

# Mast Cell Tryptase Is a Mitogen for Cultured Fibroblasts

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## Abstract

Mast cells appear to promote fibroblast proliferation, presumably through secretion of growth factors, although the molecular mechanisms underlying this mitogenic potential have not been explained fully by known mast cell-derived mediators. We report here that tryptase, a trypsin-like serine proteinase of mast cell secretory granules, is a potent mitogen for fibroblasts in vitro. Nanomolar concentrations of dog tryptase strongly stimulate thymidine incorporation in Chinese hamster lung and Rat-1 fibroblasts and increase cell density in both subconfluent and confluent cultures of these cell lines. Tryptase-induced cell proliferation appears proteinase-specific, as this response is not mimicked by pancreatic trypsin or mast cell chymase. In addition, low levels of tryptase markedly potentiate DNA synthesis stimulated by epidermal growth factor, basic fibroblast growth factor, or insulin. Inhibitors of catalytic activity decrease the mitogenic capacity of tryptase, suggesting, though not proving, the participation of the catalytic site in cell activation by tryptase. Differences in  $\text{Ca}^{++}$  mobilization and sensitivity to pertussis toxin suggest that tryptase and thrombin activate distinct signal transduction pathways in fibroblasts. These data implicate mast cell tryptase as a potent, previously unrecognized fibroblast growth factor, and may provide a molecular link between mast cell activation and fibrosis. (*J. Clin. Invest.* 1991. 88:493-499.) Key words: proteinase • proliferation • growth factor • signal transduction • chymase

## Introduction

Several lines of evidence suggest participation of mast cells in fibroblast proliferation. Increased numbers of mast cells are found in close proximity to proliferating fibroblasts in healing wounds and in fibrotic diseases of the lung and skin in humans (1-4). These observations have received support from animal models of lung fibrosis induced by ionizing radiation, bleomycin, or asbestos, which lead to mast cell hyperplasia early in the development of fibrosis (5-7). In the hypersensitivity pneumonitis model of lung fibrosis in mast cell-deficient W/W<sup>m</sup> mice, mast cells are required to produce the full pathologic response (8), suggesting a central role for mast cells in the fibroblast proliferation. Furthermore, morphologic studies of the lung parenchyma in interstitial fibrosis (1), fibrotic airway subepithelium in chronic asthma (9), and lesional skin in

scleroderma (10) provide evidence of mast cell degranulation, suggesting that granule constituents may participate in the proliferative process. This hypothesis has been examined in vitro in rodent experiments, which demonstrate cell proliferation after mast cell degranulation (11). Activation of mast cells results in extracellular release of histamine, the serine proteinases tryptase and chymase, and other mediators (12-15). Although histamine has some growth-promoting potential (16), its activity accounts only partially for the mitogenic potential of mast cell granule contents (17, 18), implying the existence of additional mast cell-derived growth factors.

Extensive work has established mitogenic activity for thrombin and other proteinases in various cell types including fibroblasts (19-21). We have explored the possible role for mast cell proteinases in fibroblast proliferation by examining the mitogenic activity of purified dog tryptase and chymase in cultured fibroblasts.

## Methods

**Materials.** Benzoyl-val-gly-arg-*p*-nitroanilide (VGR-pNA),<sup>1</sup> D-Phe-L-pipecolyl-Arg-*p*-NA, succinyl-phe-pro-phe-*p*-nitroanilide (FPF-pNA), amiloride, bovine heparin, leupeptin, diisopropylfluorophosphate (DFP), epidermal growth factor (EGF), bovine insulin, and human  $\alpha$ -thrombin (3,700 NIH U/ml) were obtained from Sigma Chemical Co. (St. Louis, MO). Basic fibroblast growth factor (bFGF) was kindly provided by Dr. D. Gospodarowicz (University of California, San Francisco). Pertussis toxin was from List Biological Laboratories (Campbell, CA). Fura-2-acetoxymethylester (AM) was obtained from Molecular Probes, Inc. (Eugene, OR). (Methyl-<sup>3</sup>H)-thymidine deoxyribose and [<sup>14</sup>C]benzoic acid were from ICN Biomedicals (Irvine, CA), and myo-(2-<sup>3</sup>H)-inositol was obtained from Amersham Corp. (Arlington Heights, IL). Mast cell tryptase (22) and chymase (23) were purified from dog mastocytoma cells.

**Cell culture.** Chinese hamster lung (CHL) fibroblasts (clone CCL39), an established diploid cell line, were obtained from the American Type Culture Collection (Rockville, MD). Rat-1 fibroblasts were obtained from the laboratory of Dr. H. Bourne (University of California, San Francisco). All cells were grown in DMEM containing 4.5 g/liter glucose, and supplemented with 10% FCS, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and 25 mM Na<sup>+</sup> bicarbonate, at 37°C in 5% CO<sub>2</sub>.

