

# Mast Cell Tryptase Causes Airway Smooth Muscle Hyperresponsiveness in Dogs

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## Abstract

Supernatants obtained by degranulation of dog mastocytoma cells greatly increased the sensitivity and the magnitude of the contractile response of isolated dog bronchial smooth muscle to histamine. The enhanced contractile response was reversed completely by H<sub>1</sub>-receptor antagonists and was prevented by an inhibitor of tryptase (a major protease released with histamine from secretory granules of mast cells). The potentiation of histamine-induced contractions was reproduced by active tryptase in pure form. The contractions due to the combination of histamine and purified tryptase were abolished by the Ca<sup>2+</sup> channel blockers nifedipine and verapamil. The bronchoconstricting effects of KCl and serotonin, which, like histamine, contract airway smooth muscle by a mechanism predominantly involving membrane potential-dependent Ca<sup>2+</sup> transport, were also potentiated by tryptase. However, the contractile effects of acetylcholine, which contracts dog airway smooth muscle by a mechanism independent of Ca<sup>2+</sup> channels, were unaffected by tryptase. These findings show a striking promotion of agonist-induced bronchial smooth muscle contraction by mast cell tryptase, via direct or indirect effects on Ca<sup>2+</sup> channels, and the findings therefore suggest a novel potential mechanism of hyperresponsiveness in dog bronchi.

## Introduction

Mast cells are believed to participate in asthma via release of preformed and newly formed mediators which contract smooth muscle. However, the bronchoconstrictor response to inhaled mast cell-derived mediators such as histamine is greater in asthmatic subjects than in normal individuals. Thus, a key ingredient for asthmatic responses to bronchoactive mediators is hyperresponsiveness, but its pathogenesis is not understood (1). The present study was stimulated by the observation that supernatants obtained by degranulation of dog mastocytoma cells greatly increase the contractile responses of dog bronchi to histamine (2). Because dog mastocytoma cells (3), like mast cells of human lung (4), release neutral serine proteases along with other preformed mediators during de-

granulation, we investigated the possibility that mast cell proteases are responsible for the observed effects of degranulation supernatant. Our results suggest that the marked supernatant-induced increases in responsiveness to histamine are due to tryptase, a proteolytic enzyme of mast cells. The effects of tryptase also extend to other agonists which contract airway smooth muscle by mechanisms involving membrane potential-dependent Ca<sup>2+</sup> transport. Thus, tryptase may play an important role in hyperresponsiveness of dog bronchi to bronchoconstricting agonists.

## Methods

*Production of mastocytoma degranulation supernatant.* Mastocytoma tumors were isolated from cutaneous lesions of dogs. We used the BR line which has been established as a stable line by serial passage as subcutaneous nodules in nude mice (5). Disaggregated cells were activated with Ca<sup>2+</sup> ionophore A23187 using a two-stage reaction, as described previously (6), so that ionophore would not be present in the supernatant added to the muscle bath. Replicate aliquots of cells ( $5 \cdot 10^6$ ) were suspended in Ca<sup>2+</sup>-free, Mg<sup>2+</sup>-free Tyrode's buffer, pH 7.4, containing 25 mM Hepes and 0.1% bovine serum albumin at 4°C. To inhibit the release of cyclooxygenase and lipoxygenase products, the cells were preincubated with BW755C ( $10^{-4}$  M; 30 min) (7, 8). The cells were then incubated with Ca<sup>2+</sup> ionophore ( $3 \cdot 10^{-6}$  M; 20 min) at 4°C, washed three times with Ca<sup>2+</sup>-free, Mg<sup>2+</sup>-free Tyrode's buffer at 4°C, and resuspended in 3 ml of complete Tyrode's buffer at 37°C. After incubation for 30 min, the reaction was stopped by reducing the temperature to 4°C, the tubes were centrifuged at 200 g for 10 min, and the supernatants were used for analysis of histamine and protease content and for studies in muscle baths. Supernatants were prepared in batches, and each series of experiments used supernatant from the same batch.

