Tryptase's potent mitogenic effects in human airway smooth muscle cells are via nonproteolytic actions

JAMES K. BROWN,^{1,2} CARY A. JONES,¹ LEEANN A. ROONEY,¹ GEORGE H. CAUGHEY,² AND IAN P. HALL³

¹Pulmonary and Critical Care Medicine Section, Medical Service, Department of Veterans Affairs Medical Center, San Francisco 94121; ²Cardiovascular Research Institute and Department of Medicine, University of California, San Francisco, CA 94143-0911; and ³Division of Therapeutics, University Hospital, Nottingham NG7 2UH, United Kingdom

Received 27 February 2001; accepted in final form 25 September 2001

Brown, James K., Cary A. Jones, Leeann A. Rooney, George H. Caughey, and Ian P. Hall. Tryptase's potent mitogenic effects in human airway smooth muscle cells are via nonproteolytic actions. Am J Physiol Lung Cell Mol Physiol 282: L197-L206, 2002.-We reported previously that mast cell tryptase is a growth factor for dog tracheal smooth muscle cells. The goals of our current experiments were to determine if tryptase also is mitogenic in cultured human airway smooth muscle cells, to compare its strength as a growth factor with that of other mitogenic serine proteases, and to determine whether its proteolytic actions are required for mitogenesis. Highly purified preparations of human lung β -tryptase (1–30) nM) caused dose-dependent increases in DNA synthesis in human airway smooth muscle cells. Maximum tryptase-induced increases in DNA synthesis far exceeded those occurring in response to coagulation cascade proteases, such as thrombin, factor Xa, or factor XII, or to other mast cell proteases, such as chymase or mastin. Irreversibly abolishing tryptase's catalytic activity did not alter its effects on increases in DNA synthesis. We conclude that β -tryptase is a potent mitogenic serine protease in cultured human airway smooth muscle cells. However, its growth stimulatory effects in these cells occur predominantly via nonproteolytic actions.

mast cell proteases; serine proteases; airway smooth muscle hyperplasia; noncatalytic effects of proteases

AIRWAY SMOOTH MUSCLE HYPERPLASIA (30) is one of several relatively irreversible structural changes that occur in the airways of asthmatic patients. Collectively, these structural changes often are referred to as airway remodeling (24). Thickening of the airway wall, resulting in part from hyperplasia of airway smooth muscle cells, has potential pathophysiological importance for several reasons. It may give rise to exaggerated decreases in airway caliber when the smooth muscle shortens by a given amount (31). Also, it may contribute to the severe, irreversible airflow obstruction that sometimes occurs in patients with refractory asthma (55). Tryptases are trypsin-like neutral serine-class proteases that are selectively expressed in mast cells

and basophils (14). Tryptase is released in airways during allergen challenge in atopic subjects (56) and is present in increased concentrations in induced sputum samples obtained from asthmatic patients (25). We previously demonstrated that tryptase is an extremely potent growth factor for cultured dog airway smooth muscle cells (10). The mitogenic potential of tryptase in human cells has been established, including in human lung and dermal fibroblasts (1, 28), dermal microvascular endothelial cells (7), and H292 lung mucoepidermoid cells (12). However, it is not known whether tryptase stimulates growth in human airway smooth muscle cells. If it does, repeated episodes of mast cell degranulation, release of tryptase extracellularly, and activation of smooth muscle growth could be important events leading to thickening of the smooth muscle layer as part of airway remodeling.

In addition to tryptase, a number of other serine proteases have the capacity to stimulate cellular growth. Trypsin has mitogenic actions in some preparations of cultured vascular smooth muscle cells (9). Chymase, like tryptase, a mast cell-associated serine protease, induces potent proliferative effects in cultured myocardial cells (27) and in dermal fibroblasts (3). A number of serine proteases in the coagulation cascade are potent growth factors in the vasculature. These include thrombin (37), factor Xa (32, 39), and factor XII (26). Thrombin also stimulates mitogenesis in human airway smooth muscle cells (41), a finding of potential importance because of likely increases in airway vascular permeability that occur in asthma (48). Indeed, the fact that growth stimulation in a given cell type may occur in response to many different serine proteases raises the possibility that these growth regulatory effects of serine proteases occur via a common, relatively nonspecific effect. Thus an important issue is to determine whether mitogenic effects of tryptase in airway smooth muscle cells occur via a mechanism that is specific to tryptase vs. via mechanisms that are shared with many other mitogenic

Address for reprint requests and other correspondence: J. K. Brown, Pulmonary and Critical Care Medicine Section (111-D), Dept. of Veterans Affairs Medical Center, 4150 Clement St., San Francisco, CA 94121 (E-mail: jkbrown@itsa.ucsf.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

serine proteases. A related need is to compare the magnitude of tryptase's growth stimulatory capacity in airway smooth muscle cells with those of other mitogenic serine proteases. To the extent that several different serine proteases induce growth responses in airway smooth muscle cells of approximately the same magnitude, the findings would support the likelihood of nonspecific, shared mitogenic mechanisms. By contrast, if growth stimulatory effects of tryptase far exceeded those of other mitogenic serine proteases in airway smooth muscle cells, the findings would suggest that tryptase may activate the cells to grow via a relatively unique mechanism.

Regarding specific mechanisms through which tryptase may activate cells, much recent emphasis has been placed on its capacity to activate protease-activated receptor-2, one member of the relatively new class of G protein-coupled, protease-activated receptors (PARs; see Ref. 21). As a family, PARs share a unique mechanism of activation. A specific protease cleaves the receptor's NH₂-terminal extracellular domain. This cleavage unmasks a new NH₂ terminus that then serves as a "tethered ligand," binding intramolecularly to the body of the receptor and effecting transmembrane signaling (21). Four different PARs now have been identified (PARs 1-4). PAR-1, -3, and -4 are receptors for thrombin (21). PAR-2 is activated by trypsin and trypsin-like proteases, including tryptase, and by tissue factor and factor Xa (13). Tryptase's ability to activate PAR-2 has been documented by demonstrating its ability to cleave peptides corresponding to the NH_2 -terminal portion of PAR-2 (36) and to stimulate PAR-2-associated phosphoinositol hydrolysis and increases in intracellular calcium (20, 36). Immunolocalization studies in respiratory tissues have identified PAR-2 expression in human airway smooth muscle (23), and expression of functional PAR-2 has been established to occur in cultured human airway smooth muscle cells (5, 29).

