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ORIGINAL

Effect of human airway trypsin-like protease on intracellular free Ca^{2+} concentration in human bronchial epithelial cells

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Abstract : It has been shown that human airway trypsin-like protease (HAT) is localized in human bronchial epithelial cells (HBEC), and trypsin activates protease-activated receptor-2 (PAR-2). Activation of PAR-2 activates G-protein followed by an increase of intracellular free Ca^{2+}, $[Ca^{2+}]_{in}$. This study was undertaken to clarify whether HAT can activate PAR-2 in HBEC or not. RT-PCR showed that HAT mRNA is expressed in HBEC, and PAR-2 mRNA is the most strongly expressed of the known PARs in HBEC. Both PAR-2 agonist peptide (PAR-2 AP) and HAT increased $[Ca^{2+}]_{in}$ in HBEC in a biphasic fashion ;a prompt, sharp increase (peak I) and a sustained low plateau (peak II). PAR-2 AP over 100-200 μM and HAT over 200-300 mU/ml (0.08-0.12 μM) induced both peak I and II, and PAR-2 AP below 100 μM and HAT below 200 mU/ml induced only peak II. Both PAR-2 AP-induced and HAT-induced peak I were induced by Ca^{2+} mobilization from intracellular stores, because they appeared even in Ca^{2+}-free medium. Both PAR-2 AP-induced and HAT-induced peak II were induced by an influx of extracellular Ca^{2+}, because they were abolished in Ca^{2+}-free medium. The Ca^{2+} response to HAT was desensitized by exposure of HBEC to PAR-2 AP. These results indicate that HBEC have a functional PAR-2, and HAT regulates cellular functions of HBEC via activation of PAR-2. J. Med. Invest. 50 : 95-107, 2003

Keywords : *human airway trypsin-like protease (HAT), human bronchial epithelial cells (HBEC), protease-activated receptor-2(PAR-2), intracellular calcium,*

INTRODUCTION

Previous investigations have indicated that several kinds of trypsin-like enzymes are biosynthesized or released in the airway (1-6). Mast cells releases trypsin-like protease (tryptase) into extracellular spaces of the airway (1). Human mast cell tryptase (MCT) has an apparent molecular weight of 144 kDa and consists of two subunits of 37(or 30.9) kDa and two subunits of 35(or 31.6) kDa (7-8). Kido *et al.* have shown in rats that Clara cells located only in the wall of the distal airway secrete a trypsin-like protease (tryptase Clara) with an apparent molecular weight of 180 kDa (2). Kawano *et al.* (4) and

Received for publication December 19, 2002 ; accepted January 8, 2003.

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Cocks *et al.* (5) have reported that a positive reaction to antibody against human trypsin (organ) is detectable in normal human airway epithelial cells and gland cells by immunohistochemistry.

Yasuoka *et al.* isolated a novel, monomeric trypsin-like protease with a molecular weight of 27 kD from the mucoid sputum of patients with chronic airway diseases and named this novel enzyme human airway trypsin-like protease (HAT) (3). Yamaoka *et al.* cloned the cDNA of HAT and indicated that HAT has a precursor form of 47 kDa, because the deduced polypeptide consisted of a 232-residue catalytic region and a 186-residue noncatalytic region with a hydrophobic putative transmembrane domain near the NH_2-terminus. Takahashi *et al.* have immunohistochemically examined the localization of HAT in the airways and have shown that a positive reaction to a monoclonal antibody to HAT is localized at ciliated bronchial epithelial cells, but not detectable at the submucosal layer of the airways (10). These results strongly suggest that precursor HAT may be synthesized in the airway epithelial cells and converted into the active HAT by limited proteolysis.

Previous investigations have suggested that trypsin-like proteases might be involved in the pathogenesis of inflammation, infection and fibrotic processes in the airway and lung. For example, MCT has been reported to play some pathophysiological roles in airway diseases like bronchial asthma (11-12) and pulmonary fibrosis (13-14). Kido *et al.* showed that tryptase Clara activates the infectivity of influenza A virus (2). However, the physiological and pathophysiological roles of HAT are unknown.

Recently, it has been clarified that certain serine proteases such as thrombin and trypsin, which have been considered to participate principally in the degradation of extracellular proteins, are also signaling molecules that regulate multiple cellular functions by activating specific receptors (5-6, 15-20). The receptors, protease-activated receptors (PARs), are a family of G-protein-coupled receptors (21). These receptors are activated by the proteolytic cleavage of a receptor-bound, NH_2-terminal tethered ligand domain, which is then able to bind to the receptor and initiate signaling (20). Four PARs (PAR1-4) have been characterized (15-19). Trypsin (16-17) and MCT (22-23) activate PAR-2. Recent evidence indicates that PAR-2 is present in epithelial cells (5-6, 18, 20), endothelial cells (24), smooth muscle cells (5, 25), fibroblasts (23) and neutrophils (26), and may participate in the regulation of functions of enterocytes (27), keratinocytes (28), neutrophils (26) and bronchial epithelial cells (5-6, 29), and can play some role in inflammatory conditions (5-6, 12, 24, 26, 28).

As described above, PARs are localized on cell surfaces and HAT, a trypsin-like protease, is also thought to be localized on cell surfaces of airway epithelial cells. Therefore, we thought that HAT regulates the cellular functions of airway epithelial cells via the activation of PARs in an autocrine or paracrine manner. It has been established that the activation of PARs activates the G-proteins followed by an increase of intracellular Ca^{2+} (21). In this work, we studied the effect of HAT on the intracellular Ca^{2+} concentration in primary human bronchial epithelial cells, to determine whether HAT regulates the cellular functions of airway epithelial cells via the activation of PARs.