*Purification of tryptase.* Tryptase was purified from BR dog mastocytoma cells as described previously (9). Tryptase activity in mastocytoma degranulation supernatants and in purified preparations was measured spectrophotometrically with the chromogenic substrate benzoyl-L-val-gly-arg-4-nitroanilide (9).

*Assay of histamine.* Histamine in supernatants and muscle bath bathing fluid was measured spectrofluorometrically (10) using an automated analyzer (ALPKEM Corp., Clackamas, OR).

*Preparation of bronchi.* Mongrel dogs were anesthetized by injection of pentobarbital (30 mg/kg). The thorax was opened, and the lungs and large airways were removed. Rings from intrapulmonary lobar and segmental bronchi were attached to strain gauges to record isometric tension, placed in chambers filled with Krebs-Henseleit solution (pH 7.4; 37°C), and aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Rings were stretched twice initially to a tension of 25 g, equilibrated for 1 h with a resting tension of 4 g, and incubated with indomethacin ( $10^{-6}$  M; 30 min), phentolamine ( $10^{-5}$  M; 15 min), and propranolol ( $10^{-6}$  M; 15 min) to eliminate the influence of prostaglandins and adrenergic mediators.

*Bronchomotor responses to mastocytoma degranulation supernatant.* To study the effect of mast cell-derived mediators on the contractile response of bronchial smooth muscle, supernatants were added to the muscle bath by removing 1.0 ml of Krebs-Henseleit solution from a 14-ml reservoir and replacing it with 1.0 ml of mastocytoma cell

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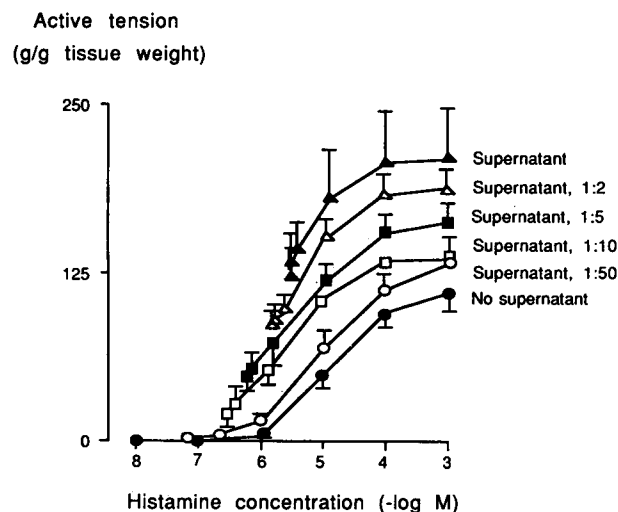
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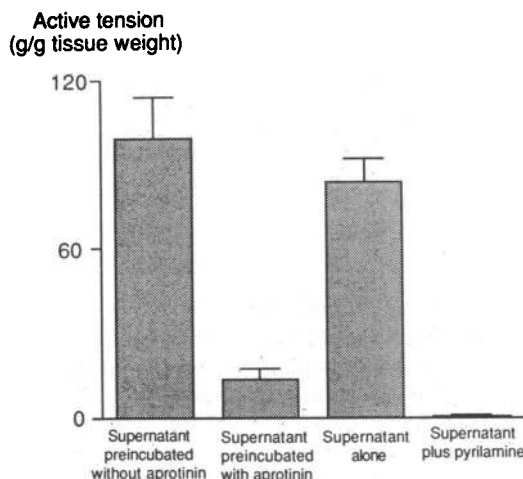
supernatant (or dilution thereof). For each of several concentrations of supernatant, the active tension produced by a range of histamine concentrations ( $10^{-8}$  to  $10^{-3}$  M) was recorded. The effects of histamine receptor antagonists on the contraction produced by 1.0 ml of supernatant were studied using a range of concentrations ( $10^{-9}$  to  $10^{-5}$  M) of the  $H_1$ -antagonists pyrilamine and diphenhydramine, and using a single concentration ( $10^{-5}$  M) of the  $H_2$ -antagonist metiamide. Similarly, the effect of trypsin inhibition on supernatant-induced contraction was examined by preincubating supernatant for 30 min with aprotinin ( $30 \mu\text{g/ml}$ ).

