Krebs buffer, including 0.1% of protease type XXIV, was instilled into the excised trachea. Both ends of the trachea were tied and incubated at 37°C for 20 min. The tied lesions were then cut out, and the lumen was washed with DMEM/F12 culture medium containing 10% fetal calf serum to stop enzyme digestion and to collect epithelial cells.

After washing, the trachea was opened longitudinally using scissors and the surface of the mucosa was scraped with a rubber policeman to collect more cells. Isolated cells were resuspended in DMEM/F12 with 10% of the fetal calf serum and seeded in 24-well tissue culture plates at a density of 2×10^5 cells/cm^2. Cells in the primary cultures were grown to confluence in a humidified atmosphere at 37°C in 95% O_2 and 5% CO_2 air. The culture medium was replaced after 24 h with serum-free DMEM/F12. Thereafter, the culture medium was replaced every 48 h.

Epithelial cells generally formed confluent monolayers 4–6 days after seeding. Several wells were cultured for electronmicroscopy on filters coated with type VI collagen. The characteristics of the cultured cells were examined by scanning and transmission electron microscopy 5 days after seeding on the filter. Scanning electron microscopy revealed microvilli and cilia on the surfaces of the cultured cells (Fig. 1). Transmission electron microscopy revealed simple or cuboidal columnar epithelium with desmosomes and tight junctions between the cells. These findings proved that the cultured cells were epithelial cells. Confluent cells were used in the following experiments.

Human epithelial cell line culture

The human epithelial cell line NCI-H441 was purchased from American Type Cell Culture. Cells of this line have characteristics of Clara cells, one of the secretory cells in the airway epithelium. A previous study showed that cytokine stimulates cGMP production via nitric oxide synthesis in this cell line.[10] The cells were passaged in accordance with the enclosed guidelines. Briefly, cells were seeded on a culture flask, passaged using trypsin/EDTA, and resuspended in RPMI 1640 including 10% fetal calf serum. A total of 55–65 passaged cells were seeded on 6-well culture plates, and when the cultured

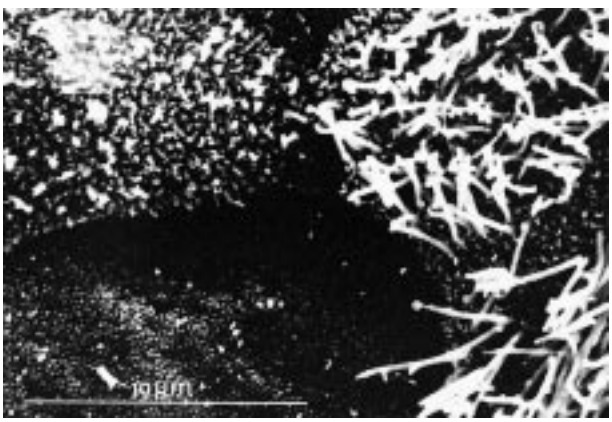

Fig. 1 Scanning electron microscopy of cultured guinea-pig tracheal epithelial cells. One of the cells possesses cilia, and every cell has microvilli on the surface of the confluent cell layer.

cells reached confluence, cells were used for the following experiments.

Determination of cAMP, cGMP and PGE_2

Guinea-pig airway epithelial cell study

Confluent primary epithelial cell cultures were washed twice by incubation with fresh serum-free DMEM/F12 for 20 min at 37°C. When indomethacin was tested, 10^{-5} mol/L indomethacin was added 40 min before IBMX administration. Thereafter, the culture mediums were replaced with 500 μL of culture medium containing 0.25 mmol IBMX and incubated for 20 min at 37°C. 3-isobutyl-1-methylxanthine was added to minimize degradation of cyclic nucleotides as described previously.[6,12] Various concentrations of agonist (AM or forskolin) were added to the cells. Then, 100 μL portions of supernatants were aliquoted for PGE_2 assay 10 min after agonist administration. After termination of incubation by the addition of ethanol, intracellular cAMP was measured with an enzyme immunoassay system and intracellular cGMP was measured with a radio immunoassay system.

NCI-H441 study

Confluent NCI-H441 cell cultures were pretreated as for the guinea-pig study except that RPMI 1640 was used instead of DMEM/F12. When indomethacin was tested, 10^{-5} mol/L indomethacin was added 40 min before IBMX administration. Thereafter, the culture medium was replaced with 1500 μL of culture medium containing 0.25 mmol IBMX and incubated for 30 min at 37°C. Various concentrations of agonist (AM, CGRP or forskolin) were added to the cells. After termination of incubation by the addition of ethanol, intracellular cAMP was measured with an enzyme immunoassay system and intracellular cGMP was measured with a radio immunoassay system. $CGRP_{8-37}$ was added 15 min before agonist administration in several wells.

