Tryptase and agonists of PAR-2 induce the proliferation of human airway smooth muscle cells

PATRICK BERGER,1,2 DIAHN-WARNG PERNG,1 HIRAN THABREW,1 STEVEN J. COMPTON,1 JENNIFER A CAINNS,1 ALAN R. MCEUEN,1 ROGER MARTHAN,2 JOSE-MANUEL TUNON DE LARA,2 AND ANDREW F. WALLS1

1Immunopharmacology Group, Southampton General Hospital, Southampton SO16 6YD, United Kingdom; and 2Laboratoire de Physiologie Cellulaire Respiratoire, INSERM E9937, Université Victor Ségalen Bordeaux2, 33076 Bordeaux Cedex, France

Received 20 February 2001; accepted in final form 21 June 2001

AIRWAY REMODELING IN BRONCHIAL asthma has been implicated in the development of poorly reversible airway narrowing and in the persistence of nonspecific bronchial hyperresponsiveness (42). Three-dimensional morphometric studies of asthmatic airways have indicated that hyperplasia of airway smooth muscle cells (SMCs) is a prominent feature of the chronic disease process (16). Airway SMCs are likely to be exposed to a range of mediators or growth factors released from inflammatory cells within the bronchial mucosa, but the key signals for cell proliferation have been little investigated.

An increased degree of mast cell activation has long been recognized in asthmatic airways (32, 39), and the number of mast cells has been recognized to increase within the smooth muscle layer (1). The mast cell can secrete a number of potent mediators, several of which have profound effects on smooth muscle. Mast cell-derived histamine and products of arachidonic acid metabolism have attracted attention for their important roles in mediating bronchoconstriction (17, 41), and histamine is a mitogen for SMC (39). The major product of human mast cell activation, however, is the trypsin-like serine proteinase trypase (EC 3.4.21.59). This enzyme is emerging as a major mediator of allergen inflammation and tissue remodeling (43), and its potential contribution to airway SMC hyperplasia deserves further study.

Asthmatic subjects have appreciably higher levels of trypase in bronchoalveolar lavage fluid than nonasthmatic controls, even during asymptomatic periods (6, 21), and concentrations may be further increased following allergen challenge (46). Relatively few natural substrates have been identified, although trypase can degrade certain regulatory peptides, including vasoactive intestinal peptide and calcitonin gene-related peptide (39, 45), implicating this enzyme in neurogenic inflammation. Tryptase can activate matrix metalloprotease-3 (18) and urokinase plasminogen activator (38), suggesting a contribution in tissue remodeling. Addition of trypase to isolated bronchial tissue from dogs (36) or from spontaneously sensitized (22) or nonsensitized patients (2) can induce hyperresponsiveness to histamine. Consistent with these findings are the results of studies in vivo with a sheep model, in which administration of this enzyme by inhalation also induced bronchial hyperresponsiveness (25), whereas inhibitors of trypase, such as APC-366, reduced allergen-induced early and late-phase bronchoconstriction and hyperresponsiveness (10). A potential role in tissue remodeling has been underscored by the finding that purified human lung trypase can act as a potent

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Tryptase and PAR-2 Activation in Human Airway SMCs

Methods

Purification and characterization of tryptase. Human mast cell tryptase was purified from lung tissue obtained post mortem. The tissue (400–500 g) was finely chopped, homogenized, and first incubated in a low-salt buffer and then subjected to a high-salt extraction procedure, as described previously (44). The supernatant was filtered through a microfiber membrane, dialyzed against distilled water (24 h, 4°C), and subjected to heparin–agarose affinity chromatography equilibrated with a low-salt buffer. Fractions were eluted using a NaCl gradient between 0.4 and 1.5 M in 10 mM MES (Sigma Chemical, Poole, UK) buffer. Tryptase-rich fractions were then subjected to a benzamidine–agarose affinity chromatography equilibrated with a high-salt buffer (2 M NaCl, 10 mM MES). Fractions were eluted with 0.15 M benzamidine and concentrated using an Amicon concentrator with a YM30 membrane that separated tryptase from benzamidine. Filtrates were then applied to a Sephacryl S-300 gel filtration column equilibrated with a high-salt buffer (2 M NaCl, 10 mM MES). Tryptase-rich fractions were again concentrated, passed through a 0.22-μm membrane filter, and stored at −80°C. The purity of the tryptase samples was confirmed by SDS-PAGE with 10% reducing gels. The preparation employed in these studies appeared as a single band on silver staining with a molecular weight of ~34 kDa. The identity of the purified protein was confirmed by immunoblotting with the tryptase-specific monoclonal antibody AAS (44).