**Mast cell proteinase catalytic assays.** Dog mast cell tryptase and chymase were assayed using the specific chromogenic peptide substrates VGR-pNA and FPF-pNA, respectively, as previously described (22, 23). Briefly, purified enzyme solutions were incubated with the respective *p*-nitroanilide substrates at 37°C, with measurement of absorbance at 394 nm performed using an 8451A Diode Array Spectrophotometer (Hewlett-Packard, Palo Alto, CA). Established specific activities for these proteinases were used to determine molar concentrations (22, 23), using the tetrameric molecular weight (140,000 D) for tryptase and monomeric molecular weight for chymase.

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1. **Abbreviations used in this paper:** bFGF, basic fibroblast growth factor; CHL, Chinese hamster lung; DFP, diisopropylfluorophosphate; EGF, epidermal growth factor; fura-2-AM, fura-2-acetoxymethylester; FPF-pNA, succinyl-phe-pro-phe-*p*-nitroanilide; VGR-pNA, benzoyl-val-gly-arg-*p*-NA.

**Measurement of DNA synthesis.** CHL cells were grown to confluence in 24-well plates and rendered quiescent by 24 h incubation in serum-free DMEM. After serum deprivation, cells were incubated for 24 h with agonists in DMEM/Ham's F-12 medium (1:1) in the presence of [ $^3$ H]thymidine (0.5  $\mu$ Ci/ml). Growth factors were added simultaneously in experiments where multiple mediators were applied. Purified trypsin or chymase, when added, were applied to cells in 270 mM NaCl, 10 mM Bis-Tris (pH 6.1) buffer. When present, bovine heparin was added in a 2.5:1 weight excess over trypsin. Controls included buffer and heparin alone. After the 24-h incubation period with [ $^3$ H]thymidine, trichloroacetic acid-precipitable material was solubilized in 0.3 N NaOH, and the incorporated radioactivity was assessed by liquid scintillation counting. Thymidine incorporation assays for Rat-1 cells followed the same protocol as above, with the exception that [ $^3$ H]thymidine (0.5  $\mu$ Ci/ml) was added to the culture medium for the final 6 h of the total 21 h of incubation with mitogens.

**Pertussis toxin sensitivity assays.** Assay of pertussis toxin-induced inhibition of DNA synthesis was performed as previously described (24). Briefly, pertussis toxin was added to quiescent CHL fibroblasts, at the concentrations indicated in Results, 4 h before stimulation and was present throughout the 24-h incubation with growth factors. [ $^3$ H]Thymidine incorporation was assessed as described above.

**Cell proliferation assays.** Stimulation of cells at low density and at high density was used to establish the characteristics of cell proliferation in response to trypsin. In the low density experiments, CHL cells were plated at 2,500 cells/cm $^2$  in multi-well plates containing DMEM with 10% FCS. After 24 h, cells were washed three times with serum-free DMEM and covered with DMEM/Ham's F-12 medium (1:1), supplemented with transferrin (5  $\mu$ g/ml) and insulin (10  $\mu$ g/ml). Growth factors, as listed in Results, were added to cells in serum-free medium at the time of medium change, and every second day subsequently with accompanying medium change. On days of medium change, cells were suspended with trypsin and counted using a hemocytometer; duplicate wells were counted for each condition.

In the high density experiments, CHL cells were grown to confluence in 10% FCS then deprived of serum for 24 h in DMEM. Quiescent cells were then stimulated with trypsin (2 nM), thrombin (10 nM), or 10% FCS. After a 36-h incubation with growth factors, cell density was determined by counting as above.

**Studies with trypsin inhibitors.** To examine the role of trypsin catalytic activity in the observed mitogenic responses, the effects of active site inhibition by leupeptin and DFP were examined.

(a) **Leupeptin studies.** The reversible, arginal-class inhibitor leupeptin was added to quiescent CHL cells concomitant with trypsin or other growth factors, as indicated below; the final leupeptin and growth factor concentrations were as given in Results. Inhibition of mitogenic activity was assessed by [ $^3$ H]thymidine incorporation.

(b) **DFP studies.** Trypsin (3  $\mu$ M) and thrombin (1  $\mu$ M) were incubated independently with DFP for 21 h at 4°C before exposure to cells. Molar ratios of DFP to enzyme ranged from  $3 \times 10^1$  to  $10^4$  for trypsin, and from  $10^{-2}$  to  $10^4$  for thrombin. The 21-h preincubations were necessary to allow hydrolysis of excess unincorporated DFP, which is cytotoxic. All incubations were performed in 270 mM NaCl, 10 mM Hepes (pH 6.1) buffer. Control DFP solutions, in concentrations corresponding to those used in the proteinase incubations, also were incubated at 4°C for 21 h. Complete DFP hydrolysis after 21 h of incubation was confirmed by the inability of the incubation solutions to inhibit the amidolytic activity of fresh trypsin or thrombin. Amidolytic activity was assessed with chromogenic substrates as described below. Aliquots of proteinases in the absence of DFP were incubated under identical conditions to assure preservation of proteinase activity during these incubations. Aliquots of the proteinase preparations above were added to quiescent CHL cells to final concentrations of 7.5 nM (trypsin) or 10 nM (thrombin). [ $^3$ H]Thymidine incorporation was measured as described above. Catalytic activity of trypsin and thrombin was measured immediately after the 21 h incubation at 4°C. Trypsin was assayed using VGR-pNA (22), and thrombin catalytic activity was assayed using the method of Witting et al. (25), with D-Phe-L-pipecolyl-Arg-p-NA (200  $\mu$ M).