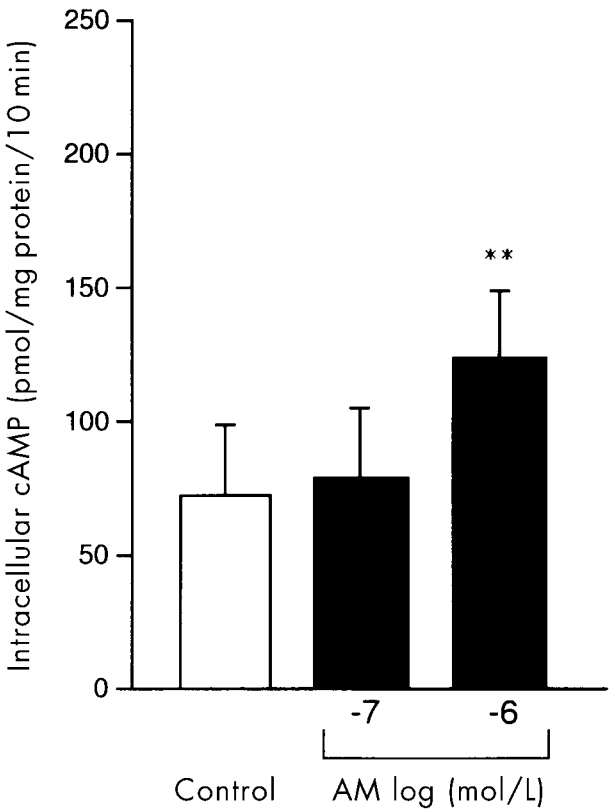

Fig. 2 Cyclic adenosine monophosphate (cAMP) level in cultured guinea-pig epithelial cells stimulated by adrenomedullin (AM). Values are means ± SD (n = 4). $^{**}P < 0.01$ compared with control.

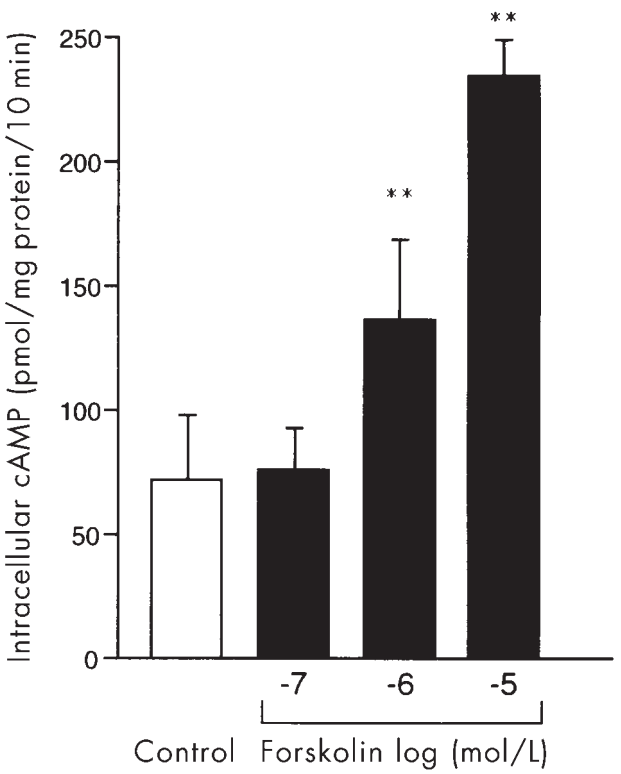

Fig. 3 Cyclic adenosine monophosphate (cAMP) level in cultured guinea-pig epithelial cells stimulated by forskolin. Values are means ± SD (n = 4). $^{**}P < 0.01$ compared with control.

Determination of nitrite content of conditioned medium

In the human cell line study, samples for the determination of nitrite content of conditioned medium of AM (10^{-7} mol/L)-stimulated cells were aliquoted 10 min, 1, 2, 4, 8, 12, and 24 h after AM administration. Nitrite content was determined by the Griess reaction.[11]

Statistical analysis

Values are expressed as means ± SD. Statistical significance was assessed using Student's *t*-test. *P*-values < 0.05 on two-tailed tests were considered significant.