It is important to note, however, that some proteases are capable of activating cells via nonproteolytic mechanisms. For example, thrombin binds to glycoprotein GP Ib α on the surface of platelets (2). The binding is of high affinity (2), clearly involves a site in the thrombin molecule different from its catalytic domain (45), and does not require a proteolytic event (45). Binding of thrombin to GP Ib α may initiate transmembrane signaling itself or may merely serve as a necessary preliminary event before thrombin-induced cleavage of PARs or other surface proteins (21). There is evidence that thrombin can induce growth in endothelial cells via both proteolytic and nonproteolytic events (4), findings that suggest the possibility that thrombin's binding to GP Ib α may, in itself, be sufficient to induce mitogenesis in these cells. For tryptase, the relative importance of proteolytic vs. nonproteolytic mechanisms for mediating its mitogenic effects have not been evaluated extensively. However, in some prior studies, although protease inhibitors clearly attenuated tryptase's growth stimulatory effects, there was substantial residual mitogenic activity, even in the presence of potent and irreversible protease inhibitors (10, 44). In one other study, on the other hand, protease inhibitors abolished tryptase's mitogenic effects completely in human lung fibroblast cells (1).

Our study had the following three goals: 1) to determine whether tryptase is a mitogen for cultured human airway smooth muscle cells; 2) to compare its relative strength as a mitogen compared with other mitogenic serine proteases and with classical growth factor receptor tyrosine kinases; and 3) to determine the importance of proteolytic vs. nonproteolytic mechanisms for tryptase's growth stimulatory effects in these cells.

MATERIALS AND METHODS

Materials. Porcine heparin (4-6 kDa), N-p-tosyl-Gly-Pro-Lys *p*-nitroanilide (GPK), *p*-amidino phenylmethanesulfonyl fluoride (p-APMSF), trypsin, Triton X-100, diisopropylfluorophosphate (DFP), and 3,4-dichloroisocoumarin (DCI) were purchased from Sigma Chemical (St. Louis, MO). Other reagents and their sources were plasminogen-free bovine thrombin, human plasma coagulation factors Xa and XII (Calbiochem, La Jolla, CA), serum-free medium (cellgro COMPLETE; Mediatech, Herndon, VA), recombinant human platelet-derived growth factor-BB homodimer (PDGF-BB), recombinant human insulin-like growth factor I (IGF-I), recombinant human basic fibroblast growth factor (bFGF), recombinant human epidermal growth factor (EGF), Moloney murine leukemia virus reverse transcriptase (MMLV RT), and Thermus aquaticus (Taq) polymerase (Life Technologies, Gaithersburg, MD). Ultraspec II was from Biotecx (Houston, TX), and succinyl-Phe-Pro-Phe-p-nitroanilide was from Bachem (Torrance, CA). Oligonucleotide primers employed in PCR reactions were synthesized by the Biomolecular Resource Center (University of California-San Francisco).

Cell culture. Primary cultures of dog tracheal smooth muscle cells were established and maintained as previously described (52). Cells were fed on alternate days and were passaged enzymatically when they approached confluence. Human cells were isolated by culture of explants of trachealis muscle obtained from individuals without respiratory disease within 12 h of death, as described previously (6). Cells isolated from three different individuals were employed in these experiments. Human cells were maintained in Ham's F-12 medium containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS. They were maintained by feeding on alternate days and were enzymatically passaged near confluence. A549 cells were obtained from American Type Culture Collection (Manassas, VA).

Tryptase and other mast cell proteases. Tryptase was isolated from human lung tissue, as described previously (28). Purity of the tryptase in these isolates has been established using chromatographic, electrophoretic, and immunologic criteria (28). This preparation is predominantly β -tryptase (personal communication, Dr. L. B. Schwartz, Medical College of Virginia, Richmond, VA). In a few experiments, we also employed tryptase from other sources for purposes of comparison. These were human lung tryptase from a commercial source (Calbiochem), from human mast cell leukemia cells (HMC-1; see Ref. 11), and from dog mastocytoma cells (18). Dog mast cell α -chymase and a novel tryptase-like serine protease known as dog mast cell protease-3 (dMCP-3; see Ref. 57) or mastin (16) also were isolated as described previously (57).

Assay of serine protease activity. Catalytic activity of tryptase was measured by assessing its ability to cleave GPK (47). Tryptase was added to 50 mM Tris buffer, pH 7.7, containing 120 mM NaCl, 20 µg/ml heparin, and 100 µM GPK (final volume: 100 µl). Rates of GPK hydrolysis were determined at room temperature for 3 min by following the change in absorbance at 405 nm using a Beckman spectrophotometer. The concentration of tryptase was calculated from the amount of cleaved substrate (molar extinction coefficient = 8,800), assuming a molecular weight of 140,000 for tetrameric tryptase (42). Catalytic activities of dMCP-3 (or mastin) and thrombin also were confirmed using this assay. Proteolytic activity of chymase was confirmed by monitoring its cleavage (at 405 nm and 37°C) of 80 µM succinyl-Phe-Pro-Phe-p-nitroanilide in 30 mM Tris buffer containing 1 M NaCl, pH 8.0 (17).

Tryptase catalytic inhibition. To assess proteolytic vs. nonproteolytic effects, we explored available techniques potentially suitable for irreversibly inhibiting tryptase's catalytic activity. We reasoned that reversible inhibitors could be inadequate for our purposes because of their possible degradation during the 48 h required to assess changes in DNA synthesis. We considered the following three different irreversible serine protease inhibitors: DFP, DCI (43), and p-APMSF (33). A disadvantage of DFP is its toxicity. DCI (100 µM) effectively inhibited catalytic activity but caused significant cytotoxicity in our cellular preparations. p-APMSF proved to be an ideal irreversible inhibitor. This compound is a specific, irreversible, noncytotoxic inhibitor of the class of serine proteases that cleaves substrates after lysine or arginine (33), and tryptase falls into this class (46). In our experiments, tryptase (10.5–27 nM) was incubated with p-APMSF (10 µM) for 100–120 min at 4°C in 10 mM BisTris buffer, pH 6.1. As a control, tryptase was incubated with p-APMSF diluent alone (1:1 acetonitrile-dimethylformamide). Incubates then were diluted 1:5 in serum-free cellgro COMPLETE media, pH 7.4, and placed on ice for 3-5 h before application to cells in 96-well plates. To test for its possible cytotoxic effects, p-APMSF that had been incubated alone was added to wells containing either serum-free media or PDGF-BB, and the effects on DNA synthesis (see below) were determined. Before application on cells, p-APMSFtreated tryptase was assayed for completeness of catalytic inhibition using the GPK assay, as described above. In some experiments, to test for irreversibility of the catalytic inhibition, we removed aliquots of media overlying cells that contained p-APMSF-treated tryptase after 24 h and confirmed that it still was unable to cleave GPK. In other experiments, trypsin (8.9–125.1 nM) was incubated with p-APMSF in an identical manner to test for mitogenic effects of p-APMSFtreated trypsin.