**Determination of intracellular pH change.** Intracellular pH change was determined from the equilibrium distribution of [ $^{14}$ C]benzoic acid as described (26). Briefly, quiescent CHL fibroblasts in 12-well plates were equilibrated for 1 h in bicarbonate-free Hepes-buffered DMEM (pH 7.4), at 37°C. [ $^{14}$ C]Benzoic acid (1  $\mu$ Ci/ml) was present for the final 30 min of equilibration. Cells were then stimulated for 10 min with trypsin or thrombin at concentrations of 7.5 and 1 nM, respectively. When present, amiloride (1 mM) was added 5 min before addition of growth factors. Stimulation of cells was stopped by aspiration of external medium and rapid washing four times with ice-cold unlabeled medium. Retained [ $^{14}$ C]benzoic acid was measured by liquid scintillation counting. Changes in intracellular pH were calculated as previously described (27).

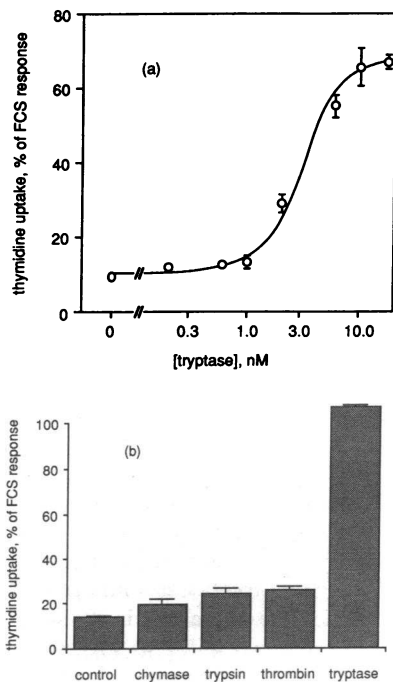
**Measurement of intracellular calcium.** Free cytosolic Ca $^{++}$  was measured in CHL cells using the fluorescent indicator fura-2. CHL cells grown to confluence on glass coverslips were loaded with fura-2-AM (1  $\mu$ M) in Hank's buffer with 20 mM Hepes (pH 7.4), for 15 min at 37°C. Cells were then washed twice with dye-free buffer and mounted in a cuvette with continuous stirring. Trypsin (7.5 nM) and thrombin (1 nM) were added to the cuvette while recording. Fluorescence was recorded in a fluorimeter (SLM 8000; SLM Instruments, Inc., Urbana, IL), changing excitation wavelengths every second between 340 and 380 nm. Emitted light above 495 nm was recorded. Background was determined after permeabilizing the cells with melittin (10  $\mu$ g/ml) and addition of 1 mM MnCl $_2$ . The ratio of light intensities upon excitation at 340 and 380 nm was used to calculate free cytosolic Ca $^{++}$  concentration, assuming a  $K_d$  of 224 nM for the Ca $^{++}$ -fura-2 complex (28).

**Measurement of inositol phosphates.** Formation of inositol phosphates was assayed as previously described (29). Confluent CHL fibroblasts in 12-well plates were incubated for 24 h in serum-free DMEM containing [ $^3$ H]inositol (2  $\mu$ Ci/ml). After equilibration in Hepes-buffered DMEM (pH 7.4) for 30 min, 10 mM LiCl was added to increase assay sensitivity through inhibition of inositol-1-phosphatase (30). LiCl-treated cells were exposed to trypsin (7.5 nM) or thrombin (1 nM) for 10 min. Cells were then extracted with 10 mM formic acid, and total radiolabeled inositol phosphates were assessed by anion-exchange chromatography and liquid scintillation counting (29).

## Results

Trypsin induced the incorporation of [ $^3$ H]thymidine in confluent quiescent CHL cells up to 22-fold above control levels. This corresponded to 65% of the response to 10% FCS (Fig. 1 a). Trypsin also initiated DNA synthesis in confluent serum-deprived Rat-1 fibroblasts. The thymidine incorporation response to trypsin observed in Rat-1 was greater than that observed in CHL cells, with the response of Rat-1 cells to 15 nM trypsin equaling the response to 10% FCS (Fig. 1 b). The half-maximal response trypsin concentration was  $\sim$  4 nM for both cell lines. In contrast to trypsin, the thymidine uptake observed in Rat-1 cells in response to thrombin, trypsin, or chymase (a chymotrypsin-like mast cell serine proteinase) was minimal (Fig. 1 b). Higher concentrations of trypsin or chymase resulted in cell rounding and detachment from the plates, whereas lower concentrations of these proteinases produced thymidine uptake that only approached control levels (Fig. 1 b). Thus, trypsin-induced DNA synthesis appeared to be a specific response. Similar effects for chymase and trypsin were observed in quiescent CHL cells (data not shown). These results suggest that proteolysis per se is not sufficient to induce DNA synthesis in the fibroblast cell lines investigated.