**Bronchomotor responses to purified trypsin.** To conserve enzyme, studies with purified trypsin were performed in 5 ml reservoirs. To stabilize trypsin (9), heparin ( $50 \mu\text{g/ml}$ ) was added to the bath 15 min before adding enzyme. Conditions were otherwise identical to those used in the supernatant muscle bath experiments. The tension produced by the combination of histamine and trypsin was measured in the presence of a range of histamine concentrations ( $10^{-8}$  to  $10^{-3}$  M). To facilitate comparison of the effects of purified trypsin with those produced by supernatant, the concentration of pure enzyme ( $90 \text{ ng/ml}$ ) in the bath was chosen to match that in the 14-ml bath containing 1 ml of supernatant. In separate experiments, the relation of trypsin concentration ( $3\text{--}90 \text{ ng/ml}$ ) to the magnitude of the contractile response was measured in the presence of  $10^{-6}$  M histamine. The effect of the  $\text{Ca}^{2+}$  channel blockers verapamil and nifedipine on trypsin ( $90 \text{ ng/ml}$ ) augmentation of smooth muscle contraction was measured in the presence of  $10^{-5}$  M histamine. To examine the influence of trypsin on smooth muscle contraction by agonists other than histamine, bronchial rings were incubated as above with purified trypsin ( $90 \text{ ng/ml}$ ) and KCl ( $2 \cdot 10^{-5}$  M) or with trypsin and a range of concentrations of serotonin ( $10^{-8}$  to  $10^{-3}$  M) or acetylcholine ( $10^{-8}$  to  $10^{-3}$  M).

**Data analysis.** Data are expressed as means  $\pm$  SEM. Statistical analyses were performed by one-way analysis of variance or by an unpaired *t* test; significance was accepted as  $P < 0.05$ .



**Figure 1.** Effect of mastocytoma degranulation supernatant on concentration-response curves to histamine in dog bronchial smooth muscle (five dogs). The increase in contraction ("active tension") above resting tension was recorded from bronchi in a 14-ml muscle bath over a range of histamine concentrations at each of several concentrations of supernatant obtained by degranulation of dog mastocytoma cells. Depicted are the responses to histamine in the absence of supernatant ( $\bullet$ ), in the presence of 1 ml of supernatant ( $\blacktriangle$ ), and in the presence of 1 ml of twofold diluted supernatant ( $\triangle$ ), fivefold diluted supernatant ( $\blacksquare$ ), 10-fold diluted supernatant ( $\square$ ), and 50-fold diluted supernatant ( $\circ$ ). All data expressed as means  $\pm$  SEM.



**Figure 2.** Effect of protease inhibitor and histamine  $H_1$ -receptor antagonist on mastocytoma degranulation supernatant-induced contraction of dog bronchi. The active tension was recorded from bronchi in a 14-ml muscle bath containing 1 ml of dog mastocytoma degranulation supernatant. Supernatant was preincubated with  $30 \mu\text{g/ml}$  aprotinin, an inhibitor of dog trypsin, before incubation with bronchi (five dogs). Control tissues were incubated with supernatant preincubated for 30 min without aprotinin (five dogs). In separate experiments (nine dogs), bronchi contracted with mastocytoma supernatant ("supernatant alone") were then exposed to the  $H_1$ -receptor antagonist pyrilamine ( $10^{-3}$  M) to measure the response to  $H_1$ -receptor blockade ("supernatant plus pyrilamine"). All data expressed as means  $\pm$  SEM.