RESULTS

Guinea-pig airway epithelial cell study

Intracellular cAMP levels were determined in cultured epithelial cells stimulated by AM (10^{-7} – 10^{-6} mol/L) or forskolin (10^{-7} – 10^{-5} mol/L) (Figs 2 and 3). The basal level of intracellular cAMP in cultured epithelial cells was 48.1 ± 21.2 pmol/mg protein. Adrenomedullin and forskolin significantly increased cAMP production at 10^{-6} mol/L (83.8 ± 3.3 and 112.7 ± 15.2 pmol/mg protein, respectively; $P < 0.01$ in each case). Although intracellular cAMP levels decreased following pretreatment with 10^{-5} mol/L indomethacin, AM (10^{-8} – 10^{-6} M) also stimulated cAMP production in a dose-dependent manner (Fig. 4). AM (10^{-8} – 10^{-6} mol/L) did not significantly affect PGE_2 release from epithelial cells (Fig. 5). In addition, intracellular cGMP levels were not significantly altered in AM-stimulated cells.

NCI-H441 study

Adrenomedullin (10^{-8} – 10^{-6} mol/L) and CGRP (10^{-15} – 10^{-9} mol/L) significantly stimulated intracellular cAMP formation in NCI-H441 cells in a dose-dependent manner (Fig. 6). A 10^{-5} mol/L concentration of $CGRP_{8-37}$ alone did not stimulate intracellular cAMP formation, but did significantly inhibit acceleration of intracellular cAMP production in AM-stimulated cells (Fig. 7). We found no significant effect of indomethacin on intracellular cAMP formation in these cells. Intracellular cGMP levels and nitrite content in conditioned medium were not significantly altered by AM stimulation (10^{-8} – 10^{-6} mol/L) of NCI-H441 cells.

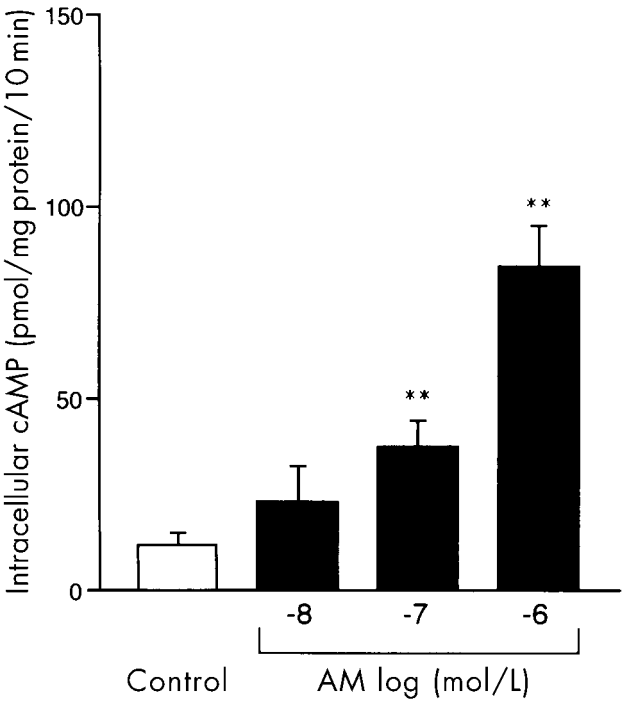

Fig. 4 Cyclic adenosine monophosphate (cAMP) level in indomethacin-pretreated cultured guinea-pig epithelial cells stimulated with adrenomedullin (AM). Values are means ± SD ($n = 4$). $^{**}P < 0.01$ compared with control.

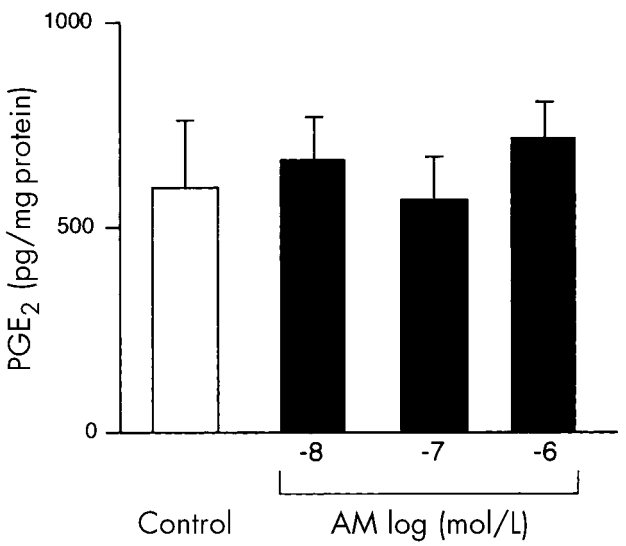

Fig. 5 Prostaglandin E_2 (PGE_2) from cultured guinea-pig epithelial cells. Values are means ± SD ($n = 5$). There was no significant increase of PGE_2 production in epithelial cells stimulated with adrenomedullin (AM).