Enzyme assay. Tryptase activity was determined using the synthetic peptide substrate N-benzoyl-d-Arg-p-nitroanilide (BAPNA), adding 10 μl of enzyme to 90 μl of 20 mM Tris buffer containing 1 M glycerol and 7.77 mM BAPNA. Absorbance was measured at 410 nm in an ELISA plate reader. Protein concentration was measured spectrophotometrically at 280 nm, using the coefficient of extinction of Smith et al. (37). The specific activity of the tryptase preparation employed was 2.9 U/mg, where 1 unit represents the amount of tryptase required to hydrolyze 1 μmol substrate per minute at 25°C. The enzyme preparation was found to be 100% active by titration with the substrate 4-methylumbelliferyl p-guanidinobenzoate (Sigma Chemical). No chymase or elastase activity was found in these samples, using the chromogenic substrates N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide and N-succinyl-L-Ala-L-Ala-L-Pro-L-Val-p-nitroanilide (both from Sigma Chemical), respectively.

Tissue preparation. Tissues were collected following lung resection for bronchial carcinoma and immediately transferred to the laboratory in DMEM (GIBCO BRL Life Technologies, Paisley, UK). From a macroscopically tumor-free part of the specimen, segments of human bronchus (3rd to 4th division) were carefully dissected under a dissecting microscope. After removal of adhering fat, parenchyma, epithelium, and submucosal tissue, the smooth muscle bands were cut into squares measuring 1–2 mm² and cultured in six-well culture plates in a humidified atmosphere at 37°C with 5% CO₂. Human airway SMCs were maintained in DMEM containing 10% (vol/vol) FCS (GIBCO), supplemented with 2 mM L-glutamine (GIBCO), 1 mM sodium pyruvate (Sigma Chemical), 1% (vol/vol) nonessential amino acid mixture (Sigma Chemical), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphoterin B (antimycotic-antibiotic solution; GIBCO). The medium was changed every 48–72 h. After 4–6 wk, confluent cells were rinsed twice with Hank’s balanced salt solution (GIBCO) and then passaged with trypsin-EDTA (GIBCO). Only cells at passages 2–4 were used for this study.

Immunocytochemistry. To assess purity of the cultured cells, an immunocytochemical method was employed using an indirect immunofluorescence technique. Cells of varying passage number were grown to 70–80% confluence on eight-well chamber slides (GIBCO). Growth was arrested using serum-free DME supplemented with 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg/ml BSA, 4.7 μg/ml linoleic and oleic acid (ITS solution, Sigma Chemical), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% (vol/vol) nonessential amino acid mixture, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphoterin B (antimycotic-antibiotic solution). After 24 h, cells were rinsed twice in PBS (GIBCO) and fixed with cold methanol for 20 min. Nonspecific staining was blocked using PBS containing 3% BSA (GIBCO) for 30 min. Monoclonal antibodies diluted in PBS with 1% BSA including anti-α-smooth muscle actin (1:200, Sigma Chemical), anti-smooth muscle myosin (1:200, Sigma Chemical), anti-cytokeratin 18 (1:500, Sigma Chemical), anti-factor VIII (1:25, Dako), or anti-fibroblast surface protein (1:100, Sigma Chemical) were incubated for 1 h. After cells were rinsed with PBS containing 0.05% Tween 20 (Sigma Chemical), the cells were incubated for 1 h with FITC-conjugated anti-mouse immunoglobulins (Dako), diluted 1:20. Counterstaining was performed using 2 μg/ml propidium iodide (Sigma Chemical). Slides were mounted with a drop of 10% glycerol in PBS and observed under a Diastar fluorescence microscope.

