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Biochemical Pharmacology 66 (2003) 519–525
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Proteinase-activated receptor-2 mediates hyperresponsiveness in isolated guinea pig bronchi

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Received 10 March 2003; accepted 11 April 2003

Abstract

The mast cell serine protease tryptase has been implicated as a critical mediator of airway hyperresponsiveness *in vitro* and *in vivo*. We have previously demonstrated that tryptase promotes hyperresponsiveness in isolated guinea pig bronchi. In this study, we have investigated the potential role of tryptase-mediated activation of proteinase-activated receptor-2 (PAR-2) in promoting airway hyperresponsiveness. *Ex vivo* exposure of guinea pig bronchi to the PAR-2 agonists H_2N-Ser-Leu-Ile-Gly-Arg-Leu-$CONH_2$ (SLIGRL) and *t*-cinnamoyl-H_2N-Leu-Ile-Gly-Arg-Leu-O-$CONH_2$ (*t*-c-LIGRLO) (0.1–10 μM) induced a concentration-dependent increase of contractile response to histamine. Treatment with 10 μM SLIGRL or *t*-c LIGRLO for 45 min increased subsequent responsiveness to histamine (0.3 mM) by 54 ± 3% and 69 ± 5%, respectively ($P < 0.05$ vs. control). In contrast, the PAR-1 agonist peptide H_2N-Ser-Phe-Leu-Leu-Arg-Asn-$CONH_2$ (SFLLRN) did not promote significant changes in the airway. Effects of the peptides were observed following at least a 30-min preincubation with the tissue. Coincubation with indomethacin or removal of epithelial cells is required for PAR-2-mediated hyperreactivity. The inactive analogue H_2N-Leu-Ser-Ile-Gly-Arg-Leu-$CONH_2$ (LISGRL; 10 μM) failed to promote hyperresponsiveness. Neuropeptide antagonists blocked the effect of the PAR-2 agonists. Selective antagonists of NK1 (L-703,606), NK2 (L-659,877), and CGRP (αCGRP 8–37) provided additive inhibition of PAR-2-mediated hyperreactivity. Pretreatment of bronchi with capsaicin (0.8 μM) also prevented the effects of SLIGRL. These results demonstrate the potential involvement of tryptase-mediated activation of PAR-2 in promoting airway hyperresponsiveness. These results further demonstrate that the PAR-2-mediated response involves a neurogenic mechanism involving neuropeptide release.

Keywords: PAR-2; Tryptase; Airway hyperresponsiveness; Neurogenic

Tryptase is a serine protease released from secretory granules of mast cells. This enzyme has been associated with cellular responses associated with asthma pathology. Tryptase has been shown to stimulate allergic mediator release from mast cells [1,2]. Tryptase also promotes airway inflammation through stimulation of the production of pro-inflammatory chemokines and expression of leukocyte adhesion molecules by epithelial cells and endothelial cells [3–5]. The enzyme also contributes to airway edema through induction of vascular permeability [2,6]. In addition, tryptase has a mitogenic effect on lung fibroblasts [7], airway smooth muscle cells [8], and epithelial cells [4].

Tryptase has also been shown to contribute to pathophysiologic responses associated with asthma. Inhaled tryptase has been shown to promote bronchoconstriction and airway hyperreactivity *in vivo* [1] and *ex vivo* [9,10]. In addition, inhibitors of tryptase have been shown to prevent bronchoconstriction and the development of airway hyperreactivity *in vivo* following allergen bronchoprovocation in both animal models of asthma [11–15] as well as in human clinical trials [16].

Efforts to understand the role of tryptase in asthma were greatly facilitated by the identification and characterization of the tryptase- and trypsin-activated proteinase-activated

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Abbreviations: PAR, proteinase-activated receptor; SLIGRL, H_2N-Ser-Leu-Ile-Gly-Arg-Leu-$CONH_2$; *t*-c-LIGRLO, *t*-cinnamoyl-H_2N-Leu-Ile-Gly-Arg-Leu-O-$CONH_2$; SFLLRN, H_2N-Ser-Phe-Leu-Leu-Arg-Asn-$CONH_2$; LSIGRL, H_2N-Leu-Ser-Ile-Gly-Arg-Leu-$CONH_2$; L-703,606, *cis*-2-[diphenylmethyl]-*N*-[(2-iodophenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine; L-659,877, cyclo(Gln-Trp-Phe-Gly-Leu-Met); αCGRP (8–37), calcitonin gene-related peptide fragment 8–37; SLPI, secretory leukocyte protease inhibitor; APC-366, *N*-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide.