DNA synthesis. To quantify DNA synthesis in the cells, we measured the incorporation of bromodeoxyuridine (BrDU) into cellular DNA using an enzyme-linked immunosorbent assay (ELISA), as we described previously (10). For studies in human cells, passage 5–9 cells were seeded at a density of 10,000 cells/well in 96-well format. After 24 h in F-12 media containing 10% FCS, the cells in each well were washed one time with PBS and then starved for 24 h in 100 μ l of serum-free F-12 media before the addition of mitogens. BrDU was introduced 24–48 h after the addition of mitogens. For dog cells, passages 1–5 were used for quantifying DNA synthesis and were starved in cellgro COMPLETE serum-free media. Mitogens were introduced in the cultures, and the BrDU (10 μ M) was added 24–48 h later.

Cell counts. Human airway smooth muscle cells were seeded at a density of 10,000 cells/well in 96-well format.

After 24 h starvation in serum-free F-12 media, mitogens or media alone were added to cells at day 0. Counts were performed 48 and 96 h later ("days 2 and 4") from triplicate wells using a hemocytometer after enzymatic detachment of cells.

PAR-2 expression in human airway smooth muscle cells. Previous investigators have demonstrated that PAR-2 is expressed in cultured human airway smooth muscle cells (29). To confirm that the cells employed in our study also express this receptor, we used PCR. RNA was isolated from five different preparations of human airway smooth cells, at a confluent or near-confluent stage, using Ultraspec II. As positive and negative controls, we also isolated RNA from A549 cells and human platelets, which express PAR-2 in abundance and not at all, respectively (8). Single-stranded cDNA was synthesized from the RNA using MMLV RT. Each reaction was carried out in 20 µl containing 2 µg RNA as template, 0.6 mM concentrations of each dNTP, random hexamers (50 ng/reaction), RNasin (20 U/reaction), 50 nM Tris (pH 8.3), 3 mM MgCl₂, 75 mM KCl, and 10 mM dithiothreitol. Reverse transcription was carried out using 200 units of MMLV RT per reaction at room temperature for 10 min followed by 42°C for 45 min and then heat inactivation for 5 min at 95°C.

For PCR, oligonucleotide primers used for amplification of PAR-2 gene fragments were as follows: forward primer (complementary to nucleotides 159-179 in exon 1), 5'-CAGCGCG-GCGTGGCTGCTGGG; reverse primer (complementary to nucleotides 446-466 in exon 2), 5'-AAGACCCACAGGGC-CATGCCG (8). The expected size of the amplified product was 287 bp (8). To test for RNA integrity and efficiency of the RT reactions in each sample, PCR also was carried out with primers for identification of β -actin. The primers used were identical to those employed previously (38), as follows: forward primer (complementary to nucleotides 3119-3136), 5'-CCGCAAATGCTTCTAGGC; reverse primer (complementary to nucleotides 3774-3754 on exon 6 of the rat β -actin gene) 5'-GGTCTCACGTCAGTGTACAGG (40). The expected product size for this reaction was 656 bp. For each reaction, 15% of the transcribed cDNA was added to a reaction mixture containing 10 mM Tris (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM concentrations of each dNTP, 0.2-0.6 µM of each primer, and 2.5 units Taq DNA polymerase. Reaction mixes were heated to 94°C for 5 min before addition of Taq polymerase to completely denature the template. Reaction mixes were overlaid with light mineral oil and incubated in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) followed by 30 cycles of amplification as follows: denaturing for 100 min at 95°C, annealing for 70 min at 57°C, and extending for 100 min at 72°C. PCR-generated DNA bands were identified by size after electrophoresis on a 2% (wt/vol) agarose gel (in 40 mM Tris and 1 mM EDTA) that was run at 10 V/cm for 1 h. Gels were stained with ethidium bromide (0.01 µg/ml) and photographed under ultraviolet illumination. In some of the reactions with the PAR-2 primers, DNA from a full-length (6.9 kb) human PAR-2 clone (generously provided by Dr. Nigel W. Bunnett, University of California-San Francisco) was used as a template.

RESULTS

Tryptase is a mitogen for human airway smooth muscle cells. Human lung tryptase in nanomolar concentrations effectively stimulated BrDU incorporation into newly synthesized DNA (Fig. 1). The mitogenic effects of tryptase were concentration dependent, and maximum stimulation occurred at concentrations of

20–30 nM. Concentrations of tryptase >20 nM elicited 33–46% of the BrDU uptake occurring in the presence of 10% FCS (data not shown). Exposure to tryptase for 4 days produced significant increases in smooth muscle cell numbers compared with numbers when cells were maintained for the same time period under basal, serum-free conditions (Fig. 2). Tryptase purified from HMC-1 mastocytoma cells (11) and from dog mastocytoma cells (18) also promoted BrDU incorporation in human cells (data not shown).

Tryptase is an extremely strong mitogen for human airway smooth muscle cells when its maximum effects on increased DNA synthesis are compared with those of peptide growth factors and other mitogenic serine proteases. In cultured human airway smooth muscle, maximum increases in BrDU incorporation induced by large concentrations of tryptase were comparable to those induced by large concentrations of PDGF-BB and bFGF, and the responses to tryptase were substantially greater than those induced by large concentrations of EGF (Fig. 3A). IGF-I did not induce detectable increases in BrDU incorporation at any concentration in the human cells (Fig. 3A). Compared with the maximum effects of tryptase, increases in BrDU incorporation in response to the other mitogenic serine proteases tested (thrombin, factor Xa, and factor XII) were relatively small.