## Results

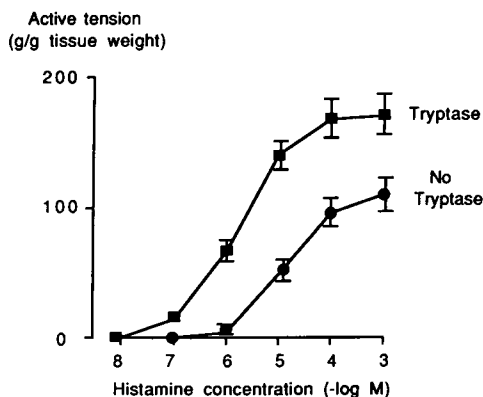
**Bronchomotor responses to mastocytoma degranulation supernatant.** When muscle was preincubated with mastocytoma supernatant, the histamine response curve was shifted strikingly to the left, and the maximum tension achieved was greater than the response to exogenous histamine alone. These augmented contractile responses to histamine were dependent on the dilution of the added supernatant (Fig. 1). In these studies, the concentration of histamine in the muscle bath in the presence of 1 ml of undiluted supernatant was  $3 \cdot 10^{-6}$  M. The supernatant-induced contractions were inhibited by the  $H_1$ -receptor antagonists pyrilamine ( $10^{-5}$  M;  $0.7 \pm 0.5\%$  of control;  $n = 9$ ;  $P < 0.01$ ) (Fig. 2) and diphenhydramine ( $10^{-5}$  M;  $0.0 \pm 0.0\%$  of control;  $n = 6$ ;  $P < 0.01$ ) with  $\text{IC}_{50}$ 's of  $0.63 \pm 0.21 \cdot 10^{-6}$  M and  $0.25 \pm 0.06 \cdot 10^{-6}$  M, respectively. In contrast, supernatant-induced contractions were increased by the  $H_2$ -receptor antagonist metiamide ( $10^{-5}$  M;  $125.3 \pm 7.3\%$  of control;  $P < 0.05$ ). These studies indicated that an ingredient of the mastocytoma supernatant potentiates histamine-induced contractions in a concentration-dependent manner via an  $H_1$ -receptor.

Because dog BR mastocytoma cells release the serine protease trypsin into degranulation supernatants (3), we tested the effects of the protease inhibitor aprotinin on supernatant-induced bronchial smooth muscle contraction. Aprotinin inhibits purified dog mastocytoma trypsin (9) but has no effect on purified chymase (11), the other major secretory serine protease of mast cell granules. Muscle bath fluid with 1 ml of undiluted supernatant contained  $90 \text{ ng/ml}$  of active trypsin in these experiments. In pilot studies we confirmed that aprotinin

incubated with mastocytoma supernatant selectively inhibits the amidolytic activity of tryptase without affecting chymase (data not shown). Preincubation of supernatant with aprotinin markedly decreased bronchial smooth muscle contraction caused by supernatant compared to the responses after preincubation of supernatant without aprotinin (Fig. 2): 1 ml of supernatant preincubated with aprotinin produced tension of only  $14.2 \pm 3.2$  g/g tissue weight compared to  $99.3 \pm 14.7$  g/g tissue weight produced by supernatant preincubated without aprotinin ( $P < 0.05$ ). The response in the presence of aprotinin is similar to the tension expected from histamine alone in the muscle bath ( $\sim 15$  g/g tissue weight, obtained by extrapolation of data from the histamine-response curve given in Fig. 1). These effects of aprotinin suggested that tryptase may be the ingredient in mastocytoma supernatants which augments histamine-induced bronchial smooth muscle contraction.