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doi:10.1016/S0006-2952(03)00292-2

receptor-2 (PAR-2) [17]. Cleavage of the extracellular amino terminal domain of PAR-2 by mast cell tryptase or trypsin [18,19] exposes a peptide sequence that serves as a tethered ligand [20–23] that binds to the second extracellular loop of the receptor to stimulate G protein-mediated signal transduction [24,25]. Cellular responses promoted by the proteolytic activation of PAR-2 can be mimicked by the use of soluble peptides corresponding to the tethered ligand exposed following tryptase-mediated cleavage [20,22,26,27]. Stimulus-response coupling involves both phosphatidylinositol hydrolysis [18,26,28] leading to cytosolic calcium mobilization [18–20,22,25,29] and the mitogen-activated protein (MAP) kinase cascade [30].

PAR-2 activation has subsequently been associated with cellular processes similar to those promoted by tryptase. PAR-2-mediated pro-inflammatory [27,31–33] and mitogenic effects [34–36] have been reported.

In previous work, we demonstrated that *ex vivo* exposure of guinea pig bronchi to tryptase promotes the development of hyperresponsiveness [10]. The development of hyperresponsiveness was characterized by an increase in the maximal contractile response of the bronchial smooth muscle to histamine and serotonin. We speculated that activation of PAR-2 by tryptase was a potential mechanism to explain the tryptase-induced development of airway hyperresponsiveness. The potential role of PAR-2 is further supported by the immunohistochemical localization of PAR-2 to the airways by a variety of cell types, including endothelial cells, epithelial cells, smooth muscle cells, and neurons [37,38].

In this work, we examine the potential role of PAR-2 in tryptase-mediated development of airway hyperresponsiveness. Synthetic peptides corresponding to the structure of the tethered ligands for the PAR receptors are assessed for the ability to promote histamine hyperreactivity in isolated guinea pig airway tissue. In addition, the mechanism of PAR-mediated hyperresponsiveness is evaluated.

1. Materials and methods

1.1. Tissue preparation

Male Hartley guinea pigs (500–800 g) were sacrificed by asphyxiation using CO_2. The lungs were removed and placed in cold oxygenated (95% O_2 + 5% CO_2) Krebs solution (pH 7.4). The bronchi were dissected and cleaned of excess fat and connective tissue. Bronchial segments, measuring 2 mm in length, were suspended in a 25 mL tissue bath at 37°, filled with Krebs oxygenated solution. Tissues were equilibrated for 1 hr and then washed once. Tissues were stretched to a resting tension of 300 mg for 1 hr in the presence of the cyclooxygenase inhibitor indomethacin (1 μM), the adrenergic antagonist phentolamine (10 μM), and the beta-adrenergic receptor agonist isoproterenol (0.1 μM). After a stable baseline was achieved, tissues were incubated with PAR receptor agonists. The effects of the agonists on the smooth muscle responsiveness were evaluated by their subsequent response to histamine (0.01 μM to 0.3 mM). The NK_1 antagonist L-703,606, the NK_2 antagonist L-659,877, and the CGRP receptor antagonist αCGRP (8–37), were added to the tissue bath immediately prior to addition of PAR peptides and co-incubated both for 45 min before histamine was added to the preparation. Tissues were then washed at least twice during a period of 1 hr until they returned to baseline and then contracted maximally to histamine (0.3 mM). In experiments to deplete tachykinins from C fibers, capsaicin (0.8 μM), was added to the tissues for 15 min, and washed extensively for a period of at least 1 hr, after which capsaicin (0.4 μM) was added to the tissue to confirm depletion prior to the addition of SLIGRL.

1.2. Experimental procedure

Tissues were exposed to different peptides concentrations (0.1–30 μM) for varying exposure times (15–50 min) to study concentration and incubation time effects. Each isolated segment received a single concentration of the peptide for a period of time followed by histamine concentration response curve as described above.