J Appl Physiol • VOL 91 • SEPTEMBER 2001 • www.jap.org
Mitogenesis and cell proliferation assays. Cells were seeded in 96-well microtiter plates at a density of 10^5 cells/ml. When close to confluence (70–80%), cells were rinsed twice with Hanks’ balanced salt solution and growth was arrested using serum-free DMEM (described above). Cells that achieved complete confluence were not used because of the potential for contact inhibition of cell growth and decreased cell viability. After 24 h of serum deprivation, various stimuli were added. Cells were incubated with purified lung tryptase, added in the absence or presence of heparin (in a weight ratio of 1:1 to stabilize enzymatic activity) (34) or with recombinant β II tryptase isolated from a Pichia pastoris expression system (27) (a kind gift from Dr. Mary Haak-Frendscho and Andrew Niles, Promega, Madison, WI). To investigate the dependency on an intact catalytic site, tryptase (with added heparin) was incubated in the presence or absence of specific inhibitors such as leupeptin (100 μM, Sigma Chemical), benzamidine (100 μM, Sigma Chemical), and APC-366 (12) (100 μM, a kind gift from Axys Pharmaceuticals, South San Francisco, CA). The effect of heat treatment was also investigated, by heating tryptase at 56°C for 60 min. The potential involvement of PAR-2 in airway SMC proliferation was investigated using the agonist peptide SLIGKV-NH₂ and, as a control, the reverse peptide VKGILS-NH₂ (both synthesized by MWG-Biotech). Bovine trypsin, a serine protease that cleaves PAR-2, was also tested. FCS (10%) and PDGF (15 ng/ml) acted as positive controls.

In a further series of experiments, the effects of adding various inhibitors of signal transduction pathways were studied. Pertussis toxin (50 ng/ml; Sigma Chemical) was added for 24 h before tryptase challenge. Calphostin C (1 μM) and genistein (100 μM; both from Sigma Chemical) were incubated for 1 h before challenge. In studies of protein kinase C (PKC) activity, the effects of the phorbol ester PKC activators phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate (both from Sigma Chemical) were investigated. After incubation for 12, 24, or 48 h with various stimuli or inhibitors (at 37°C in 95% air and 5% CO₂), 0.5 μCi (methyl-3H)thymidine (Amersham) was added to each well for an additional 8 h. Cells were harvested on a 0.7-m pore glass fiber filter and counted in scintillant on a 2000CA TriCarb liquid scintillation analyzer. To determine cell number, a cell proliferation assay was performed using the di-methylthiazol-carboxymethoxyphenyl-sulfophenyl-tetrazolium inner salt (MTS) and the electron coupling agent phezine ethosulfate according to the manufacturer’s instructions (Promega). In separate experiments, growth-arrested SMCs were challenged for 48 h with tryptase, trypsin, peptide agonist of PAR-2, or other stimuli. To each well, 25 μl of MTS dye solution were added and incubated at 37°C for 1 h. The quantity of formazan product was measured spectrophotometrically at 490 nm and was taken as being directly proportional to the number of living cells in culture. The cell numbers were then calculated using a standard curve constructed using known amounts of cells.

Statistical analysis. Proliferation assays were performed with cells derived from tissue from a minimum of four different patients. Experiments from each patient were conducted in triplicate, and data represented the mean of three wells. The relative degree of [3H]thymidine incorporation was assessed in each group of samples as a percentage of the control with buffer alone. Comparisons between different groups were made by one-way ANOVA; when significant, Student’s t-tests were performed with Bonferroni correction for multiple comparisons. P < 0.05 was taken as significant.

Cell preparations. All cells stained positively for smooth muscle actin and myosin, and there was no apparent variation in staining intensity between cells of different passage numbers. No immunostaining was seen with antibodies specific for cytokeratin, factor VIII, or fibroblast surface protein (data not shown).