**Bronchomotor responses to purified tryptase.** To establish more definitively the role of tryptase suggested by the supernatant experiments, we studied the actions of highly purified tryptase in the muscle bath. Tryptase, in a concentration matching that in the supernatant bath (90 ng/ml), did not affect resting tension, and neither heparin alone nor the vehicle for tryptase alone had an effect on the histamine concentration-response curve ( $P > 0.5$ ). However, tryptase caused a marked leftward shift of the histamine concentration-response curve, decreasing the  $ED_{50}$  for histamine, and also increased the maximum tension after histamine (Fig. 3 and Table I). These effects were similar to those produced by the supernatant containing identical tryptase activity. Smooth muscle contractile responses at a fixed concentration of histamine ( $10^{-6}$  M) increased with increasing concentrations of tryptase in the bath (Fig. 4).

Because proteases may release mast cell histamine (12), we studied the effect of tryptase on the histamine content in the smooth muscle chamber. Incubation of bronchial rings with tryptase (90 ng/ml) increased the histamine content in the bathing medium slightly but not significantly (from  $0.43 \pm 0.28 \cdot 10^{-8}$  M to  $6.30 \pm 2.80 \cdot 10^{-8}$  M;  $n = 6$ ,  $P > 0.05$ ). These concentrations of histamine did not contract bronchial muscle in the bath (Fig. 1) and therefore can not explain the tryptase-induced muscle effects. Furthermore, the increased



**Figure 3.** Effect of purified tryptase (90 ng/ml) on concentration-response curve to histamine in bronchial smooth muscle (five dogs). Histamine-induced active tension was measured in bronchial rings exposed (■) or unexposed (●) to tryptase. Data expressed as means  $\pm$  SEM.

**Table I.** Effect of Tryptase on Agonist-induced Bronchial Smooth Muscle Contraction

	ED <sub>50</sub> *	Maximum tension
	10 <sup>-6</sup> M	g/g tissue weight
Histamine	19.11 $\pm$ 3.50	110.6 $\pm$ 10.4
Histamine + tryptase	2.03 $\pm$ 0.60 ( $P < 0.05$ ) <sup>†</sup>	170.4 $\pm$ 12.7 ( $P < 0.01$ ) <sup>†</sup>
Serotonin	2.16 $\pm$ 0.40	79.5 $\pm$ 6.9
Serotonin + tryptase	0.46 $\pm$ 0.09 ( $P < 0.05$ ) <sup>†</sup>	99.7 $\pm$ 2.8 ( $P < 0.05$ ) <sup>†</sup>
KCl (20 mM)	Not done	92.8 $\pm$ 5.7
KCl + tryptase	Not done	155.0 $\pm$ 9.7 ( $P < 0.01$ ) <sup>†</sup>
Acetylcholine	10.89 $\pm$ 2.48	157.7 $\pm$ 3.6
Acetylcholine + tryptase	11.00 $\pm$ 3.90 ( $P > 0.05$ ) <sup>†</sup>	160.3 $\pm$ 3.8 ( $P > 0.05$ ) <sup>†</sup>

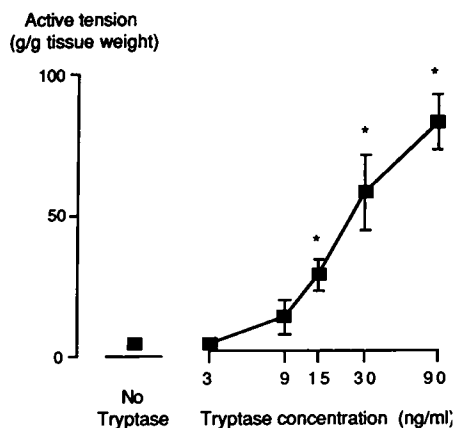
\* Estimated concentration of agonist producing half-maximum tension.

<sup>†</sup> Agonist + tryptase compared with agonist alone.

responses after tryptase were not mimicked by concentrations of histamine as high as  $10^{-3}$  M (Fig. 1), and tryptase alone did not increase resting tension, as would be expected if significant concentrations of histamine were released.