MATERIALS AND METHODS

Materials

Benzamidine-Sepharose 6B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and SP-Toyopearl 650M (a cation-exchange gel) was from TOSOH (Tokyo, Japan). M-Per™ (a mammalian protein extraction reagent) was purchased from Pierce (Illinois, USA), and Triton X-100, PEG-6000, bovine serum albumin (BSA) and U73122, an inhibitor of phospholipase C, were from Sigma (St Louis, MO, USA). Methylcoumarinamide (MCA)-substrates for the assay of trypsin-like activity were obtained from Peptide Institute (Osaka, Japan). Heparin sodium salt from porcine intestinal mucosa was obtained from Calbiochem (La Jolla, CA, USA). Gradient gels (Multigel 10/20) and a silver-staining kit were obtained from Daiichi Pure Chemicals (Tokyo, Japan), and prestained low range protein standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY, USA). A type II collagen (Vitrogen 100) was obtained from Collagen Co. (CA, USA). Tissue culture plates were purchased from Becton Dickinson Labware (Franklin Lakes, NJ). 1-(2-(5'-carboxyoxazol-2'-yl)-6-aminobenzofuran-5-oxy)-2(2'amino-5-methylphenoxy) ethane-N, N, N', N'-tetraacetic acid, pentaacetoxy methylester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR, USA). The human PAR-2 agonist peptide, con-

sisting of the amino acid sequence (SLIGKV-NH_2) was obtained from BACHEM (Bubendorf, Switzerland). The primers for RT-PCR of mRNA of HAT and PAR1-4 were synthesized in our laboratory. Bradykinin was obtained from Wako Pure Chemicals (Osaka, Japan).

Human Bronchial Epithelial Cells (HBEC)

Primary human bronchial epithelial cells (HBEC) were collected by brushing the bronchial mucosa of healthy volunteers under bronchoscopy with local anesthesia. Before the study, written informed consent was obtained from all volunteers after a full explanation of the procedures involved. The cells obtained by brushing were collected in 50 ml polypropylene tubes containing 20 ml RPMI-1640 with 10% FBS, and then washed with RPMI-1640 by centrifugation at 400 g at 4°C for 10 min. The rinsed pellets of cells were resuspended in LHC-9 (30)/RPMI-1640 medium in which a mixture of an equal volume of LHC-Basal medium and RPMI-1640 medium was supplemented with bovine pituitary extract (BPE), hydrocortisone, human recombinant epidermal growth factor (rEGF), epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, ethanolamine, o-phophoethanolamine and gentamycin. The cells were plated in 24-well culture plates coated with Vitrogen, and incubated at 37°C in a humidified 5% CO_2 in air atmosphere. The medium was changed every other day until the cells had grown to 90% confluence. Then, the cells were passaged with 0.125% trypsin/0.1% EDTA in PBS and seeded for use in the next study. Experiments were performed with cell passages 1 (P1) and P2.

Other respiratory cells

Normal human small airway epithelial cells (SAEC) and normal human lung fibroblasts (NHLF) were obtained from BioWhittaker (Walkersville, MD, USA). BEAS-2B, a transformed human bronchial epithelial cell line, and A549, a human alveolar cell line, were obtained from American Type Culture Collection (Rockville, MD, USA). Human alveolar macrophages (HAM) were collected by segmental bronchoalveolar lavage from normal volunteers as previously reported (31). SAEC and BEAS-2B were cultured in LHC-9/RPMI-1640 as described for the HBEC, and HAM, NHLF and A549 were cultured in Dulbecco's modified Eagle's medium (DMEM)/10% FBS.

Preparation of recombinant HAT

Recombinant HAT (rHAT) was expressed in insect cells infected with baculovirus carrying HAT cDNA as previously reported (9). The rHAT was purified by a modification of the method used to purify the native HAT (3).

Briefly, a cell pellet from about 400 ml of the culture broth of insect cells was suspended in 100 ml of M-Per™, and this mixture was stirred at room temperature for 2 hrs, then centrifuged at 10,000 rpm for 20 min at room temperature. The pellet (pellet I) was preserved for a second purification. The supernatant was dialyzed against 2.5 liters of 50 mM Na acetate buffer (pH 4.0)/0.01%PEG6000 for 2 hrs, and centrifuged at 10,000 rpm for 20 min. The pellet (pellet II) was also preserved for a second purification, and the supernatant was dialyzed against 50 mM Tris-HCl buffer (pH 8.0)/0.5 M NaCl/0.01% PEG6000 for 4 hrs and the latter dialysis buffer was changed after 2 hrs. Each half of the supernatant was applied to a column (bed volume 5 ml) of Benzamidine-Sepharose 6B equilibrated with the latter dialysis buffer. After the column was successively washed with 50 ml of the latter dialysis buffer, 25 ml of 10 mM Na phosphate buffer (pH 8.0), rHAT adsorbed on the column was eluted with 10 mM HCl (pH2.0) in a 0.5-ml fraction. The active fractions from each column were collected, and pH of this fraction was adjusted to 6-7 by adding a 1/19 volume of 200 mM Na phosphate buffer.

The above-described pellets I and II were combined, and suspended in 50 ml of M-Per™/4% Triton X-100/2% bile salts. The mixture was stirred at room temperature for 2 hrs, and centrifuged at 10,000 rpm for 2 min. The supernatant was dialyzed against 1 liter of 50 mM Na acetate buffer (pH 4.0)/0.01% PEG6000 at room temperature for 4 hrs, the latter dialysis buffer was changed after 2 hrs, and then centrifuged at 10,000 rpm for 20 min at 4°C. The resultant supernatant was applied to a column (bed volume 4 ml) of SP-Toyopearl 650 M equilibrated with the dialysis buffer. After the column was washed with 40 ml of the equilibration buffer, rHAT was eluted with 35 ml of 50 mM Tris-HCl buffer (pH 8.0)/0.5 M NaCl/0.01% PEG6000. After the eluate was dialyzed against 1 liter of the elution buffer at room temperature for 4 hrs with a buffer change after 2 hrs, it was subjected to affinity chromatography using Benzamidine-Sepharose 6B as described above.

By the first and second purification, about 600μg of rHAT was obtained. This purified material showed a single band located in the position corresponding to a molecular weight of about 27 kDa, under

both reducing and non-reducing conditions on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) carried out by the method of Laemmli (32) (Fig. 1). The endotoxin level in the purified rHAT was very low and routinely less than 15 pg/200 μg enzyme protein.

About 15 ml of the purified rHAT solution composed of rHAT and Na phosphate buffer (pH 6-7) was concentrated by ultrafiltration with an Amicon membrane (YM-10), and the rHAT was washed with 10 ml of PBS 3 times by ultrafiltration. rHAT was finally dissolved in 12 ml of PBS/BSA(100 μg/ml) unless otherwise stated, and sterilized by filtration through a Millipore PVDF membrane (0.22 μm, low protein-binding).