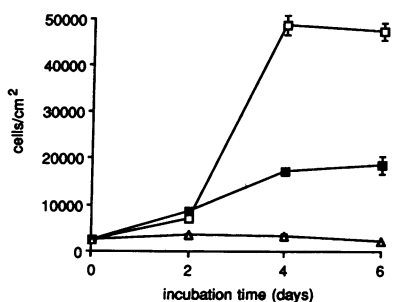
To confirm that cell growth accompanies DNA synthesis after trypsin stimulation, cell numbers were determined after CHL cell exposure to trypsin under two different cell density conditions. First, CHL cells were seeded in 10% FCS at low density, then shifted to serum-free medium after 24 h. Cells



**Figure 1.** Concentration-dependent reinitiation of DNA synthesis in fibroblasts by dog trypase. (a) Quiescent, confluent CHL fibroblasts were incubated for 24 h with purified dog trypase, from 0.25 to 22.5 nM, in the presence of [ $^3$ H]thymidine. (b) Serum-deprived confluent Rat-1 fibroblasts were incubated for 18 h with chymase (3 nM), trypsin (100 nM), trypase (15 nM), and thrombin (10 nM). Results of incorporation of [ $^3$ H]thymidine are normalized to the response to 10% FCS. Mean $\pm$ SE of 5 to 20 independent determinations are shown.

maintained in the presence of insulin and transferrin alone did not proliferate (Fig. 2). In contrast, cell number increased 8-fold after 6 d in the presence of 2 nM trypase, with a subsequent plateau in cell density (Fig. 2). In the presence of 1 nM thrombin, CHL cell number increased 20-fold in the same period (Fig. 2). CHL cells did not reach confluence under these conditions, but confluence was achieved by 6 d with incubation in 10% FCS or 10 nM thrombin. Higher concentrations of trypase did not increase growth rate or final cell density. The mechanism for a cessation of trypase-induced cell growth at subconfluence remains unknown.

To examine induction of cell proliferation by trypase at high cell density, confluent serum-deprived CHL cells were stimulated with 7.5 nM trypase, 10 nM thrombin or 10% FCS. Cell counts assessed 36 h after stimulation demonstrated that trypase was equivalent to thrombin or 10% FCS in promoting growth (Table I). In each condition cell number increased over controls by  $\sim$ 60%. Similar results were obtained for confluent, serum-deprived Rat-1 cells, where cell number increased by 39.7 and 42.4% after trypase and FCS exposure,



**Figure 2.** Effect of trypase on cell proliferation in low density CHL fibroblasts. CHL cells were seeded at 2,500 cells/cm<sup>2</sup> in medium containing 10% FCS. After 24 h, cells were washed and covered with serum-free medium supplemented with transferrin and insulin. Fresh medium supplemented with transferrin and insulin was added to cells alone (open triangles), or with 2 nM trypase (closed squares) or 1 nM thrombin (open squares), every second day. Cells were counted in a Neubauer chamber; triplicate wells were counted for each condition. Data represent mean $\pm$ SD.

insulin. Fresh medium supplemented with transferrin and insulin was added to cells alone (open triangles), or with 2 nM trypase (closed squares) or 1 nM thrombin (open squares), every second day. Cells were counted in a Neubauer chamber; triplicate wells were counted for each condition. Data represent mean $\pm$ SD.

**Table I.** Cell Proliferation in Confluent Fibroblasts after Mitogen Exposure

Stimulus	Cells ( $\times 10^5$ /cm <sup>2</sup> )*	
	CHL	Rat-1
Control	2.43 $\pm$ 0.15	4.03 $\pm$ 0.38
7.5 nM Trypase	3.80 $\pm$ 0.31 <sup>†</sup>	5.63 $\pm$ 0.18 <sup>†</sup>
10 nM Thrombin	3.90 $\pm$ 0.45 <sup>†</sup>	3.81 $\pm$ 0.25
10% FCS	3.98 $\pm$ 0.40 <sup>†</sup>	5.74 $\pm$ 0.29 <sup>†</sup>

Cell counts 36 h after exposure to mitogens.

\* Mean $\pm$ SEM for triplicate determinations. <sup>†</sup> A significant difference from control ( $P < 0.05$ ) by Student *t* test.