Effects of tryptase and trypsin on DNA synthesis and SMC proliferation. Tryptase stimulated a concentration-dependent increase in DNA synthesis in quiescent human airway SMCs (Fig. 1A). In the presence of heparin (added to stabilize tryptase activity), the mi-

Fig. 1. DNA synthesis and proliferation of human airway smooth muscle cells (SMCs) stimulated by tryptase. A: [3H]thymidine incorporation in growth-arrested human airway SMCs incubated for 24 h with various concentrations of purified human lung tryptase in the absence (shaded bars) or presence (solid bars) of heparin (1:1, wt/wt). FCS, in the absence (solid bars) or presence (open bars) of 100 μM leupeptin. Values are means ± SE from 5 separate experiments each performed in triplicate. *P < 0.05 compared with control. B: cell number was assessed by the MTS assay (see text) 48 h after addition of heat-inactivated tryptase (Heat T), 30 mU/ml of purified (T30) or recombinant (r-T30) tryptase, 15 ng/ml platelet-derived growth factor (PDGF), or 10% FCS, in the absence (solid bars) or presence (open bars) of 100 μM leupeptin. Values are means ± SE from 6 separate experiments each performed in triplicate.
The mitogenic effect of purified tryptase was associated with an increase in cell number, as determined by the MTS assay, and SMC proliferation was stimulated to a similar extent with recombinant tryptase at 30 mU/ml (Fig. 1B).

To investigate dependency on an intact catalytic site, tryptase was preincubated with 100 μM of the protease inhibitors leupeptin, benzamidine, or APC-366. These inhibitors reduced the enzymatic activity of tryptase to various extents and abolished the tryptase-induced enhancement in thymidine incorporation at 30 mU/ml (Table 1). For each inhibitor tested, thymidine incorporation in the presence of tryptase was not significantly different from control values without tryptase (one-way ANOVA). Because leupeptin appeared to be the most potent tryptase inhibitor under these conditions, its effect was tested on SMC proliferation induced by tryptase, PDGF, or FCS (Fig. 1B). Leupeptin significantly reduced the tryptase-induced SMC proliferation but did not significantly alter SMC proliferation induced by either PDGF or FCS. In the same way, abolition of tryptase activity by heating tryptase for 1 h at 56°C resulted in the complete loss of tryptase-stimulated DNA synthesis and cell proliferation. Trypsin, another protease that can activate PAR-2, was also able to enhance thymidine incorporation at 24 h in a concentration-dependent manner (Fig. 2). There was a trend for increased SMC proliferation at 48 h, but this did not reach significance (data not shown).

**Effects of peptide agonist of PAR-2 on DNA synthesis and SMC proliferation.** To investigate the potential role of PAR-2 activation in SMC mitogenesis, the agonist peptide SLIGKV was added for 24 h at concentrations ranging from 10^{-6} to 10^{-4} M. SLIGKV at 10^{-4} M significantly enhanced thymidine incorporation compared with that with the same concentration of the reverse peptide VKGILS (Fig. 3A). The enhancement with SLIGKV in thymidine uptake was associated with cell proliferation, as indicated by the MTS assay after 48 h (Fig. 3B).

**Time course of mitogenic responses.** The effect of purified, recombinant, and heat-inactivated tryptase on thymidine uptake was investigated after 12, 24, and 48 h (Fig. 4A). At 24 and 48 h, both purified and recombinant tryptase induced a significant increase in thymidine incorporation, whereas the heat-inactivated tryptase was without effect. The PAR-2 agonist SLIGKV also stimulated increased thymidine incorporation at 24 and 48 h (Fig. 4B). The reverse peptide VKGILS added as a control did not induce mitogenesis.

**Signal transduction mechanisms.** To investigate the signal transduction pathways involved in tryptase-induced DNA synthesis, SMC were preincubated with the various inhibitors listed in Table 2. Pertussis toxin, a G protein inhibitor, inhibited completely the thymidine uptake induced by tryptase. Both the PKC inhibitor calphostin C and the tyrosine kinase inhibitor genistein abolished tryptase-stimulated-DNA synthesis. The phorbol ester PKC activators phorbol 12-myristate 13-acetate at 10 nM and phorbol 12,13-dibutyrate at 100 nM increased thymidine uptake by SMC (945 ± 234% and 1,360 ± 251% of control values, respectively).