Assay of Trypsin-like Activity

Assay of trypsin-like activity was measured by the method of Yasuoka *et al.* (3). Briefly, the assay mixture (1.5 ml) containing 50 mM Tris-HCl (pH 8.6), Boc-Phe-Ser-Arg-MCA at 100 μM, BSA at 100 μg/ml, and 100 μl of the test sample was incubated at **37°C for 60 min, and the reaction was stopped by the addi**tion of 1 ml of 30% acetic acid. Then, the fluorescence

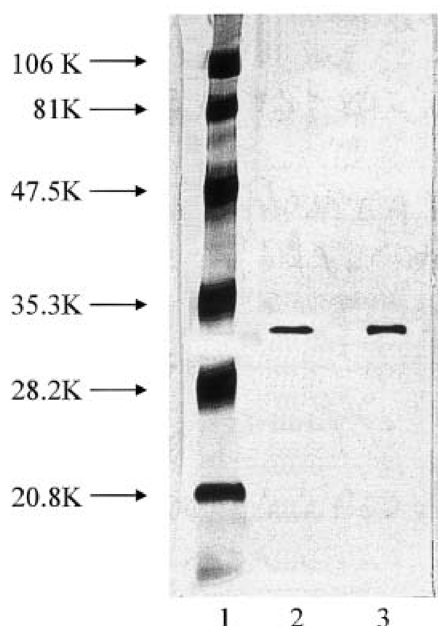

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified recombinant HAT (rHAT).
SDS-PAGE was carried out by the method of Laemmli (34). About 0.3 μg of the purified rHAT was subjected to SDS-PAGE on a multigel 10/20 under denaturing and reducing conditions (lane 2) and non-reducing conditions (lane 3). The gels were silver-stained using a kit obtained from Daiichi Pure Chemicals. The gels were calibrated with prestained SDS-PAGE low range standards (Bio-Rad Laboratories) (lane 1 : phosphorylase B (106 kDa), BSA (81 kDa), Ovalbumin (47.5 kDa), carbonic anhydrase (35.3 kDa), soybean trypsin inhibitor (28.2 kDa), lysozyme (20.8 kDa).

intensity of the released aminomethyl-coumarin (AMC) was measured with fluorescence spectrophotometer (F-3010 Hitachi Co, Japan) at 440 nm with excitation at 380 nm. The amount of AMC released was calculated from a standard curve. One unit of enzyme was defined as amount that produced 1 μmole of AMC per min.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis of mRNA for PAR-1, 2, 3 and 4, and mRNA for HAT

1 RT-PCR for mRNA for PAR-1, 2, 3 and 4 in HBEC

Each of the respiratory cells were seeded into 6-cm tissue culture dishes to 100% confluence. The cells on each dish were lysed in 1 ml of ISOGEN, a mixture of guanidium isothiocyanate and phenol (Nippon Gene, Tokyo, Japan). From the lysed cells, total cellular RNA was extracted with chloroform and precipitated with isopropanol.

A mRNA-selective PCR Kit Ver.1.1 (*Takara Shuzo Co., Shiga, Japan*) was used to amplify selectively the mRNA-derived products. Total RNA(1 μg) was reverse transcripted using this kit and some of the reverse transcription products (cDNA) were used as a template for PCR amplification. Briefly, the PCR was performed for 25-30 cycles (denaturation at 85°C for 45 sec, annealing at 58°C for 45 sec and elongation at 72°C for 2 min). Sense primer and antisense primer for PCR of each PAR-1, PAR-2, PAR-3 and PAR-4 were synthesized according to the methods of Kahn *et al.* (33), and their base sequences were as follows

PAR-1. Sense primer : CAG TTT GGG TCT GAA TTG TGT CG
Antisense primer : TGC ACG AGC TTA TGC TGC TGA C
PAR-2. Sense primer : TGG ATG AGT TTT GTG CAT CTG TCC
Antisense primer : CGT GAT GTT CAG GGC AGG AAT G
PAR-3. Sense primer : TCC CCT TTT CTG CCT TGG AAG
Antisense primer : AAA CTG TTG CCC ACA CCA GTC CAC
PAR-4. Sense primer : AAC CTC TAT GGT GCC TAC GTG C
Antisense primer : CCA AGC CCA GCT AAT TTT TG

The lengths of the expected PCR products were as follows : PAR-1, 592 bp ; PAR-2 491 bp ; PAR-3 512 bp ; PAR-4 542 bp. Products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide

solution and visualized by UV transillumination.

2 RT-PCR for mRNA for HAT in respiratory cells

Total RNA was extracted as described above from each of the respiratory cells. RT-PCR was carried out as described above. Sense and antisense primer for this RT-PCR were synthesized, and the sequences were as follows. Sense primer : 5'-CATTG TCGTC GCAGG GGTAG-3', Antisense primer : 5'-TCAGC CTCAG TGCCT CCAAG-3'. The length of the expected PCR product was 519 bp.

Measurement of intracellular Ca^{2+} concentration

The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_{in}$) was evaluated by microfluorometry with the fluorescent dye, Fura-2/AM, at an excitation wavelength of 340 nm and 380 nm, and at an emission wavelength of 510 nm with a specially designed chamber and an ARGUS-50/CA system (Hamamatsu Photonics, Tokyo, Japan)(34). In each experiment, the ratio of fluorescence at 340 nm to that 380 nm was determined in 15-20 individual cells that were selected with the system.

The HBEC in LHC-9/RPMI-1640 were seeded on Vitrogen-coated coverslips (13 mm×13 mm) in a 6-well culture plate, and were grown to the 60-70% confluence. After 5 days, the culture medium was changed to RPMI-1640/0.01% BSA and the cells were cultured for 1 day. On the day when the experiment was carried out, HBEC on each coverslip were loaded with 2 μM Fura-2/AM, unless otherwise stated, in a Hepes buffer (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 10 mM glucose, pH 7.4) containing 0.01% BSA for 1 hr at 37 °C, and washed twice with the same buffer. The coverslips were inserted into the chamber kept at 37°C and were perfused with the same buffer containing test samples for 30-60 min.