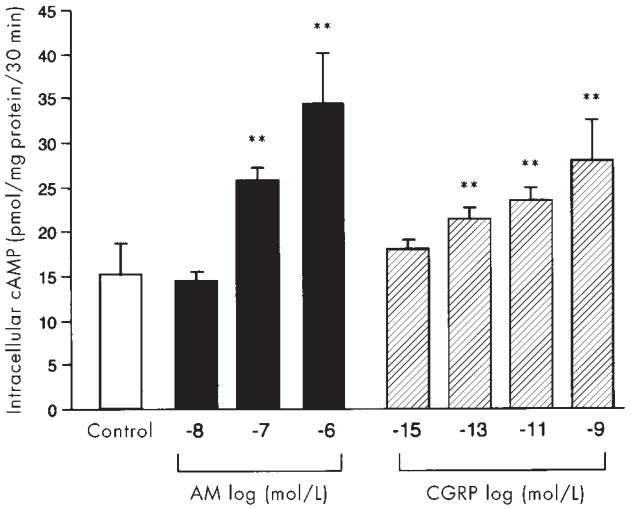

Fig. 6 Cyclic adenosine monophosphate (cAMP) level in NCI-H441 cells stimulated by adrenomedullin (AM) or calcitonin gene-related peptide (CGRP). Values are means ± SD (n = 6). **P < 0.01 compared with control.

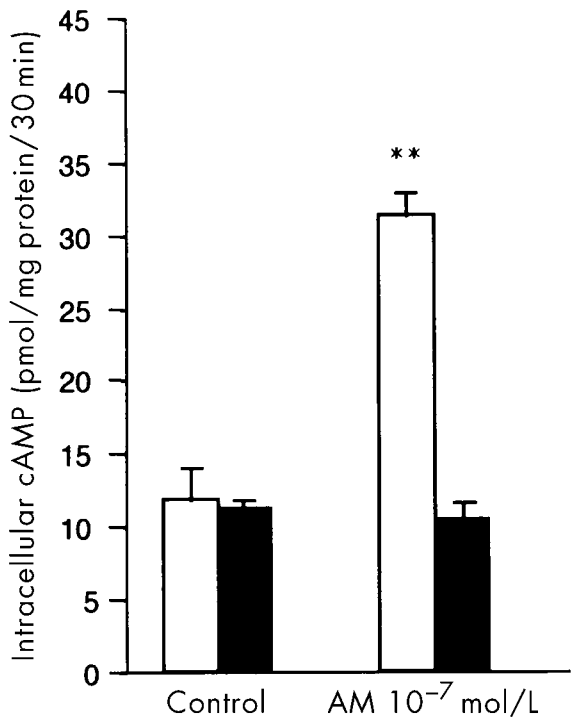

Fig. 7 The effect of calcitonin gene-related peptide 8–37 ($CGRP_{8-37}$) pretreatment on cAMP level in NCI-H441 cells stimulated by adrenomedullin (AM). –$CGRP_{8-37}$, (□); +$CGRP_{8-37}$, (■). Values are means ± SD (n = 6). **P < 0.01 compared with control. $CGRP_{8-37}$ (10^{-5} mol/L) alone did not stimulate intracellular cAMP formation, but did significantly inhibit acceleration of intracellular cAMP production in AM-stimulated cells.

Discussion

Adrenomedullin is known to increase cAMP levels in various kinds of cells including vascular smooth muscle cells,[12] vascular endothelial cells,[13] mesangial cells,[14] hepatic pericytes[15] and alveolar macrophages.[6] In the present study, we found that AM stimulates cAMP production in cultured airway epithelial cells to the same extent as forskolin, an adenylate cyclase activator, in both guinea-pig tracheal epithelial cells and a human bronchiolar epithelial cell line.

Adrenomedullin has a disulfide bond between cysteins 16–21 and exhibits slight homology with CGRP, including the disulfide ring structure which is required for binding to its specific receptors.[1] Calcitonin gene-related peptide, one of the potent mediators in the lung, is widely distributed in sensory neurons and nerve fibers, some of which terminate just beneath the airway epithelium.[16] Autoradiographic localization of CGRP binding sites in lung sections showed that CGRP receptors are abundant in airway, including epithelium, vessels, alveolar wall and smooth muscle cells.[17] Moreover, a recent study showed that CGRP receptor antagonist inhibits physiological function of AM (e.g. pretreatment with $CGRP_{8-37}$ inhibits AM-induced vasodilatation).[18] It is therefore important to compare AM with CGRP to clarify the pharmacological characteristics of the former.