Serotonin and KCl, like histamine, contract dog airway smooth muscle predominantly via Ca<sup>2+</sup> transport through voltage-dependent channels (13–15). We found that the responses of dog bronchi to serotonin and KCl, like those to histamine, were also potentiated by tryptase (Table I). Thus, in five dogs, the  $ED_{50}$  for serotonin was decreased by tryptase and the maximum contraction produced by serotonin was increased. In five dogs, tryptase also markedly increased the active tension produced by KCl ( $2 \cdot 10^{-5}$  M; Table I).

In separate experiments in five dogs, the time course of tryptase potentiation of KCl-induced contraction was examined. After incubation of muscle with tryptase (90 ng/ml),



**Figure 4.** Effect of concentration of purified tryptase on histamine-induced contraction of bronchial rings (five dogs). Effect of histamine alone ( $10^{-6}$  M) is shown on the left; effect of histamine ( $10^{-6}$  M) plus different concentrations of tryptase is shown on the right; \* $P < 0.01$  (compared to histamine alone). Data expressed as means  $\pm$  SEM.

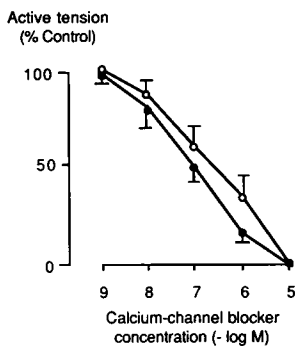


Figure 5. Effect of calcium channel blockers verapamil (○) and nifedipine (●) on bronchial smooth muscle contraction induced by a combination of tryptase (90 ng/ml) and histamine ( $10^{-5}$  M) in bronchial smooth muscle in five dogs. Data expressed as percent of the tension produced in the absence of blockers (means $\pm$ SEM).

responses to rechallenges with KCl ( $2 \cdot 10^{-5}$  M) following washout of tryptase were significantly increased when measured after 20 min ( $156.0 \pm 9.8$  g/g tissue weight;  $P < 0.001$ ), 1 h ( $164.3 \pm 10.0$  g/g tissue weight;  $P < 0.001$ ), and 2 h ( $138.2 \pm 8.6$  g/g tissue weight;  $P < 0.01$ ) compared to the tension ( $96.4 \pm 4.9$  g/g tissue weight) developed in response to KCl in control tissues which had not been exposed to tryptase. Tension returned to the control state within 3 h ( $93.6 \pm 5.2$  g/g tissue weight;  $P > 0.05$ ). Thus, the effects of tryptase persisted after removal of enzyme from the bath, with a slow rate of return to the control level of responsiveness.

Because acetylcholine contracts airway smooth muscle by mobilizing intracellular  $Ca^{2+}$  stores (13, 14), a mechanism that differs from that of histamine, serotonin and KCl (14, 15), we studied the effect of tryptase (90 ng/ml) on the concentration-response curve to acetylcholine. In five dogs, the  $ED_{50}$  for acetylcholine and the maximum tension produced by acetylcholine were unaffected by tryptase (Table I). These findings demonstrate that the potentiating effects of tryptase extend to other agonists which contract smooth muscle by stimulating  $Ca^{2+}$  transport via voltage-dependent channels, but do not extend to acetylcholine, which contracts smooth muscle by a different mechanism.

To further investigate the possibility that tryptase-augmented muscle contraction is mediated via an influence on  $Ca^{2+}$  channels, we studied the effects of verapamil and nifedipine, which block that portion of  $Ca^{2+}$  influx which is due to membrane depolarization in smooth muscle (16). We found that verapamil and nifedipine reverse the large contractions produced by the combination of tryptase (90 ng/ml) and histamine ( $10^{-5}$  M) with  $IC_{50}$  values of  $0.59 \pm 0.27 \cdot 10^{-6}$  M and  $0.12 \pm 0.04 \cdot 10^{-6}$  M, respectively, and that both  $Ca^{2+}$  channel blockers reversed the contractions completely at concentrations of  $10^{-5}$  M (Fig. 5).