When the effect of rHAT on the HBEC was tested in a Ca^{2+}-free condition, a 10 mM Hepes buffer which contained 1 mM EGTA instead of 1 mM $CaCl_2$ was used instead of the Hepes buffer.

The HAT or PAR-2 AP added to HBEC was prepared in the Hepes buffer, warmed to 37°C and then perfused into the chamber. For desensitization experiments, the solutions were perfused through sequentially, without any separate washes between treatments.

RESULTS

Expression of PAR-2 mRNA in HBEC

Total RNA obtained from the HBEC was subjected to RT-PCR analysis for the detection of mRNA of PAR-1, 2, 3 and 4. In the present experimental conditions using PAR-1, 2, 3, 4 primer, only the expression of PAR-2 mRNA was clearly detectable in the HBEC (Fig. 2 A). This result strongly suggested that primary HBEC contained more PAR-2 than other known PARs at their cell membranes, because the expression of PAR-2 mRNA was the most pronounced in the HBEC compared with mRNA of other known PARs.

Expression of HAT mRNA in various respiratory cells

The existence of HAT in the various respiratory cells was investigated by analyzing the HAT mRNA content with RT-PCR. HAT mRNA was clearly detectable in only native bronchial epithelial cells, namely HBEC and SAEC, while it was not detectable in BEAS-2B (a human bronchial cell line), human alveolar macrophages (HAM), an alveolar cell line (A549) and NHLF, as shown in Fig. 2B. This result is in accordance with the histochemical findings of Takahashi *et al.* (10) that HAT is localized at bronchial epithelial cells.

Stabilization of rHAT

This experiment was carried out using rHAT dissolved in 0.15 M NaCl (pH 7.4) at a concentration of 40 μg/ml. The stability of rHAT was tested at 37°C at a concentration of 0.4 μg/ml in 50 mM MES (pH 6.2), 50 mM Na phosphate buffer (pH 7.4) and 50 mM Tris-HCl buffer (pH 8.6). When rHAT was incubated in the absence of BSA at pH 7.4, its activity declined to 33%, 10% and 5% of the original activity after incubation for 1, 2 and 3 hrs, respectively, and was completely lost after incubation for 24 hrs (Fig. 3). When rHAT was incubated in the presence of 0.01% BSA at pH 7.4, its original activity remained even at 24 and 48 hrs after incubation (Fig. 3). The stabilizing effect of BSA on rHAT was almost the same at concentrations of 0.01%, 0.1% and 1.0%, and also almost the same at any pH tested (data not shown).

Schwarz *et al.* reported that even when mast cell tryptase was incubated at 37°C in 10 mM Tris-HCl buffer (ph 7.4) containing 0.1% BSA and 0.15 M NaCl, its activity rapidly declined to 50% and about 10%

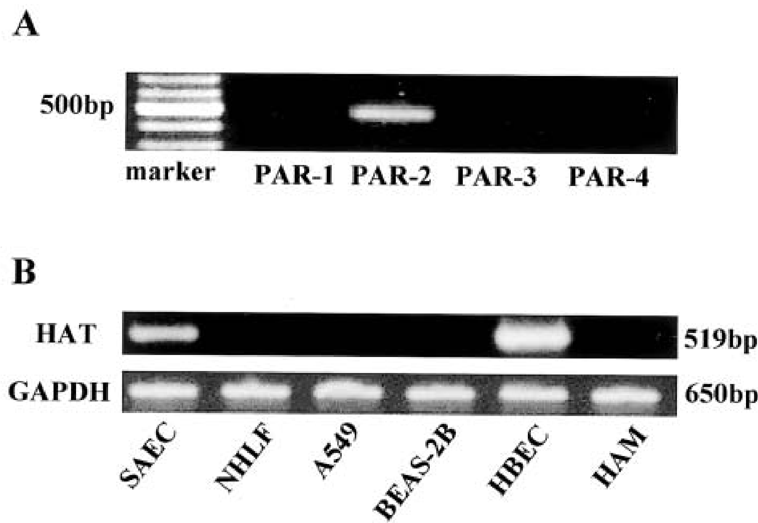

Fig. 2. Analysis of the expression of protease-activated receptor (PAR)-1, 2, 3 and 4 mRNA in the HBEC and of the expression of HAT mRNA in various respiratory cells by RT-PCR.
(A) The HBEC were proliferated in LHC9/RPMI-1640 to 100% confluence in a 60-mm dish. Total RNA was extracted from the HBEC, and subjected to RT-PCR for PAR-1, 2, 3 and 4 as described in Materials and Methods. Briefly, total RNA (1 μg) was subjected to RT reaction, and some of the products (cDNA) were amplified for 27 cycles with each specific primer. The products were electrophoresed on 1.5% agarose gels. molecular markers ; 100 bp step ladder (Promega). (B) Various respiratory cells were proliferated in each growth medium in a 60-mm dish. These cells except BEAS-2B and A549 were in passage 1 (P1) or P2 while BEAS-2B were in P45. Total RNA was extracted from these cells, and subjected to RT-PCR for HAT mRNA as described in Materials and Methods. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control of HAT mRNA.
Abbreviations ; SAEC : normal human small airway epithelial cells, NHLF : normal human lung fibroblasts, A549 : a human pulmonary epithelial cell line, BEAS-2B :a human bronchial epithelial cell line, HBEC : human bronchial epithelial cells, HAM : human alveolar macrophages