Fig. 1. Concentration-dependent effects of tryptase on DNA synthesis in human airway smooth muscle cells. A range of concentrations of tryptase (0.8–60 nM) was applied to cells, and DNA synthesis was quantitated by bromodeoxyuridine (BrDU) enzyme-linked immunosorbent assay (ELISA), as described in MATERIALS AND METHODS. BrDU incorporation is expressed as a percentage of the response to the highest concentration of tryptase ([tryptase]) applied to cells. Each point is a mean value from 4 separate experiments, each performed in duplicate, over the range of tryptase concentrations shown. The concentration of tryptase that elicited half-maximal stimulation was 4.9 nM, as determined using SigmaPlot (Jandel, San Rafael, CA).



Fig. 2. Tryptase-induced proliferation of human airway smooth muscle cells. Compared proliferative responses of human airway smooth muscle cells to human lung tryptase (5 nM), recombinant human platelet-derived growth factor (PDGF)-BB (100 ng/ml), and 10% FCS at *days 2* and 4 after addition of mitogens. Cell counts on *day 2* and *day 4* are expressed as the degree of increases in cell number above those that occurred in the presence of serum-free media alone. Results are means \pm SD of triplicate determinations in one experiment. *Day 4* values were significantly greater than those occurring in response to serum-free medium alone for tryptase, PDGF, and 10% FCS (P < 0.05, paired *t*-test).

We previously have demonstrated that tryptase is a mitogen for airway smooth muscle cells from one other species, dogs (10). Therefore, during the course of these experiments, we used dog tracheal smooth muscle cells and similar techniques to compare maximal growth stimulatory effects of tryptase with those of other mitogens in cultured canine cells. The human lung tryptase employed in these experiments induced large increases in BrDU incorporation, once again similar in magnitude to those induced by PDGF-BB (Fig. 3B). Unlike in human airway smooth muscle cells (Fig. 3A), in the dog cells we found that IGF-I also was a strong mitogen (Fig. 3B). Although thrombin clearly induced increases in BrDU incorporation in dog cells, the responses to tryptase were approximately sixfold greater (Fig. 3B). Dog mastin, a serine protease closely related to tryptase, and chymase, a serine protease with chymotrypsin-like specificity, were not mitogenic in these cells. Together, these results suggest that tryptase is a strong mitogen for both human and dog airway smooth muscle cells.

It has been reported previously that trypsin has the potential to induce mitogenic effects via its properties as a serine protease (9), perhaps via its ability to cleave and activate insulin receptors (49). Therefore, during the course of these experiments, we were interested in testing for trypsin's potency as a mitogen for cultured airway smooth muscle cells and to

AJP-Lung Cell Mol Physiol • VOL 282 • FEBRUARY 2002 • www.ajplung.org



compare it with tryptase's potency. Because of the structural similarity of receptors for insulin and IGF-I (53), we employed IGF-I, rather than PDGF, as the peptide growth factor for these experiments and therefore employed dog, rather than human, airway smooth muscle cells because IGF-I did not increase BrDU incorporation in the human cells (Fig. 3A). Trypsin alone, in concentrations of 1-10 nM, did induce small but detectable increases in BrDU incorporation (Fig. 4). Higher concentrations of trypsin invariably caused the cells to detach from their supporting matrix, an effect that we never observed with tryptase over the range of concentrations employed in these experiments. Therefore, we were not able to examine full concentration-response relationships between trypsin and mitogenesis. Previous reports

Fig. 3. Maximum increases in DNA synthesis in human (A) and dog (B) airway smooth muscle cells in response to serine proteases and peptide growth factors. Cellular DNA synthesis was measured using the BrDU ELISA after exposure of human airway smooth muscle cells to large concentrations of either human lung tryptase (20–47 nM, n = 5), bovine thrombin (10 U/ml, n = 6), human factor Xa (50 nM, n = 3), human factor XII (6 μ g/ml, n = 3), human epidermal growth factor (EGF; 100 ng/ml, n = 8), human PDGF-BB (100 ng/ml, n = 8), human basic fibroblast growth factor (bFGF; 100 ng/ml, n = 8), or human insulin-like growth factor I (IGF-I; 100 ng/ml, n = 3). Responses in dog cells were tested with human tryptase (4 nM, n = 9), bovine thrombin (10 U/ml, n = 3), dog mast cell protease-3 (dMCP-3 or mastin; 550 ng/ ml, n = 2), chymase (550 ng/ml, n = 2), human PDGF-BB (100 ng/ml, n = 5), or human IGF-I (50 ng/ml, n = 19). Responses shown are means \pm SE and are expressed as a percentage of the response to 10% FCS.

have indicated that low "threshold" concentrations of tryptase may potentiate responses to peptide growth factors of the receptor tyrosine kinase class in lung fibroblast cells (28). Therefore, we sought to determine, in dog airway smooth muscle cells, whether threshold concentrations of trypsin and tryptase both potentiated responses to IGF-I and, if so, to a similar degree. Threshold concentrations of tryptase caused synergistic or more-than-additive increases in BrDU accumulation when applied in combination with IGF-I (Fig. 4A). By contrast, when threshold concentrations of trypsin were applied to cells, alone or together with IGF-I, the magnitude of the resulting responses was an additive combination of the responses to each mitogen, and there was no indication of synergistic potentiation (Fig. 4B).



Fig. 4. Compared effects of threshold concentrations of tryptase (A) and trypsin (B) on IGF-Iinduced BrDU incorporation in dog airway smooth muscle cells. IGF-I dose responses were measured alone or in the presence of threshold doses of tryptase (0.25-0.5 nM) or trypsin (1-10nM). Data are expressed as mean \pm SE from 5 experiments, each performed in triplicate. [IGF-I], IGF-I concentration.

AJP-Lung Cell Mol Physiol • VOL 282 • FEBRUARY 2002 • www.ajplung.org

Fig. 5. Compared effects of *p*-amidino phenylmethylsulfonyl fluoride (*p*-APMSF) treatment on mitogen-induced increases in DNA synthesis in cultured dog (*A*) and human (*B*) tracheal smooth muscle cells. Cells were growth arrested in serum-free media and then exposed to PDGF (50 ng/ml) or tryptase (2.1–5.4 nM) alone (control) or to PDGF or tryptase that had been preincubated with the irreversible inhibitor *p*-APMSF (plus *p*-APMSF). DNA synthesis was measured as BrDU incorporation using an ELISA. Results are means \pm SE; n = 6 or 10 experiments with PDGF or tryptase, respectively. *Significant difference compared with control response (P <0.05, paired *t*-test).