## Discussion

The marked potentiation of histamine-induced bronchial contraction by mastocytoma degranulation supernatants demonstrates a novel and potentially important interaction between mast cell products and airway smooth muscle. The similar smooth muscle responses to tryptase-containing supernatant and to matching concentrations of pure tryptase suggest that the principal ingredient responsible for supernatant effects is tryptase. Because supernatant-augmented histamine contractions were abolished by an inhibitor of tryptase, it is likely that the effects require catalytically active enzyme. The persistent augmentation of KCl-induced contractions after removal of

tryptase from the muscle bath is consistent with a mechanism involving proteolytic cleavage rather than simple binding; the return to the control state indicates that the action of tryptase does not involve irreversible membrane damage.

The observation that tryptase potentiates the contractile effects of histamine, serotonin, and KCl suggests that the enzyme exerts its effects at a point in the stimulus-contraction pathway shared by the three chemically distinct agonists. Because the smooth muscle effects of these three agonists depend on the movement of external  $Ca^{2+}$  into the cell, and because  $Ca^{2+}$  channel blockers abolish contractions induced by the combination of tryptase and histamine, we suggest that tryptase may affect airway smooth muscle responsiveness by modifying  $Ca^{2+}$  channels. This hypothesis is supported by the failure of tryptase to augment the contractions produced by acetylcholine, a bronchoconstricting agonist whose smooth muscle effects are mediated by a mechanism independent of voltage-dependent  $Ca^{2+}$  channels. Tryptase could affect the channel itself. Alternatively, it could cleave a regulatory protein on the cell surface. More studies are needed to investigate these possibilities and to determine the molecular basis of the action of tryptase on airway smooth muscle.

The relevance *in vivo* of the levels of tryptase used in the muscle bath is difficult to establish with certainty. However, the highest concentration used (90 ng/ml) is within the range of those detected in the serum of patients with systemic anaphylaxis or mastocytosis (17). The predominance of tryptase in lung mast cells (18) adds to the likelihood that this enzyme may play an etiologic role in airway smooth muscle hyperresponsiveness. Human airways contain  $33 \mu\text{g}$  of tryptase per  $\text{cm}^3$  in the submucosa of segmental bronchi, assuming a mast cell density of  $3 \cdot 10^6$  cells per  $\text{cm}^3$  (19) and  $11 \mu\text{g}$  of tryptase per  $10^6$  cells (18). Therefore, the concentration of tryptase in human airway tissue is 2–3 orders of magnitude greater than the concentration used in these experiments.

Several previous studies provide evidence of airway hyperresponsiveness *in vivo* in dogs inhaling  $O_3$  (20), leukotriene  $B_4$  (21), or allergen (22). These stimuli all result in the release of thromboxane, which in turn increases bronchomotor responses via vagal motor pathways (23). Effects of thromboxane and tryptase differ as follows: (a) thromboxane causes a small shift to the left in the response to electrical field stimulation (23), while tryptase causes a striking leftward shift and an increase in maximum tension in response to agonists (histamine, serotonin, KCl) that cause contraction via  $Ca^{2+}$  channels; (b) thromboxane acts through pre- or postsynaptic effects on motor nerves which then release acetylcholine, while our evidence suggests that tryptase may act on smooth muscle to affect voltage-dependent  $Ca^{2+}$  channels.

Thus, it seems that different mediators may alter dog airway smooth muscle responsiveness in at least two different ways: tryptase, released by activated mast cells, may potentiate muscle responses via direct or indirect effects on voltage-dependent  $Ca^{2+}$  channels; other mediators, such as thromboxane, may cause changes in vagal nervous pathways. It is possible that one or both mechanisms may contribute to the hyperresponsiveness that plays such an important role in the pathogenesis of asthma.

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