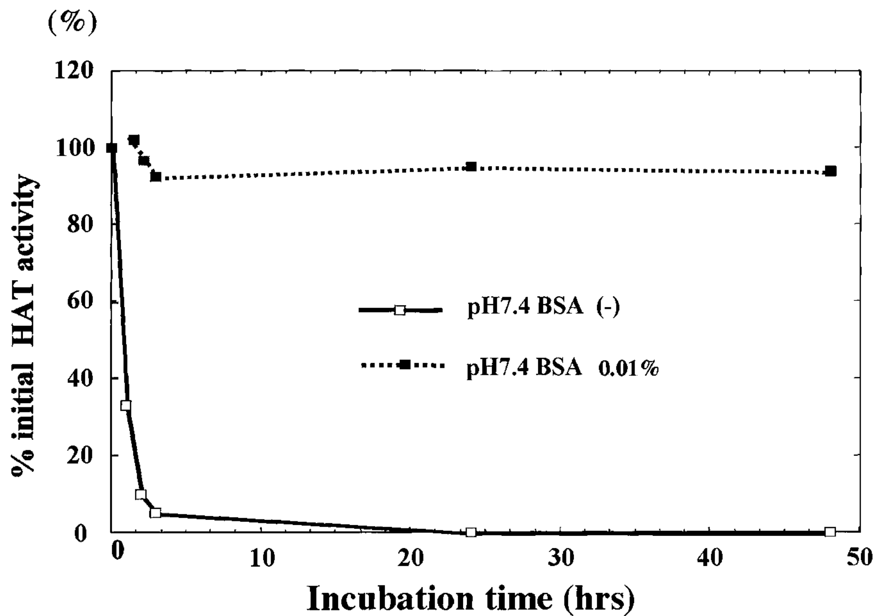

Fig. 3. Stabilization of rHAT by BSA. The rHAT dissolved in 2 ml of 50 mM Na phosphate buffer (pH 7.4) at a concentration of 0.4 μg/ml was incubated at 37°C at the absence or presence of 0.01% of BSA. The trypsin-like activity was measured at the time indicated in the figure. The activity at each time was expressed as % of the original activity. Points show means of 3 experiments.

after incubation for 6-8 min, and 60 min, respectively, and that heparin completely preserved its activity during a 2-hr incubation at 37°C at 50 μg/ml (35). However, when rHAT was incubated at 50 mM MES (pH 6.2), 50 mM Na phosphate buffer (pH 7.4) and 50 mM Tris-HCl buffer (pH 8.6) in the presence of 0.1 to 100 μg/ml of heparin, its activity declined rapidly as it was incubated at the absence of heparin (data not shown). Therefore, we used 0.01% BSA as a stabilizer for rHAT in the following experiments.

Effect of PAR-2 agonist peptide (PAR-2 AP, SLIGKV-NH_2) on intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) in HBEC

The result shown in Fig. 2A indicated that primary HBEC contained more PAR-2 than other known PARs at their cell membranes. Previous investigators reported that when PARs of various kinds of cells are activated by their agonists, serine proteases or agonist peptides, the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_{in}$) in the cells increased via a G-protein-mediated mechanism. They utilized the increase of $[Ca^{2+}]_{in}$ as an indicator of the activation of PARs by PAR agonists (12, 15-19, 26, 28, 29, 36). Therefore, the increase of $[Ca^{2+}]_{in}$ is thought to be useful in estimating whether functional PAR-2 exists in HBEC stimulated with agonist serine protease or PAR-2 agonist peptide.

When HBEC were stimulated with 500 μM PAR-2 AP, two peaks of elevation of $[Ca^{2+}]_{in}$ were found (Fig. 4A) ; the first high, sharp peak (peak I) promptly appeared and rapidly declined within 5 min, and the second low peak (peak II) appeared gradually following the first peak within 5-10 min after the addition of PAR-2 AP, and was sustained during the incubation period of 40 min. When HBEC were stimulated with 100 μM PAR-2 AP, peak I was not detectable while peak II was detectable (Fig. 4B).

In most of the experiments, this PAR-2 AP-induced biphasic increase of $[Ca^{2+}]_{in}$ shown in Fig. 4A was detectable when HBEC were stimulated with 200 μM or over 200 μM of PAR-2 AP. The amplitude of peak I increased in a dose-dependent manner in the range of 200-500 μM of PAR-2 AP. Most previous investigators observed the rapid, sharp $[Ca^{2+}]_{in}$ increase corresponding to peak I when they simulated various kinds of cells with PAR agonist serine proteases or PAR agonist peptides, and considered that this peak is induced via a G-protein-mediated mechanism. These results indicate that our HBEC have functional PAR-2.

In most of the experiments, when HBEC were stimulated with 10-150 μM of PAR-2 AP, peak I was not detectable, while peak II was continuously detectable.

However, the minimum concentration of PAR-2 AP to induce the peak I differed in range of 100-200 μM depending on the HBEC used.

When HBEC were stimulated with 200-500 μM PAR-2 AP in Ca^{2+}-free Hepes buffer, peak I appeared but peak II did not (data not shown).

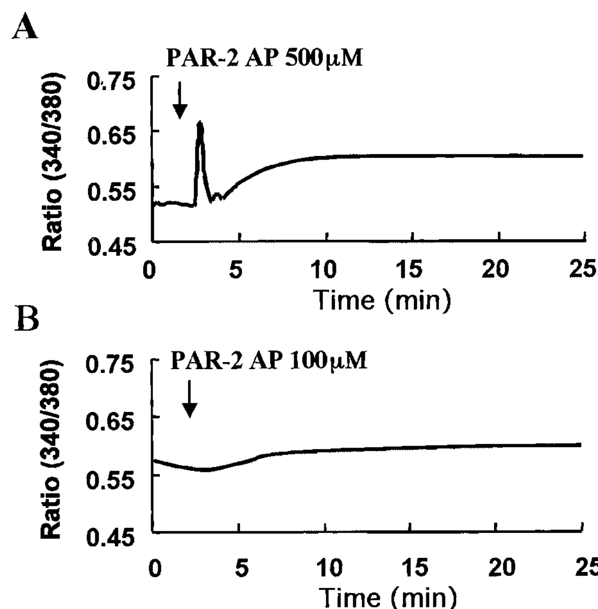

Fig. 4. Effect of human PAR-2 agonist peptide (AP) on the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_{in}$ in the HBEC. After the HBEC had been proliferated on 13×13 mm cover slips in a 6-well plate to 70% confluence in LHC9/RPMI-1640, they were cultured in RPMI/0.01%BSA for 1 day. Then, they were loaded with 2 μM fura-2/AM for 1 hr in 10 mM Hepes buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose (pH 7.4), and stimulated at 37°C with rHAT for 40 min. The $[Ca^{2+}]_{in}$ in the HBEC was evaluated by microfluorometry at an exitation wavelength of 340 nm and 380 nm, and at an emission wavelength of 510 nm, and was expressed as the ratio of fluorointensity at 340 nm to that at 380 nm. (a) 500 μM PAR-2, (b) 100 μM PAR-2. Traces represent results in single cells from 3 experiments.