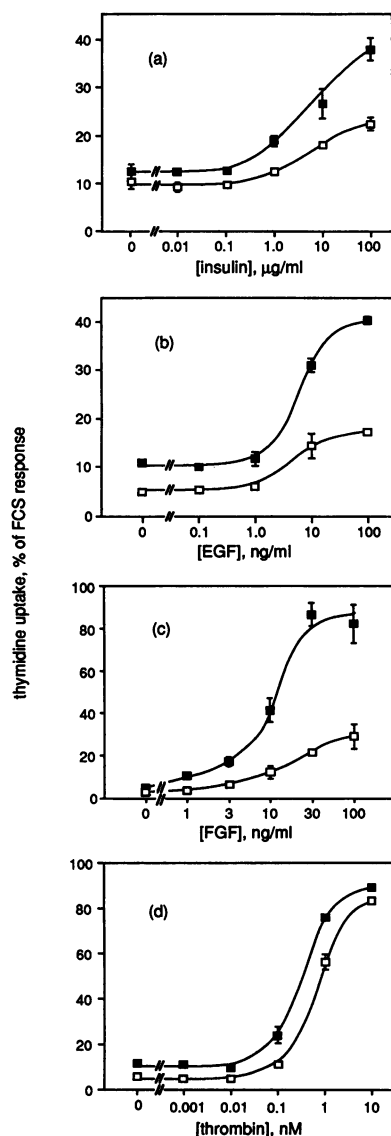
respectively (Table I). Thrombin did not significantly increase Rat-1 cell number over baseline, in agreement with the failure of thrombin to stimulate thymidine incorporation in these cells (Fig. 1 b). These results indicate that trypase not only stimulates S-phase entry in fibroblasts, but also promotes completion of the cell cycle, resulting in cell division.

To examine the interaction between trypase and other growth factors, CHL fibroblasts were incubated with trypase in combination with EGF, bFGF, insulin, or thrombin. Threshold response concentrations of trypase (0.75 nM) in combination with either insulin, EGF, or bFGF elicited increasing synergistic responses (Fig. 3, a–c). Synergy was produced without an apparent shift in the concentration producing the half-maximal response for all three growth factors. In contrast, the [ $^3$ H]-thymidine incorporation response to concomitant low concentration trypase plus thrombin was additive rather than synergistic (Fig. 3 d).

Trypase is found in mast cell preparations noncovalently linked to, and stabilized by, heparin, a constituent of mast cell secretory granules (31). Heparin is known to modulate the effects of a number of mitogens, including members of the heparin-binding growth factor family, and may also have independent growth regulatory function (32). The [ $^3$ H]thymidine uptake response of CHL cells to 7.5 nM trypase was not altered by the addition of heparin when added to trypase solutions in a weight ratio to trypase of 2.5:1. Heparin alone at an equivalent concentration had no effect on thymidine incorporation (data not shown).

The possibility that proteinases exert their mitogenic effects through a mechanism that is dependent upon their catalytic activity continues to be an issue of great interest (21, 33). To determine whether preserved catalytic activity of trypase is required for the mitogenic effect, we studied the effect of proteinase catalytic inhibition on DNA synthesis using leupeptin and DFP, two established trypase inhibitors (22).

Incubation of CHL fibroblasts with trypase in the presence of leupeptin resulted in a concentration-dependent inhibition of [ $^3$ H]thymidine uptake (Fig. 4). Leupeptin concentrations above 300  $\mu$ M suppressed [ $^3$ H]thymidine uptake by greater than 80%. At the concentrations used, nonspecific effects of leupeptin were not observed, as demonstrated by the unaltered response to EGF in the presence of leupeptin (Fig. 4). Catalytic activity of trypase after incubation with leupeptin (1 mM) was reduced to less than 0.5% of initial activity (data not shown), consistent with previous results (22). These results are compatible with the requirement for an intact catalytic site for trypase-induced stimulation of DNA synthesis. However, leupeptin is a

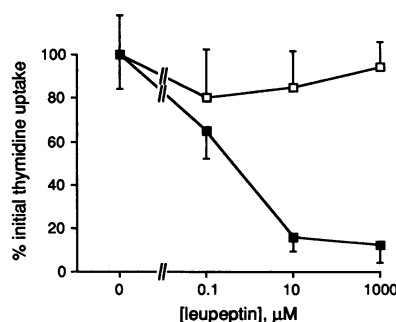


**Figure 3.** Reinitiation of DNA synthesis in CHL fibroblasts by tryptase in combination with insulin, EGF, bFGF, and thrombin. Results represent 24-h  $^3\text{H}$ -thymidine uptake responses for insulin (a), EGF (b), bFGF (c), and thrombin (d), alone (open symbols), and in combination with 0.75 nM purified tryptase (closed symbols). Radiolabel incorporation data are normalized to the response to 10% fetal bovine serum. Data are mean  $\pm$  SD from a representative experiments done in duplicate.

reversible inhibitor and could potentially affect other cellular proteins while in solution during the 24-h stimulation period.

As an alternative to leupeptin, we used DFP, an irreversible, covalent serine proteinase inhibitor, which can completely inhibit the catalytic activity of tryptase (22). When free in solution, this inhibitor is cytotoxic. Taking advantage of the instability of free DFP in aqueous solution, we incubated proteinases (both tryptase and thrombin) with DFP, and continued the incubations for 21 h at 4°C to allow for the hydrolysis of unincorporated DFP. Complete hydrolysis of DFP was confirmed by a lack of inhibition of proteinase amidolytic activity by the incubated DFP solutions; amidolytic activity of tryptase and thrombin in the absence of DFP did not diminish during incubation at 4°C (data not shown).