To characterize further the effects of trypsin on the cultured cells, we examined the effects of inhibiting trypsin's proteolytic activity on its ability to induce mitogenesis in the dog cells. Treating trypsin (8.9–64.4 nM) with *p*-APMSF appeared to prevent trypsin-induced cellular detachment. *p*-APMSF-treated trypsin, even in a large concentration (64.4 nM), induced relatively small increases in BrDU incorporation (6.5 \pm 1.5%; *n* = 2; data not shown). Treatment with an even larger concentration of trypsin (125.1 nM) with *p*-APMSF reduced its proteolytic activity to only ~60% of control values and did not prevent trypsin-induced cellular detachment.

Tryptase's mitogenic effects in human airway smooth muscle cells are largely nonproteolytic. Treatment of tryptase with *p*-APMSF invariably inhibited its proteolytic activity by 99% or more. In human airway smooth muscle cells, growth stimulatory effects of tryptase were not altered by prior treatment by *p*-APMSF (Fig. 5B). Results shown are from 10 different experiments, each performed in triplicate, and in none of these experiments was there an apparent difference between p-APMSF-treated and -untreated tryptase. By contrast, in dog cells, the tryptase response clearly was attenuated by p-APMSF (Fig. 5A). The total and irreversible inhibition of tryptase's proteolytic activity after treatment with *p*-APMSF was confirmed upon the addition of *p*-APMSF-treated tryptase to cells and after a period of 24 h under conditions identical to those employed in the BrDU assay. In the same experiments, we tested the effects of *p*-APMSF treatment on responses to PDGF in both human and dog cells. Concentrations of PDGF employed in these experiments were adjusted such that PDGF-induced mitogenic responses approximated those achieved in response to tryptase. Treatment of PDGF with *p*-APMSF had no

significant attenuatory effects on responses to PDGF in either human or dog cells (Fig. 5). In the human cells, human lung tryptase from a commercial source (Calbiochem) also was a potent mitogen, and *p*-APMSF treatment had no significant attenuating effects on its mitogenic activity (data not shown).

Human airway smooth muscle cells employed in these experiments express PAR-2. PCR yielded DNA bands of the expected size from each of the five different preparations of human airway smooth muscle cells, although there was variability in band intensity compared with that of β -actin (Fig. 6). Using the PAR-2 primers, we also identified intense bands of the appropriate size using RNA preparations from A549 cells, but no bands were seen using RNA from human platelets (data not shown). We also identified an intense



Fig. 6. Demonstration of protease-activated receptor (PAR)-2 expression in human airway smooth muscle cells. RNA was isolated from 5 different preparations of confluent human airway smooth muscle cells, and RT-PCR was carried out to test for PAR-2 expression, as described in MATERIALS AND METHODS. A: DNA bands, stained with ethidium bromide, that are of the appropriate size for the 287-bp product expected for PAR-2 cDNA fragments when the PAR-2 primers were used. B: RT-PCR for β -actin carried out on the same RNA samples as in A. The relatively equal intensity of the bands demonstrates comparable RNA integrity and efficiency of the RT reactions among the different cellular preparations employed.

band of the appropriate size when a *Hin*d III-linearized human PAR-2 cDNA was used as template with the PAR-2 primers (data not shown). The findings indicate that the preparations of human cells employed in our experiments express PAR-2, as reported previously in other preparations of cultured human airway smooth

muscle cells by other investigators (5, 29).

DISCUSSION

In this study, we found that mast cell tryptase produced concentration-dependent increases in DNA synthesis in cultured human airway smooth muscle cells (Fig. 1), as in cultured dog tracheal smooth muscle cells reported previously (10). Tryptase-induced increases in DNA synthesis resulted in demonstrable and significant increases in cell numbers in the human cells (Fig. 2). The increases in DNA synthesis occurred over the 1–30 nM range of tryptase concentrations, a concentration range with potential relevance in vivo based on previous estimates of mast cell densities in the smooth muscle layer of human bronchi (35) and of tryptase concentrations per human lung mast cell (22). In our experiments, the maximum increases in DNA synthesis induced by tryptase were comparable to those in response to several classical peptide growth factors and substantially greater than to other mitogenic serine proteases (Fig. 3). Bolstering the strength of these findings is our prior extensive validation of the BrDU ELISA as an accurate means of quantifying DNA synthesis in cultured airway smooth muscle cells (10). As reported by prior investigators in other preparations of human airway smooth muscle cells (29), the human cells employed in our experiments appeared to express PAR-2 (Fig. 6). However, a major and somewhat surprising finding of our experiments was that tryptase-induced mitogenic responses in the human cells were largely via nonproteolytic mechanisms (Fig. 5B); therefore, PAR-2 activation likely was not involved in mediating tryptase's growth stimulatory effects in these cells.

In a previous study, we showed that tryptase-induced mitogenesis in cultured dog airway smooth muscle cells was attenuated by a protease inhibitor (10), and we confirmed this finding in the present study (Fig. 5A). For that reason, at the outset of the current study, we were curious to compare the magnitude of mitogenic responses to tryptase with those of other mitogenic serine proteases to determine whether or not tryptase's effects reflected a general, nonspecific growth stimulatory response to serine proteases. We tested several other serine proteases. Chymase, a mast cell protease with chymotrypsin-like activity, has mitogenic effects on intramyocardial cells (27) and 3T3 fibroblasts (3). The cellular mechanism is not known but may relate to chymase's potent angiotensin-converting enzyme activity (50). Human coagulation factor Xa is a potent growth factor for vascular smooth muscle and endothelial cells, perhaps mediated via activation of effector cell protease receptor-1 (EPR-1; see Ref. 39). Current evidence suggests that factor Xa

binds with high affinity to EPR-1 and then may initiate growth via a secondary event that requires its (factor Xa's) catalytic site (39). Factor XII stimulates growth of hepatocytes and aortic smooth muscle, endothelial, and alveolar type 2 cells (28). Intriguingly, the factor XII molecule contains EGF-homologous domains, although recent evidence argues against EGF receptor activation as the mechanism for factor XII's growth stimulatory activity (28). Potent mitogenic effects of thrombin in the vasculature may occur via proteolytic activation of PARs (21) and possibly via nonproteolytic binding and activation of GP Ib α (2). In our experiments, thrombin clearly was a mitogen in both human and dog airway smooth muscle cells (Fig. 3, A and B), as described previously in human cells by other investigators (41). However, tryptase stimulated maximal increases in DNA synthesis to a much greater extent than any of these other serine proteases. The findings suggest that tryptase's growth stimulatory effects in cultured airway smooth muscle cells do not reflect a nonspecific effect of many different serine proteases. Instead, a mechanism relatively unique to tryptase likely is involved. Specificity of the mechanisms through which different proteases induce growth stimulatory effects also is suggested by the prior observation that tryptase was not a mitogen for cultured vascular smooth muscle cells (28), where the coagulation proteases would be expected to have relatively potent growth stimulatory effects.