Effect of HAT on $[Ca^{2+}]_{in}$ in HBEC

In the present microfluorometry assay system of $[Ca^{2+}]_{in}$, no prominent detachment of HBEC was observed within an incubation period of 60 min even when HAT was added at 1,000 mU/ml (approximately 0.4 μM). Therefore, the effect of HAT on $[Ca^{2+}]_{in}$ was tested at a concentration of 1,000 mU/ml or below.

At first, the effect of HAT was examined in Hepes buffer (pH 7.4) containing 1 mM Ca^{2+}. When HBEC were stimulated with 600 mU/ml of HAT, two peaks of elevation of $[Ca^{2+}]_{in}$ were found (Fig. 5A) ; the first high, sharp peak (peak I) appeared and rapidly declined within 5-10 min, and the second low peak (peak II) appeared gradually following the first peak within 5-15 min after the addition of HAT, and was sustained during the incubation period of 40 min. The duration of HAT-induced peak I was rather longer than that of PAR-2 AP-induced peak I

On the other hand, when HBEC were stimulated with 60 mU/ml of HAT, peak I was not detectable while peak II was detectable (Fig. 5C).

The HAT-induced biphasic increase of $[Ca^{2+}]_{in}$

shown in Fig. 5A was detectable when HBEC were stimulated with a rather higher concentration of HAT. The minimum concentration to induce peak I differed in the range of 200-300 mU/ml (0.08-0.12 μM) depending on the HBEC used. The amplitude of peak I increased in a dose-dependent manner in the HAT concentration range of 300-1,000 mU/ml (data not shown).

In the presence of 1-200 mU/ml of HAT, peak I was not detectable, while peak II was continuously detectable.

Next, the effect of HAT was examined in Ca^{2+}-free Hepes buffer. When HBEC were stimulated with 600 mU/ml, peak I appeared but peak II did not (Fig. 5 B). The amplitude of peak I was rather lower in Ca^{2+}-free conditions than in the Ca^{2+}-positive conditions. When HBEC were stimulated with 60 mU/ml of HAT, no prominent increase of $[Ca^{2+}]_{in}$ was detectable (Fig. 5D).

Both the HAT-induced peak I and II were almost completely abolished by heating HAT at 95°C for 5 min.

Desensitization of the Ca^{2+} response to HAT by PAR-2 AP

At first, desensitization experiments were carried out in Hepes buffer containing Ca^{2+}.

HBEC were exposed to 500 μM PAR-2 AP. Then, they were successively exposed to 500 μM PAR-2 and finally to 1 μM bradykinin at 5 min intervals, without an intervening wash. As shown in Fig. 6A, the HBEC showed a lower response to the second PAR-2 AP than to the first PAR-2 AP, while they responded well to a subsequent treatment with bradykinin. Thus, the first PAR-2 AP treatment induced desensitization of the calcium response to the second PAR-2 AP treatment.

After HBEC were exposed to 500 mU/ml HAT, they were successively exposed to 500 mU/ml HAT and finally to 1 μM bradykinin at 5 min intervals. The HBEC showed a lower response to the second HAT than to the first HAT treatment, while they responded well to a subsequent treatment with bradykinin, as shown in Fig. 6 B.

After HBEC were exposed to 500 μM PAR-2 AP, they were successively exposed to 500 mU/ml HAT and 1 μM bradykinin at 5 min intervals. As shown in Fig. 6C, PAR-2 AP induced desensitization of the calcium response to HAT ; most of the cells showed no significant Ca^{2+} response to HAT. Nevertheless, they responded well to a subsequent treatment with 1 μM bradykinin.

The same experiment was carried out in Ca^{2+}-free Hepes buffer (data not shown). In this condition, HAT (500 mU/ml) markedly decreased the calcium response to PAR-2 AP (500 μM). PAR-2 AP (500 μM)

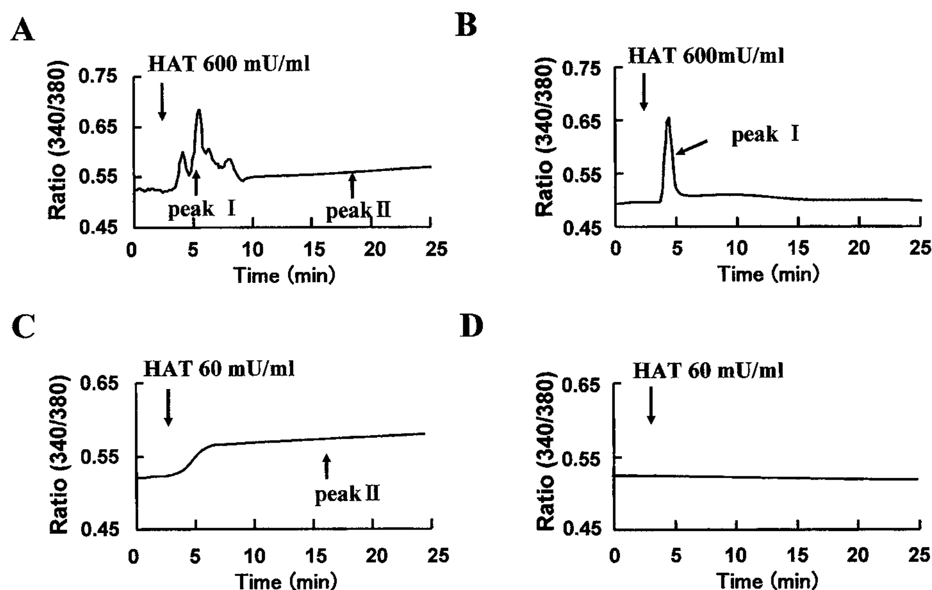

Fig. 5. Effect of rHAT on the $[Ca^{2+}]_{in}$ in the HBEC.
Unless otherwise stated, the experimental procedures were essentially the same as those for Fig. 4. When the effect of rHAT on the $[Ca^{2+}]_{in}$ in the HBEC was tested in a Ca^{2+}-free condition, a 10 mM Hepes buffer which contained 1 mM EGTA instead of 1 mM $CaCl_2$ was used instead of the Hepes buffer. (A) stimulated with 600 mU/ml rHAT in the presence of extracellular Ca^{2+}, (B) stimulated with 600 mU/ml rHAT in the absence of extracellular Ca^{2+}, (C) stimulated with 60 mU/ml rHAT in the presence of extracellular Ca^{2+}, (D) stimulated with 60 mU/ml rHAT in the absence of extracellular Ca^{2+}. Traces represent results in single cells from 3 experiments.

markedly decreased the calcium response to HAT (500 mU/ml) as in the experiment using Hepes buffer containing Ca^{2+}.