While the catalytic activity of tryptase was inhibited 98.7% by DFP, the reinitiation of DNA synthesis was reduced by only 60.9% (Table II). For thrombin, catalytic activity was inhibited greater than 99.2%, while the mitogenic response of CHL cells to DFP-thrombin was inhibited by 85.2%, in agreement with published data (34) (Table II). These data, together with the leupeptin inhibition results, suggest that expression of the full mitogenic activity of tryptase is dependent upon an uninhibited



**Figure 4.** Effect of leupeptin on the CHL fibroblast thymidine uptake response to tryptase (7.5 nM; closed symbols) and EGF (100 ng/ml; open symbols). Cells were incubated for 24 h in the presence or absence of the leupeptin concentrations shown, with tryptase or EGF.  $^3\text{H}$ Thymidine incorporation results were normalized to results obtained in the absence of leupeptin. Data represent mean  $\pm$  SD from triplicate determinations.

ited catalytic site, although a catalytic site-independent component may exist. It remains to be proven that a proteolytic event is a necessary component of tryptase-induced mitogenesis.

Notwithstanding the finding that tryptase is a mitogen for Rat-1 cells while thrombin is not, the similarities in the structure and catalytic activity of these two mitogens suggest the possibility of a shared mechanism of cell activation. To investigate this possibility, we compared the participation of both mitogens in known signal transduction pathways and responses.

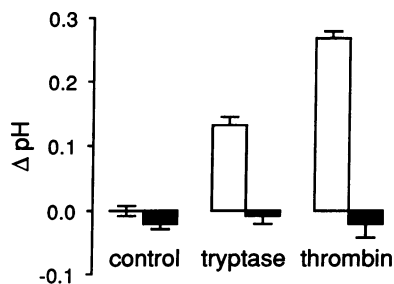
Activation of the amiloride-sensitive  $\text{Na}^+/\text{H}^+$  antiporter is a virtually universal response of fibroblasts to mitogens, including thrombin, EGF, bFGF, and platelet-derived growth factor (27, 35, 36). Measured in bicarbonate-free conditions, stimulation of  $\text{Na}^+/\text{H}^+$  exchange results in a transient intracellular alkalinization. Tryptase, like thrombin, increased intracellular pH transiently in CHL fibroblasts, an effect that was abolished by amiloride pretreatment of the cells (Fig. 5). Significant alkalization was evident by 5 min, was maximal 10 min after addition of tryptase, and decreased over the next 20 min (data not shown).

Important early cellular responses to growth factors include stimulation of phospholipase C (resulting in inositol phosphate formation), mobilization of cytosolic  $\text{Ca}^{++}$ , and activation of GTP-binding proteins (35, 37, 38). These responses are known to occur after fibroblast exposure to thrombin. Therefore, we compared the effects of tryptase and thrombin on formation of inositol phosphates, on changes in cytosolic  $\text{Ca}^{++}$  concentration, and on reinitiation of DNA synthesis in the presence of pertussis toxin. The concentrations of tryptase and thrombin

**Table II.** Diisopropylfluorophosphate Inhibition of Tryptase and Thrombin: DNA Synthesis and Catalytic Activity Responses

	Tryptase*	Thrombin†
Inhibition of DNA synthesis‡	60.9 $\pm$ 2.7%	85.2 $\pm$ 0.9%
Inhibition of catalytic activity	98.7%	99.2%

\* Tryptase incubated at 3  $\mu\text{M}$ ; molar ratio of DFP to tryptase equaled 300:1. † Thrombin incubated at 1  $\mu\text{M}$ ; molar ratio of DFP to thrombin equaled 100:1. ‡ Assayed by radiolabeled thymidine uptake (see Methods); compared with enzyme incubated 21 h at 4°C, in the absence of DFP; mean  $\pm$  SE for triplicate determinations. || Assayed by cleavage of chromogenic substrate (see Methods); compared with enzyme incubated 21 h at 4°C, in the absence of DFP.



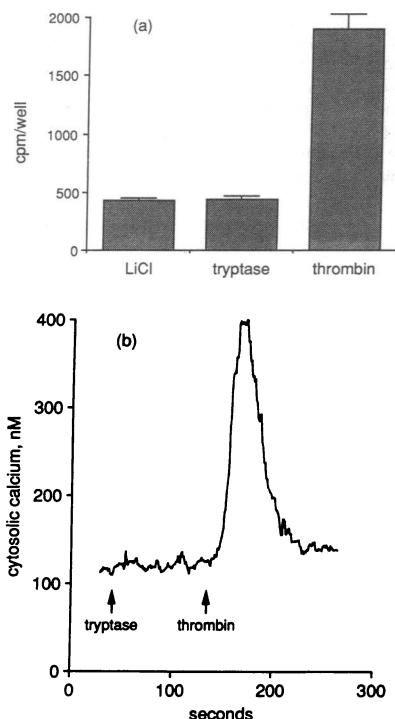
**Figure 5.** Effect of trypsin and thrombin on intracellular pH. The figure shows the results of 10-min incubation of CHL cells with trypsin (7.5 nM) or thrombin (1 nM), in the presence (closed bars) or absence (open bars) of 1 mM amiloride pretreatment. Measurement of

intracellular [ $^{14}\text{C}$ ]benzoic acid and calculation of changes in intracellular pH were performed as described in Methods. Data are mean  $\pm$  SD for triplicate determinations.

used in the following experiments were chosen because they result in similar [ $^3\text{H}$ ]thymidine uptake responses (see Figs. 1 *a* and 3 *d*).