**DISCUSSION**

We have demonstrated that human tryptase can provide a potent stimulus for both DNA synthesis and cell proliferation in human airway SMC. Similar re-

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**Table 1. Effect of protease inhibitors on the enzymatic activity of tryptase and on DNA synthesis in human airway SMC, incubated in the presence or absence of 30 mU/ml tryptase**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percentage of Tryptase Inhibition</th>
<th>DNA Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without tryptase</td>
<td>With tryptase</td>
</tr>
<tr>
<td>No inhibitor</td>
<td>100 ± 0.0</td>
<td>267 ± 62.5(^a)</td>
</tr>
<tr>
<td>Leupeptin (100 μM)</td>
<td>98.2 ± 0.3</td>
<td>109 ± 22.5</td>
</tr>
<tr>
<td></td>
<td>109 ± 22.5</td>
<td>78.4 ± 11.3(^†)</td>
</tr>
<tr>
<td>Benzamidine (100 μM)</td>
<td>45.3 ± 4.1</td>
<td>119 ± 36.2</td>
</tr>
<tr>
<td></td>
<td>119 ± 36.2</td>
<td>132 ± 19.9(^†)</td>
</tr>
<tr>
<td>APC-366 (100 μM)</td>
<td>43.9 ± 8.1</td>
<td>91.6 ± 17.7</td>
</tr>
<tr>
<td></td>
<td>91.6 ± 17.7</td>
<td>135 ± 12.8(^†)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of experiments. Results are expressed as the percent inhibition of tryptase activity toward the substrate BAPNA (see text) and as the relative degree of [3H]thymidine incorporation (as a percentage of the control with no inhibitor and no tryptase added) after 24 h. Tryptase was preincubated for 1 h in ice with 100 μM of each protease inhibitor. SMC, smooth muscle cell. \(^a\)P < 0.05 compared with control without tryptase; \(^†\)P < 0.05 compared with tryptase without inhibitor.
responses were evoked with trypsin and the peptide agonist of PAR-2, SLIGKV, consistent with the idea that tryptase may be acting through this receptor. Our studies also indicate some of the signal transduction mechanisms that could be involved in tryptase-induced proliferation of SMC.

Purified lung tryptase at a concentration of 30 mU/ml increased by more than twofold the degree of [3H]thymidine incorporation in growth-arrested human airway SMCs incubated for 24 h with various concentrations of the agonist SLIGKV (solid bars) or the reverse peptide control VKGILS (open bars). Values, expressed as a percentage of the control (hatched bar), are means ± SE from 6 separate experiments each performed in triplicate. B: cell number was assessed by the MTS assay after 48-h challenge with SLIGKV (black bar) or VKGILS (white bar). Values are means ± SE from 5 separate experiments each performed in triplicate. *P < 0.05 compared with control.

![Fig. 3. DNA synthesis and proliferation of human airway SMC stimulated with proteinase-activated receptor-2 (PAR-2) agonist peptide. A: [3H]thymidine incorporation was measured in growth-arrested human airway SMCs incubated for 24 h with various concentrations of the agonist SLIGKV (solid bars) or the reverse peptide control VKGILS (open bars). Values, expressed as a percentage of the control (hatched bar), are means ± SE from 6 separate experiments each performed in triplicate. B: cell number was assessed by the MTS assay after 48-h challenge with SLIGKV (black bar) or VKGILS (white bar). Values are means ± SE from 5 separate experiments each performed in triplicate. *P < 0.05 compared with control.](image)

Fig. 4. Time course of tryptase and PAR-2-mediated DNA synthesis in human airway SMCs. [3H]thymidine incorporation was measured in growth-arrested human airway SMCs incubated for 12 (n = 4), 24 (n = 6), or 48 h (n = 5) with heat-inactivated tryptase (○) or 30 mU/ml of purified (●) or recombinant (▲) human tryptase (A) or with the synthetic peptides VKGILS (□) or SLIGKV (■) (B). Values are means ± SE from separate experiments each performed in triplicate. *P < 0.05 compared with control cells at each time point.