Characterization of Ca^{2+} response to HAT in HBEC

To characterize the mechanism of increase of $[Ca^{2+}]_{in}$ in HBEC by HAT, we examined the effects of pertussis toxin, a G-protein-coupled receptor inhibitor, and U73122, a phospholipase C inhibitor, on the HAT-induced $[Ca^{2+}]_{in}$ increases in HBEC.

The HBEC were pre-incubated with 50 μM pertussis toxin for 2 hrs, and then incubated with 600 mU/ml HAT and 50 μM pertussis toxin. The pretreatment of HBEC with pertussis toxin had no significant effect on both 600 mU/ml HAT-induced peak I and peak II (Fig. 7B).

The HBEC were pre-incubated with 4 μM U73122, and then incubated with 600 mU/ml HAT and 4 μM U73122. The U73122 treatment almost completely abolished peak I, but had no significant effect on peak II (Fig. 7C).

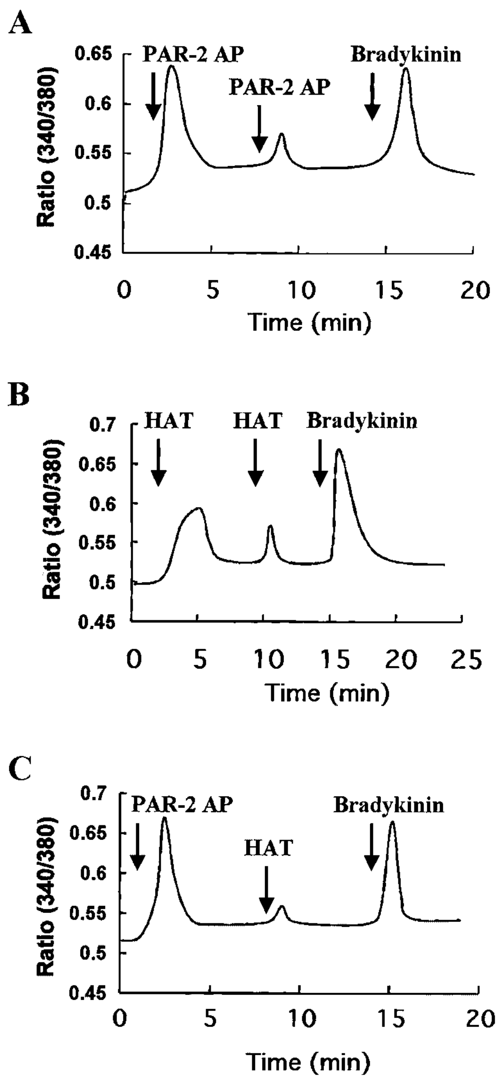

Fig. 6. Desensitization of the Ca^{2+} response to HAT by PAR-2 AP.
HBEC were exposed to the first agonist in the Hepes buffer containing Ca^{2+}. Then they were successively exposed to the second agonist and finally to 1 μM bradykinin at 5 min intervals, without an intervening wash. (A) HBEC were subjected to two consecutive 500 μM PAR-2 AP exposures, and finally exposed to 1 μM bradykinin, (B) HBEC were subjected to two consecutive exposures to 500 mU/ml HAT, and finally exposed to 1 μM bradykinin, (C) HBEC were successively exposed to 500 μM PAR-2 AP, 500 mU/ml HAT, and 1 μM bradykinin. Traces represent results in single cells.

DISCUSSION

In this study, the expression of HAT mRNA was detectable only in primary bronchial epithelial cells such as HBEC and SAEC, but not in BEAS-2B, a transformed bronchial epithelial cell, as well as in alveolar macrophages, lung fibroblasts and an alveolar cell line (A549). This result supported the immunohistochemical findings by Takahashi *et al.* (10) showing that HAT is synthesized in the airway

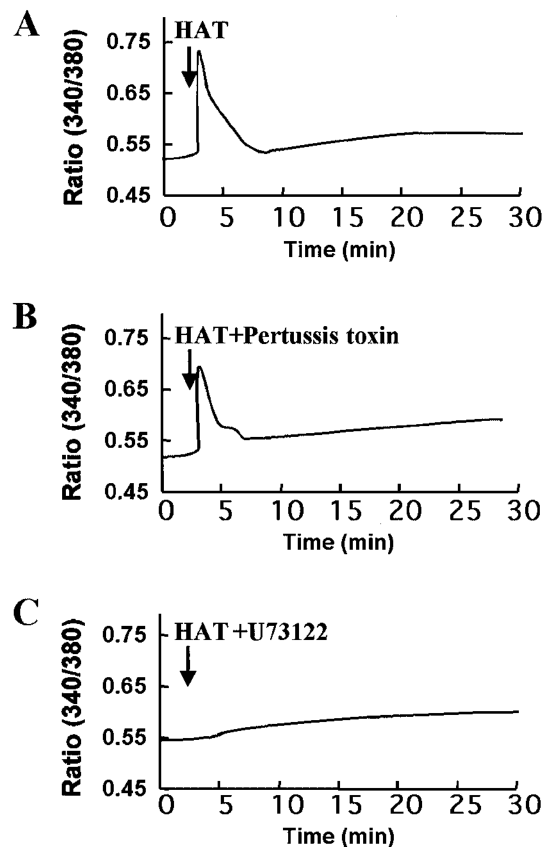

Fig. 7. Effects of pertussis toxin and U73122 on the rHAT-induced $[Ca^{2+}]_{in}$ increase in the HBEC
(A) HBEC were stimulated with 600 mU/ml HAT. (B) HBEC were pre-incubated with 50 μM pertussis toxin for 2 hrs, and then incubated with 600 mU/ml HAT and 50 μM pertussis toxin. (C) HBEC were pre-incubated with 4 μM U73122 for 30 min, and then incubated with 600 mU/ml HAT and 4 μM U73122.