Trypsin is a mitogen in some cells (9); therefore, during the course of our experiments, we were interested in determining its potential growth stimulatory effects in airway smooth muscle cells. Not surprisingly, we were unable to test the full range of trypsin concentrations in our BrDU ELISAs because trypsin concentrations >10 nM detached the smooth muscle cells from their substrate. These observations, coupled with the fact that tryptase never caused such detachment, are consistent with a general lack of specificity at the substrate P3 locus for trypsin's proteolytic actions vs. the relatively narrow range of extended peptide substrates cleaved by tryptase (51). Trypsin may induce growth stimulatory effects via its insulinomimetic effects (49). Thus tryptic cleavage of the α -subunit of the insulin receptor resulted in autophosphorylation of the β -subunit and initiation of transmembrane signaling (49). Cleavage of the α -subunit may have relieved its tonic inhibition of β -subunit phosphorylation and thus allowed signaling and growth induction to proceed just as if the receptor were occupied by insulin (49). In our experiments, we considered the possibility that the IGF-I receptor might be a site of action for tryptase effects because of this known insulinomimetic activity of trypsin, the structural similarities of insulin and IGF-I receptors (53), and the potent growth-promoting effects of IGF-I in cultured dog airway smooth muscle cells (Fig. 3B). We defined concentrations of trypsin that produced a small mitogenic response without detaching cells from the substratum. Concentrations of tryptase were chosen to match these small mitogenic responses. We then sought to determine the effects of

threshold concentrations of each protease on responses to IGF-I. Interestingly, trypsin and IGF-I induced increases in DNA synthesis that were additive (Fig. 4*B*). The finding is consistent with activation by trypsin and IGF-I of the same cell surface receptor, possibly the IGF-I receptor, although more information would be needed to establish this mechanism. In contrast, responses to tryptase and IGF-I were synergistic (Fig. 4*A*), and the findings suggested that these two agents employed separate modes of activation and possibly separate, rather than a shared, receptor(s) (28).

In our experiments, we used *p*-APMSF to inhibit tryptase's proteolytic actions irreversibly (10, 33). Other more commonly used inhibitors of tryptase's proteolytic activity include leupeptin, antipain, and benzamidine (15). All have the potential problems of reversibility and low potency (15). Compounds such as bis(5amidino-2-benzimidazolyl)methane (15) and APC366 (19) have improved potency but still are reversible. p-APMSF (33) is well suited for use in relatively lengthy proliferation assays in vitro because it effectively and irreversibly blocks virtually all catalytic activity of tryptase at low concentrations without inducing cytotoxic effects (33). In our experiments, we established that *p*-APMSF inhibited tryptase's catalytic activity by 99% or more in every experiment, and the inhibition persisted throughout the time course of the mitogenic assays. Our principal finding was that treatment of tryptase with *p*-APMSF significantly attenuated, but failed to abolish, increases in DNA synthesis in cultured dog airway smooth muscle cells (Fig. 5B) on the one hand but did not significantly alter tryptase-induced responses in human cells on the other hand (Fig. 5A). In the human cells, it seems unlikely that a very small amount of residual catalytic activity in *p*-AMSF-treated-tryptase could account for its persistently potent mitogenic activity, particularly when the relationships between tryptase concentration and mitogenic response are taken into consideration (Fig. 1). The findings raise the possibility that tryptase has the capacity to induce its mitogenic effects in airway smooth muscle cells via both proteolytic and nonproteolytic mechanisms, with each mechanism being of different degrees of importance depending on the specific cell type and experimental conditions.

Although tryptase-induced activation of PAR-2 clearly is one potential proteolytic mechanism for tryptase's effects (1, 20, 36), we can only speculate about possible nonproteolytic mechanisms. In bovine airway smooth muscle cells, potent proliferative responses to β -hexosaminidase and other mannosyl-rich glycoproteins are mediated by specific mannose receptors (34). The purified tryptase from human lung used in our studies most likely is composed of a combination of β_{1-} and β_{2} -isoforms that have two and one NH₂linked glycosylation site(s) per monomer, respectively (54). The crystal structure of tetrameric human β -tryptase has several glycosylated residues on the outer surface of the molecule in a location where such residues likely would be accessible for binding to cell surface mannose receptors (42). Such a mechanism would account for nonproteolytic activation of cellular growth by tryptase in airway smooth muscle cells.

In summary, tryptase is an extremely potent mitogen in cultured human airway smooth muscle cells. Therefore, its repeated release in the airways after mast cell degranulation (56) could contribute importantly to the smooth muscle hyperplasia and remodeling that occur in the airways of asthmatic patients (30). Many have assumed that all biological effects of extracellular tryptase are mediated via its catalytic site, but the tryptase tetramer has a complicated structure with the potential to exert both nonproteolytic and proteolytic actions (42). To our knowledge, all current efforts to develop tryptase inhibitors focus on attenuating its catalytic effects exclusively (15, 19). In this regard, our finding that tryptase's growth stimulatory effects in human airway smooth muscle cells occur largely via nonproteolytic mechanisms is important. The finding suggests that more complete information about the specific cellular mechanisms for tryptase's growth stimulatory effects may be needed before compounds can be developed that would be predicted to inhibit tryptase-induced mitogenesis in vivo with a full degree of effectiveness.

This work was supported by the Research Service of the Department of Veterans Affairs, National Heart, Lung, and Blood Institute Program Project Grant HL-24136, and the National Asthma Campaign (United Kingdom).