The total inositol phosphates generated after exposure of [ $^3\text{H}$ ]inositol-loaded CHL cells to proteinases was assessed by anion-exchange chromatography, as previously described (29). No increase in inositol phosphate formation was detected after trypsin stimulation, consistent with a lack of phospholipase C activation (Fig. 6 *a*). In contrast, thrombin produced a marked increase in inositol phosphates, as previously reported (39).

An important cellular response to phospholipase C activation is the mobilization of intracellular  $\text{Ca}^{++}$ , mediated through inositol-1,4,5-trisphosphate (37). Alternatively, cytosolic  $\text{Ca}^{++}$  concentration can be elevated through influx of extracellular  $\text{Ca}^{++}$  (36, 40). To determine whether trypsin alters  $\text{Ca}^{++}$  homeostasis through a phospholipase C-independent pathway, we examined the effect of trypsin on intracellular  $\text{Ca}^{++}$  concentration, using the fluorescent  $\text{Ca}^{++}$ -indicator fura-2 (28). No increase in cytosolic  $\text{Ca}^{++}$  concentration was observed after addition of 7.5 nM trypsin to CHL cells (Fig. 6 *b*);



**Figure 6.** Effect of trypsin on inositol phosphate generation (*a*) and cytoplasmic  $\text{Ca}^{++}$  concentration (*b*) in CHL cells. (*a*) Inositol phosphates generated after 10 min stimulation with trypsin (7.5 nM) or thrombin (1 nM) in the presence of 10 mM LiCl. Results represent mean cpm/well ( $\pm$ SD) for three determinations. (*b*) Free cytosolic  $\text{Ca}^{++}$  concentration measured in CHL cells using the fluorescent indicator fura-2. Trypsin (7.5 nM) and thrombin (1 nM) were added sequentially during continuous recording. The tracing shown is representative of three independent measurements.

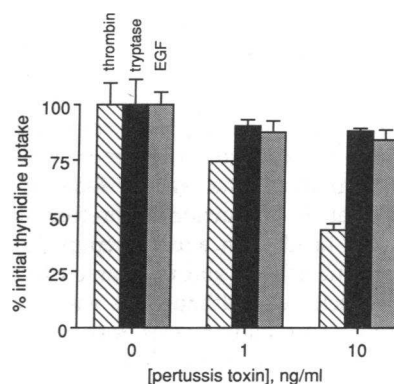
however, a prominent  $\text{Ca}^{++}$  mobilization response was observed with 1 nM thrombin, as previously reported (41). These results indicate that cytosolic  $\text{Ca}^{++}$  is not used as a second messenger in signal transduction by trypsin.

Pertussis toxin-sensitive GTP-binding proteins have been shown to mediate the mitogenic signal of certain growth factors, including thrombin (24, 38). Trypsin-induced stimulation of DNA synthesis in CHL fibroblasts was unaffected by pertussis toxin over a concentration range of 1–10 ng/ml, as was the response to EGF (Fig. 7), a mitogen thought to act through a pertussis toxin-insensitive pathway in fibroblasts (42). Pertussis toxin exposure produced significant inhibition of the thrombin-mediated mitogenic response (Fig. 7), in agreement with Chambard et al. (24).

## Discussion

These data establish trypsin as a potent mitogen for fibroblasts *in vitro*. Trypsin induces the synthesis of DNA in fibroblasts with concomitant cell proliferation. Prompt activation of fibroblasts occurs upon trypsin exposure, as evidenced by  $\text{Na}^+/\text{H}^+$  antiporter activation within 5 min of trypsin addition. Even at threshold concentrations, trypsin markedly potentiates the mitogenic response of fibroblasts to bFGF, EGF, and insulin. The marked reduction of trypsin-induced mitogenesis by proteinase inhibitors suggests that an intact, unoccupied catalytic site is required to exhibit the full response. In these respects, trypsin resembles thrombin, a serine proteinase with similar catalytic features (22, 43). However, although both enzymes stimulate DNA synthesis and cell proliferation in fibroblasts, and both activate the amiloride-sensitive  $\text{Na}^+/\text{H}^+$  antiporter, the two enzymes appear to activate different signaling pathways. The activation of fibroblasts by trypsin involves neither the breakdown of phosphatidylinositol phosphates nor an increase in cytosolic  $\text{Ca}^{++}$ , both of which occur upon stimulation with thrombin. Furthermore, unlike thrombin, trypsin does not appear to activate cells through a pathway involving pertussis toxin-sensitive G-proteins. Although trypsin induces an eightfold increase in CHL cell number over a 6-day growth period, confluence is not achieved. The mechanism for this limit in proliferation is not known.