(ciliated) epithelial cells. No expression of HAT mRNA in the BEAS-2B is considered to be due to the fact that transformed bronchial epithelial cells lose various kinds of native properties of bronchial epithelial cells during transformation and repeated culture. RT-PCR analysis of the expression of mRNA of PAR-1, 2, 3 and 4 in the HBEC showed that HBEC contains much more PAR-2 mRNA than mRNA of other known PARs. Ubl *et al.* showed the expression of PAR-2 mRNA in HBEC using RT-PCR (39) and the expression of PAR-2 in bronchial epithelial cells was immunohistochemically shown by previous studies (5-6). The biochemical structure of HAT differs from that of mast cell tryptase (MCT), and the substrate specificity against fluorogenic synthetic substrates of HAT was different from that of MCT (3, 38). However, HAT and MCT showed similar substrate specificities against native proteins. HAT showed limited proteolysis against fibrinogen (3, 39), single-chain urinary-type plasminogen activator (40) and vasoactive peptide (unpublished), in the same way as MCT (41-43). On the other hand, PAR-2 is activated not only by trypsin (16-17) but also by MCT (12, 22, 23). These results strongly suggested that HAT can activate PAR-2 at the airway epithelium.

The PAR-2 agonist peptide (PAR-2 AP) corresponds to a new NH_2-terminus which is produced when PAR-2 is activated by limited proteolysis of its extracellular NH_2-terminus with trypsin, and acts as a tethered ligand (20). The present study showed that human PAR-2 AP over 150-200 μM induced a prompt increase of $[Ca^{2+}]_{in}$, which occurred within 2-5 min, indicating that our HBEC contain a functional PAR-2. We named this prompt increase of $[Ca^{2+}]_{in}$ as peak I.

HAT over 200-300 mU/ml also induced a prompt increase of $[Ca^{2+}]_{in}$ in HBEC. The time course of HAT-induced peak I was very similar to that of PAR-2 AP-induced peak I, although the duration of the former was slightly longer than that of the latter. Both HAT-induced peak I and PAR-2 AP-induced peak I are thought to be caused by Ca^{2+} mobilization from intracellular stores, because they appeared even when HBEC were incubated in Ca^{2+}-free Hepes buffer.

The present study showed that both PAR-2 AP and HAT induced a second low $[Ca^{2+}]_{in}$ peak (peak II), which appeard gradually following peak I. Peak II was induced not only by a high dose of HAT (over 200-300 mU/ml) and a high dose PAR-2 AP (over 150-200 μM), but also by a low dose of HAT (1-200 mU/ml) and a low dose PAR-2 AP (10-150 μM). Both PAR-2 AP-induced and HAT-induced peak II were abolished when the HBEC were incubated in Ca^{2+}-free Hepes buffer. These results strongly suggested that peak II is induced by an influx of extracellular Ca^{2+}.

Previously, Bohm *et al.* (36) reported that trypsin induced biphasic $[Ca^{2+}]_{in}$ elevations in both intestinal epithelial cells and in kidney epithelial cells transfected with cDNA encoding human PAR-2 ; trypsin stimulated a prompt increase in $[Ca^{2+}]_{in}$ due to the mobilization of intracellular Ca^{2+}, followed by a sustained plateau due to the influx of extracellular Ca^{2+}. The former increase and the latter sustained plateau correspond to peak I and peak II in our HBEC, respectively.

Our result showing that the increase of intracellular Ca^{2+} concentration induced by HAT is very similar to that induced by PAR-2 AP in HBEC strongly suggested that HAT may induce the increase of intracellular Ca^{2+} concentration in these cells via the activation of PAR-2. Furthermore, this effect of HAT is thought to be due to its enzymatic action, because it was abolished by heat treatment of HAT.

The desensitization of the Ca^{2+} response to PAR-2 agonist protease (trypsin) or PAR-2 AP with these agonists in the cells which contain PAR-2 was shown by previous investigators ; Bohm *et al.* showed that exposure of the intestinal and kidney epithelial cells to the PAR-2 AP or trypsin results in desensitization of the Ca^{2+} response in these cells (36). Trypsin and PAR-2 AP increased the levels of intracellular calcium in human airway smooth muscle cells, with evident desensitization after treatment with either agonist (12).

In the present study, exposure of the HBEC to 500 μM PAR-2 AP before 500 μM PAR-2 AP or 500 mU/ml HAT treatment almost completely abolished the Ca^{2+} response to the latter agonist treatment, and exposure of the HBEC to 500 mU/ml HAT markedly decreased the Ca^{2+} response to the second HAT treatment. In all experiments, the Ca^{2+} response to bradykinin was constantly detectable. These results indicate that both HAT and PAR-2 AP increase intracellular calcium by acting via the PAR-2 receptor, while bradykinin increases intracellular calcium via a different receptor mechanism, supporting the idea that HAT induces the elevation of $[Ca^{2+}]_{in}$ in HBEC via the activation of PAR-2.

The HAT-induced peak I was not inhibited by 50 μM pertussis toxin but was inhibited by 4 μM U73122, a phospholipase C inhibitor. These results indicated that HAT may trigger Ca^{2+} release from intracellular

Ca^{2+} stores (peak I), probably via the activation of PAR-2 followed by a pertussis toxin-insensitive G-protein-mediated activation of phospholipase C.

On the other hand, the HAT-induced peak II was not inhibited by 50 μM pertussis toxin and 4 μM U73122. These results strongly suggested that peak II is induced by an influx of extracellular Ca^{2+} in a mechanism related to the activation of PAR-2, but different from the pathway which induces peak I, and that HAT and the activation of PAR-2 induce the $[Ca^{2+}]_{in}$ elevation at least via two different mechanisms in the airway epithelial cells. The intracellular free calcium ion is related to transmission of signals from extracellular stimuli to various kinds of cellular functions. The difference in the physiological significance of peak I and II is unknown.

Finally, the results of the present study indicate that HAT may activate PAR-2 in human airway epithelial cells. Recent reports showed that PAR-2 is related to the regulation of various kinds of cellular functions, such as prostaglandin synthesis (5-6), ion transport (29) and cytokine production (37) of airway epithelial cells. We also obtained a result showing that HAT enhances cytokine production in HBEC via activation of PAR-2 (unpublished). These results strongly suggest that HAT may be related to regulation of cellular functions of airway epithelial cells via activation of PAR-2.

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