REFERENCES

- Akers IA, Parsons M, Hill MR, Hollenberg MD, Sanjar S, Laurent GJ, and McAnulty RJ. Mast cell tryptase stimulates human lung fibroblast proliferation via protease-activated receptor-2. Am J Physiol Lung Cell Mol Physiol 278: L193–L201, 2000.
- 2. Akiba S, Kawauchi T, and Sato T. Acceleration of Ca2+ ionophore-induced arachidonic acid liberation by thrombin without the proteolytic action toward the receptor in human platelets. *Eur J Biochem* 259: 643–650, 1999.
- Algermissen B, Hermes B, Feldman-Boeddeker I, Bauer F, and Henz BM. Mast cell chymase and tryptase during tissue turnover: analysis on in vitro mitogenesis of fibroblasts and keratinocytes and alterations in cutaneous scars. *Exp Dermatol* 8: 193–198, 1999.
- Bar-Shavit R, Benezra M, Eldor A, Hy-Am E, Fenton JW, Wilner DG, and Vlodavsky I. Thrombin immobilized to extracellular matrix is a potent mitogen for vascular smooth muscle cells: nonenzymatic mode of action. *Cell Regul* 1: 453–463, 1990.
- Berger P, Tunon-De-Lara M, Savineau J, and Marthan R. Signal transduction in smooth muscle. Selected Contribution: tryptase-induced PAR-2-mediated Ca²⁺ signaling in human airway smooth muscle cells. J Appl Physiol 91: 995–1003, 2001.
- Billington CK, Joseph SK, Śwan C, Scott MG, Jobson TM, and Hall IP. Modulation of human airway smooth muscle proliferation by type 3 phosphodiesterase inhibition. Am J Physiol Lung Cell Mol Physiol 276: L412–L419, 1999.
- Blair RJ, Meng H, Marchese MJ, Ren S, Schwartz LB, Tonnesen MG, and Gruber BL. Human mast cells stimulate vascular tube formation. J Clin Invest 99: 2691-2700, 1997.
- Bohm SK, Kong W, Bromme D, Smeekens SP, Anderson DC, Connolly A, Kahn M, Nelken NA, Coughlin SR, Payan DG, and Bunnett NW. Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem J* 314: 1009–1016, 1996.
- 9. Bono F, Lamarche I, and Herbert JM. Induction of vascular smooth muscle cell growth by selective activation of the protein-

ase activated receptor-2 (PAR-2). Biochem Biophys Res Commun 241: 762–764, 1997.

- Brown JK, Tyler CL, Jones CA, Ruoss SJ, Hartmann T, and Caughey GH. Tryptase, the dominant secretory granular protein in human mast cells, is a potent mitogen for cultured dog tracheal smooth muscle cells. Am J Respir Cell Mol Biol 13: 227-236, 1995.
- Butterfield JH, Weiler DA, Hunt LW, Wynn SR, and Roche PE. Purification of tryptase from a human mast cell line. *J Leukoc Biol* 47: 409–419, 1990.
- Cairns JA and Walls AF. Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intracellular adhesion molecule-1 expression. J Immunol 156: 275–283, 1995.
- Camerer E, Huang W, and Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci USA 97: 5255–5260, 2000.
- Caughey GH. Of mites and men: trypsin-like proteases in the lungs. Am J Respir Cell Mol Biol 16: 621–628, 1997.
- Caughey GH, Raymond WW, Bacci E, Lombardy RJ, and Tidwell RR. Bis(5-amidino-2-benzimidazolyl)methane and related amidines are potent, reversible inhibitors of mast cell tryptases. J Pharmacol Exp Ther 264: 676–682, 1993.
- Caughey GH, Raymond WW, Blount JL, Hau LW, Pallaoro M, Wolters PJ, and Verghese GM. Characterization of human gamma-tryptases, novel members of the chromosome 16p mast cell tryptase and prostasin gene families. J Immunol 164: 6566– 6575, 2000.
- Caughey GH, Viro NF, Lazarus SC, and Nadel JA. Purification and characterization of dog mastocytoma chymase: identification of an octapeptide conserved in chymotryptic leukocyte proteinases. *Biochim Biophys Acta* 952: 142–149, 1988 (published erratum appears in *Biochim Biophys Acta* 996: 263, 1989).
- Caughey GH, Viro NF, Ramachandran J, Lazarus SC, Borson DB, and Nadel JA. Dog mastocytoma tryptase: affinity purification, characterization and amino-terminal sequence. *Arch Biochem Biophys* 258: 555–563, 1987.
- Clark JM, Abraham WM, Fishman CE, Forteza R, Ahmed A, Cortes A, Warne RL, Moore WR, and Tanaka RD. Tryptase inhibitors block allergen-induced airway and inflammatory responses in allergic sheep. Am J Respir Crit Care Med 152: 2076–2083, 1995.
- Corvera CU, Déry O, McConalogue K, Bohm S, Khitin L, Caughey GH, Payan DG, and Bunnett NW. Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. J Clin Invest 100: 1383–1393, 1997.
- Coughlin SR. Thrombin signaling and protease-activated receptors. Nature 407: 258–264, 2000.
- Craig SS and Schwartz LB. Tryptase and chymase, markers of distinct types of human mast cells. *Immunol Res* 8: 130–148, 1989.
- D'Andrea MR, Derian CK, Leturcq D, Baker SM, Brunmark A, Ling P, Darrow AL, Santulli R, Brass LF, and Andrade-Gordon P. Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. J Histochem Cytochem 46: 157-164, 1998.
- Elias JA. Airway remodeling in asthma. Unanswered questions. Am J Respir Crit Care Med 161: S168–S171, 2000.
- 25. Fahy JV, Liu J, Wong H, and Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and healthy subjects. *Am Rev Respir Dis* 147: 1126–1131, 1993.
- 26. Gordon EM, Venkatesan N, Salazar R, Tang H, Schmeidler-Sapiro K, Buckley S, Warburton D, and Hall FL. Factor XII-induced mitogenesis is mediated via a distinct signal transduction pathway that activates a mitogen-activated protein kinase. *Proc Natl Acad Sci USA* 93: 2174–2179, 1996.
- Hara M, Matsumori A, Ono K, Kido H, Hwang M, Miyamoto T, Iwasaki A, Okada M, Nakatani K, and Sasayama S. Mast cells cause apoptosis of cardiomyocytes and proliferation of other intramyocardial cells in vitro. *Circulation* 100: 1443– 1449, 1999.
- Hartmann T, Ruoss SJ, Raymond WW, Seuwen K, and Caughey GH. Human tryptase as a potent, cell specific mitogen: role of signaling pathways in synergistic responses. Am J Physiol Lung Cell Mol Physiol 262: L528–L534, 1992.