![Fig. 4. Time course of tryptase and PAR-2-mediated DNA synthesis in human airway SMCs.](image)
TRYPTASE AND PAR-2 ACTIVATION IN HUMAN AIRWAY SMCs

Table 2. Signal transduction mechanisms involved in human airway SMC mitogenesis induced by tryptase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>n Without Tryptase</th>
<th>With Tryptase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Pertussis toxin (50 ng/ml for 24 h)</td>
<td>6</td>
<td>97.2 ± 7.3</td>
</tr>
<tr>
<td>Calpain C (1 μM for 1 h)</td>
<td>6</td>
<td>133.0 ± 27.3</td>
</tr>
<tr>
<td>Genistein (100 μM for 1 h)</td>
<td>6</td>
<td>161.0 ± 27.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of experiments. Results are expressed as the degree of [3H]thymidine incorporation (as a percentage of control with no inhibitor and no tryptase added). Cells were preincubated with inhibitors of protein G (pertussis toxin), protein kinase C (calpain C), and tyrosine kinases (genistein) and then challenged with 30 mU/ml tryptase for 24 h. *P < 0.05 compared with control without tryptase; †P < 0.05 compared with tryptase without inhibitor.

our studies strongly suggest that tryptase-induced SMC proliferation is mediated through the activation of PAR-2. Tryptase, which like tryptase (26) is able to activate this receptor (28), was found to stimulate an increase in thymidine incorporation in a manner very similar to that elicited with tryptase. The maximal response was of a similar order for both proteases. Moreover, an increase in cell number was induced, and once again the response was inhibited by adding the enzyme in the presence of the protease inhibitor leupeptin. Both tryptase and trypsin can cleave the extra-

or on cells or tissue structures, thus rendering it enzymatically more stable (34).

The actions of tryptase appeared to be dependent on an intact catalytic site. Tryptase-induced DNA synthesis and SMC proliferation were inhibited by the synthetic tryptase inhibitor APC-366 and also by leupeptin and benzamidine, both of which have been shown previously to inhibit tryptase-induced proliferation of fibroblasts (9, 33) and epithelial cells (8). In the present study, leupeptin reduced thymidine incorporation by 113%, benzamidine by 81%, and APC-366 by 79%. The inactivation of the enzymatic activity by heating also abolished the ability of tryptase to stimulate DNA synthesis and SMC proliferation.

Because purified tryptase is enzymatically unstable in physiological solutions but may be stabilized by heparin in a 1:1 ratio (35), the actions of tryptase on SMC were compared in the presence or absence of heparin. The addition of heparin did indeed enhance the mitogenic effect of tryptase on SMC, consistently increasing the degree of thymidine incorporation. There was a bell-shaped curve in both cases, but the maximal thymidine incorporation was achieved at a lower concentration of tryptase when heparin was absent. We found that heparin was able to inhibit by about one-third the degree of thymidine incorporation in unstimulated cells, in accord with previous investigations of the effect of heparin on SMC mitogenesis (23). It seems likely that in the present studies heparin will have had a dual effect, enhancing the ability of tryptase to stimulate cell proliferation by stabilizing this enzyme but also interacting with SMC to inhibit cell proliferation.

In conclusion, we have found that mast cell tryptase can provide a potent stimulus for DNA synthesis and the proliferation of human airway SMCs in culture. The actions of tryptase were dependent on an intact catalytic site, and the similarities in findings with tryptase and other agonists of PAR-2 suggest that the activation of this receptor could be central to an understanding of the mechanism of tryptase-induced SMC mitogenesis. Other potential actions of tryptase and other PAR-2 agonists on airway SMCs deserve investigation, but the ability to contribute to airway remod-
eling could be particularly important in asthma, a condition associated with hyperplasia of airway SMC (16) and increased secretion of tryptase in the airways (6, 21, 46). Tryptase has been demonstrated to possess a range of other inflammatory actions, acting as a stimulus for the degranulation of mast cells and eosinophils, for granulocyte accumulation, and for cytokine release from various cell types (reviewed in Ref. 43). A report that activation of PAR-2 can provide prostaglandin E₂-mediated bronchoprotection in the airways (11) contrasts with the observation that tryptase can induce hyperresponsiveness in vitro (2) and indicates that tryptase may be involved in a complex interplay of processes in vivo. Nevertheless, tryptase has the potential to be a key mediator of disease in bronchial asthma, and it is a promising target for therapeutic intervention.

Financial support is gratefully acknowledged from the Société Pneumologique de Langue Française, Banque Nationale de Paris, Centre Hospitalier Universitaire de Bordeaux, France, and the National Asthma Campaign, UK.

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