In the present study, both AM and CGRP increased intracellular cAMP in a dose-dependent manner, and CGRP had potent effects on acceleration of intracellular cAMP production at a lower concentration than AM. Our study also showed that $CGRP_{8-37}$ alone had no effect on cAMP production, while pretreatment with $CGRP_{8-37}$ inhibited AM-stimulated cAMP production by NCI-H441 cells. These results suggest that AM functionally competes with CGRP for binding sites in airway epithelial cells.

The airway epithelial cell, which releases many kinds of cytokines, chemokines, and chemical mediators such as GM-CSF, IL-1β,[19] IL-6, IL-8,[20] IL-8 superfamily regulated on activation normal T-expressed and secreted (RANTES),[21] PGE_2, LTB4,[22–26] and so on, is one of the most important modulator cells in airway inflammation. Prostaglandin E_2 stimulates intracellular cAMP production,[19] and airway epithelial cells secrete PGE_2 both in the basal and in the stimulated condition.[23–26] In the present study, in the absence of indomethacin, the dose dependent response of cAMP production in AM-stimulated guinea-pig airway epithelial cells was unclear. It is possible that endogenous PGE_2 augments the basal level of cAMP and this

augmentation of cAMP makes the response of AM in a low concentration unclear. In other words, using indomethacin, the dose dependent response of cAMP formation in AM-stimulated guinea-pig airway epithelial cells was observed more clearly. However, in NCI-H441 cells, we could find very little influence of indomethacin in cAMP formation.

It is possible that endogenous PGE_2 in NCI-H441 cells contributes less to cAMP formation in the basal level than in guinea-pig epithelial cells. Another thing related to PGE_2 which is important in the present study is the possibility that AM stimulates cyclooxygenase (COX) to produce COX products such as PGE_2, and that such products altered cAMP levels. However, we found no significant increase in release of PGE_2 by AM-stimulated epithelial cells and even in the presence of COX inhibitor, indomethacin, AM stimulated the intracellular cAMP level in airway epithelial cells. Adrenomedullin thus appears to stimulate cAMP production in epithelial cells via a cyclooxygenase-independent mechanism.

In aortic endothelial cells, it is known that AM stimulates not only cAMP production but also Ca^{2+} mobilization and nitric oxide synthase (NOS) activity, which results in augmentation of cGMP production.[13,27] Airway epithelial cells are known to possess constitutive nitric oxide synthase (cNOS). NCI-H441 cells have already been reported to possess cNOS, and produce nitric oxide when stimulated by A23187.[10] In the present study, we measured nitric oxide in the conditioned medium by Griess reaction. Although with this method the nitric oxide contents can be measured in the range 6×10^{-6} mol/L to 200×10^{-6} mol/L, nitric oxide was not detected in the conditioned medium from epithelial cells stimulated by various concentrations of AM.

We also measured intracellular cGMP levels to evaluate the contribution of the nitric oxide – cGMP system to a signal transduction in airway epithelial cells stimulated by AM. The cGMP RIA system used in the present study, which can detect cGMP in the range 20–1280 fmol/mL, failed to detect cGMP contents in NCI-H441 cells stimulated by AM. Our finding that neither nitric oxide nor cGMP was detected in AM-stimulated cells suggests that AM stimulated cAMP production but not cGMP production in airway epithelial cells, unlike in aortic endothelial cells. However, in the cerebral arteries it has been reported that AM increases cerebral blood flow independent[28] of nitric oxide or prostaglandin formation, as in our result.

Recent studies[29,30] suggest that AM stimulates nitric oxide production in the presence of IL-1β. However, several reports showed that AM stimulates nitric oxide production without IL-1β.[31,32] Although the airway epithelium can produce IL-1β, in the present study total incubation time seemed to be too short for airway epithelial cells to produce IL-1β . If IL-1β is essential for the production of nitric oxide in AM-stimulated airway epithelial cells, then this could be one explanation for nitric oxide production not being observed in our study.

We used concentrations of AM somewhat higher than the plasma level of AM.[33] It seems likely that local AM concentration in the airway is higher than plasma concentration; moreover, a recent study showed that the plasma level of AM is markedly elevated during asthmatic attacks.[34]

Although cyclooxygenase products contribute to some extent to cAMP formation in some species, AM independently stimulates intracellular cAMP formation in airway epithelial cells.

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