- Hauck RW, Schulz C, Schömig A, Hoffman RK, and Panettieri RA Jr. α-Thrombin stimulates contraction of human bronchial rings by activation of protease-activated receptors. Am J Physiol Lung Cell Mol Physiol 277: L22–L29, 1999.
- Hossain S. Quantitative measurement of bronchial muscle in men with asthma. Am Rev Respir Dis 107: 99–109, 1973.
- James AL, Pare PD, and Hogg JC. The mechanics of airway narrowing in asthma. Am Rev Respir Dis 139: 242-246, 1989.
- 32. Ko FN, Yang YC, Huang SC, and Ou JT. Coagulation factor Xa stimulates platelet-derived growth factor release and mitogenesis in cultured vascular smooth muscle cell of rat. J Clin Invest 96: 1493–1501, 1996.
- Laura R, Robison DJ, and Bing DH. (p-Amidinophenyl)methanesulfonyl fluoride, an irreversible inhibitor of serine proteases. *Biochemistry* 19: 4859–4864, 1980.
- Lew DB, Songu-Mize E, Pontow SE, Stahl PD, and Rattazzi MC. A mannose receptor mediates mannosyl-rich glycoprotein-induced mitogenesis in bovine airway smooth muscle cells. J Clin Invest 94: 1855–1863, 1994.
- Matin R, Tam EK, Nadel JA, and Caughey GH. Distribution of chymase-containing mast cells in human bronchi. J Histochem Cytochem 40: 781–786, 1992.
- 36. Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, Hoxie JA, Schechter N, Woolkalis M, and Brass LF. Interactions of mast cell tryptase with thrombin receptors and PAR-2. J Biol Chem 272: 4043-4049, 1997.
- 37. Molloy CJ, Pawlowski JE, Taylor DS, Turner CE, Weber H, Peluso M, and Seiler SM. Thrombin receptor activation elicits rapid protein tyrosine phosphorylation and stimulation of the Raf-1/MAP Kinase pathway preceding delayed mitogenesis in cultured rat aortic smooth muscle cells. J Clin Invest 97: 1173–1183, 1996.
- Mullhaupt B, Feren A, Fodor E, and Jones A. Liver expression of epidermal growth factor RNA. J Biol Chem 269: 19667–19670, 1994.
- Nicholson AC, Nachman Rl Altieri DC, Summers BD, Ruf W, Edgington TS, and Hajjar DP. Effector cell protease receptor-1 is a vascular receptor for coagulation factor Xa. *J Biol Chem* 271: 28407–28413, 1996.
- Nudel U, Zakut R, Shani M, Neuman S, Levy Z, and Yaffe D. The nucleotide sequence of the rat cytoplasmic beta-actin gene. Nucleic Acids Res 11: 1759–1771, 1983.
- Panettieri RA Jr, Hall IP, Maki CS, and Murray RK. Alphathrombin increases cytosolic calcium and induces human airway smooth muscle cell proliferation. Am J Respir Cell Mol Biol 13: 205–216, 1995.
- 42. Pereira PJ, Bergner A, Macedo-Ribero S, Huber R, Matschiner G, Fritz H, CPS, and Bode W. Human β-tryptase is a ring-like tetramer with active sites facing a central pore. *Nature* 392: 306–311, 1998.
- Powers JC and Kam CM. Isocoumarin inhibitors of serine peptidases. *Methods Enzymol* 244: 442–457, 1994.
- Ruoss SJ, Hartmann T, and Caughey GH. Mast cell tryptase is a mitogen for cultured fibroblasts. J Clin Invest 88: 493–499, 1991.
- 45. Schaeffer P, Riera E, Dupuy E, and Herbert J. Nonproteolytic activation of the thrombin receptor promotes human umbilical vein endothelial cell growth but not intracellular Ca2+, prostacyclin, or permeability. *Biochem Pharmacol* 53: 487–491, 1997.
- Schwartz LB. Tryptase: a mast cell serine protease. Methods Enzymol 244: 88–100, 1994.
- 47. Schwartz LB and Bradford TR. Regulation of tryptase from human lung mast cells by heparin. Stabilization of the active tetramer. *J Biol Chem* 261: 7372–7379, 1986.
- Shiels IA, Bowler SD, and Taylor SM. Airway smooth muscle proliferation in asthma: the potential of vascular leakage to contribute to pathogenesis. *Med Hypotheses* 45: 37–40, 1995.
- 49. Shoelson SE, White MF, and Kahn CR. Tryptic activation of the insulin receptor. Proteolytic truncation of the alpha-subunit releases the beta-subunit from inhibitory control. J Biol Chem 263: 4852–4860, 1988.

- Takai S, Jin D, Sakaguchi M, and Miyazaki M. Chymasedependent angiotensin II formation in human vascular tissue. *Circulation* 100: 654–658, 1999.
- Tanaka T, McRae BJ, Cho K, Cook R, Fraki JE, Johnson DA, and Powers JC. Mammalian tissue trypsin-like enzymes. *J Biol Chem* 258: 13552–13557, 1983.
- Tom-Moy M, Madison JM, Jones CA, de Lanerolle P, and Brown JK. Morphologic characterization of cultured smooth muscle cells isolated from the tracheas of adult dogs. *Anat Rec* 218: 313–328, 1987.
- 53. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM, and Ramachandran J. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* 313: 756–761, 1985.
- 54. Vanderslice P, Ballinger SM, Tam EK, Goldstein SM, Craik CS, and Caughey GH. Human mast cell tryptase: multiple cDNAs and genes reveal a multigene serine protease family. *Proc Natl Acad Sci USA* 87: 3811–3815, 1990.
- Wenzel SE. Proceedings of the ATS workshop on refractory asthma. Current understandings, recommendations, and unanswered questions. Am J Respir Crit Care Med 162: 2341-2351, 2000.
- 56. Wenzel SE, Fowler A, and Schwartz LB. Activation of pulmonary mast cells by bronchoalveolar allergen challenge. In vivo release of histamine and tryptase in atopic subjects with and without asthma. *Am Rev Respir Dis* 137: 1002–1008, 1988.
- 57. Yezzi MJ, Hsieh IE, and Caughey GH. Mast cell and neutrophil expression of dog mast cell protease-3. A novel tryptaserelated serine protease. J Immunol 152: 3064–3072, 1994.