The specific signal transduction mechanisms responsible for the mitogenic effect of trypsin remain unresolved. However, two lines of evidence reinforce the specificity of trypsin-



**Figure 7.** Pertussis toxin sensitivity of thymidine incorporation in response to thrombin (1 nM; striped bars), trypsin (7.5 nM; black bars), and EGF (100 ng/ml; gray bars) in CHL fibroblasts. Pertussis toxin was added to cells, at the concentrations indicated, 4 h before stimulation and was present throughout the 24-h incubation

with growth factors. [ $^3\text{H}$ ]Thymidine incorporation results were normalized to 100% for each agonist in absence of pertussis toxin and represent mean  $\pm$  SD for triplicate determinations.

induced mitogenesis. First, two additional serine proteinases, trypsin and mast cell chymase, were also tested for their ability to induce DNA synthesis in fibroblasts. Trypsin has known mitogenic capacity in fibroblasts, particularly chick embryo fibroblasts, which appear exquisitely responsive to a number of proteinases (21, 44, 45). Chymase, a chymotrypsin-like mast cell granule proteinase, has been implicated in extracellular matrix degradation and architectural alteration in fibroblasts after mast cell degranulation (46). In Rat-1 cells (Fig. 1 b) and CHL fibroblasts (data not shown), neither trypsin nor chymase increased [ $^3\text{H}$ ]thymidine uptake, whereas tryptase produced striking responses at concentrations similar to, or lower than, those of trypsin and chymase. Second, a marked difference was observed in the [ $^3\text{H}$ ]thymidine incorporation responses of Rat-1 cells to tryptase and thrombin. While both tryptase and thrombin stimulated DNA synthesis in CHL cells, only tryptase produced a response in Rat-1 cells.

The mechanisms underlying mitogenic activity of proteinases, such as thrombin, remain unclear despite considerable work in this field. The participation of proteolytic events in the activation of cells by proteinases has been proposed, though not conclusively established (21). As reported herein, inhibitors of catalytic activity (leupeptin and DFP) produce substantial reductions in the mitogenic response of fibroblasts to tryptase (Fig. 4, and Table II).

The observation that leupeptin and DFP diminish the mitogenic activity of tryptase is consistent with a number of hypotheses. One is that a specific proteolytic event is required for the initiation of signal transduction leading to DNA synthesis and cell proliferation. A second is that binding to the tryptase catalytic site is required, but that the binding protein (possibly a cell surface receptor) acts as a pseudosubstrate, not subject to cleavage. In the latter case, the proteinase would induce a critical conformational change in the transduction protein in the absence of a proteolytic event, thereby initiating cell activation. A third possibility is a binding interaction with a cell surface molecule involving a part of tryptase distinct from the active site.

Clearly, these hypotheses need not be mutually exclusive. Activation by tryptase may require both catalytic and noncatalytic events. It is interesting that the degree of mitogenic inhibition produced by leupeptin and DFP differs. Leupeptin, a tripeptide inhibitor, produces virtually complete mitogenic and catalytic inhibition, whereas DFP produces a lesser degree of mitogenic inhibition of 60%, even though catalytic activity is inhibited almost completely. Given the size difference between these two inhibitors, the difference in magnitude of mitogenic inhibition could be explained by the presence of a binding epitope on tryptase that extends beyond the immediate substrate binding pocket. This binding region, although adjacent to the catalytic site, may not depend on a catalytic event for cell activation. Evidence exists for thrombin that a catalytic site-independent epitope may participate in production of a mitogenic signal in some target cells (47). In addition, a recent report (33) suggests dissociation of catalytic activity and mitogenic effect for thrombin in smooth muscle cells, although, as with the tryptase results presented here, the study reveals only partial inhibition of mitogenic activity by proteinase inhibitors. This would support a mixed catalytic site-dependent and -independent function of proteinases in mitogenic activation. The lack of any significant effect of trypsin or chymase on DNA synthesis in CHL and Rat-1 cells supports the conclusion that nonspe-

cific cell surface proteolytic events are not sufficient to induce S-phase entry in these fibroblasts.

Our in vitro results provide strong support for the hypothesis that mast cell activation can lead to fibroblast proliferation in vivo, and for the possibility that tryptase plays an important role as mediator of this response. This hypothesis is also supported by existing knowledge concerning the activity, concentration, and tissue distribution of this proteinase. Tryptase is released from mast cells as a heparin-associated tetramer of catalytically active subunits, and is unusual among serine proteinases in being active in serum and highly resistant to inactivation by circulating inhibitors (48, 49). Furthermore, the concentrations of tryptase sufficient in our experiments to stimulate fibroblast growth directly or in synergy with other growth factors are predicted to be achieved readily in the microenvironment of the degranulating mast cell (50, 51). Finally, the wide distribution of tryptase-containing mast cells in humans suggests that tryptase may play a part in the development of fibrotic disorders affecting a variety of tissues, including lung, skin, and gut.

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