The isometric contractions of the bronchial segments were recorded usings a Radnotti isometric force transducer (Radnotti Inc.) connected to a MacLab analog to digital converter system (AD Instruments Inc.) and a Macintosh Performa 630 CD computer. Statistical significance of dose response data was determined by ANOVA followed by Fisher's PLSD and Bonferroni/Dunn analysis.

1.3. Drugs

Peptides used in this research were synthesized at Amgen Inc. APC-366 and secretory leukocyte protease inhibitor (SLPI) were also produced by Amgen. Histamine, capsaicin, phentolamine, indomethacin, and isoproterenol were purchased from Sigma Chem. Co. L-703,606, L-659,877, and αCGRP (8–37) were obtained from Sigma-RBI.

2. Results

2.1. Effects of the PAR-1 and PAR-2 agonists on bronchial hyperreactivity

Isolated guinea pig bronchi were treated with 10 μM SLIGRL (specific PAR-2 agonist), LSIGRL (inactive PAR-2 analog), or SFLLRN (selective PAR-1 agonist) [39] for a period of 50 min. The tissues were subsequently contracted with histamine (0.3 mM). Tissues treated with SLIGRL showed an increased maximal response to histamine (48 ± 4%) when compared to untreated bronchi. In contrast, tissues exposed to the inactive peptide LSIGRL and

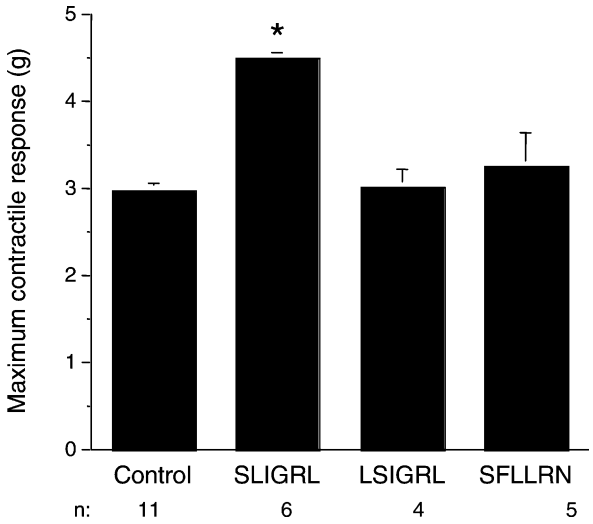

Fig. 1. PAR-2-specific induction of hyperresponsiveness to histamine in isolated guinea pig bronchi. Isolated bronchi were treated with SLIGRL (PAR-2 agonist), LSIGRL (inactive PAR-2 analog), or SFLLRN (PAR-1 agonist) (10^{-5} M) for 30 min at 37°. Tissues were subsequently contracted with histamine (3×10^{-4} M). Bronchial response to histamine is expressed as maximum contractile response (g, mean ± SEM) ($^{*}P < 0.05$ vs. histamine response of control tissue; N = 4–11).

the PAR-1 agonist SFLLRN showed no difference from control responses (Fig. 1). Treatment with synthetic PAR-2 agonist *t*-c-LIGRLO also increased the maximal response to histamine (69 ± 5%).

In comparison, we previously showed that exposure of bronchi to tryptase (90 ng/mL for 45 min) increased contractility to histamine by 84% [10]. These results demonstrate PAR-2-specific development of airway hyperreactivity.

2.2. Dose- and time-dependent effects of the PAR-2 agonist SLIGRL on bronchial hyperreactivity

The dose-dependent effects of the PAR-2 agonist SLIGRL on airway hyperreactivity is shown in Fig. 2. Following pretreatment with varying concentration of SLIGRL, bronchial tissue contraction is stimulated with histamine. The dose-dependent effect of SLIGRL is shown when the subsequent response to histamine is graphed as a function of grams of contractile response (Fig. 2a). The maximal response to SLIGRL was reached at a concentration of 10 μM. At this concentration, treatment with SLIGRL increased contractility to histamine (0.3 mM) by 54 ± 3%. In contrast, when the histamine response is graphed as a function of percent of maximal response (Fig. 2b), responses of control and SLIGRL-treated tissues are identical. This observation shows that SLIGRL does not promote hyperreactivity by affecting the affinity of the histamine receptor for its ligand.

The effects of the PAR-2 agonist SLIGRL were time dependent (Fig. 3). Isolated bronchi treated with SLIGRL (10 μM) showed that a minimum incubation time of

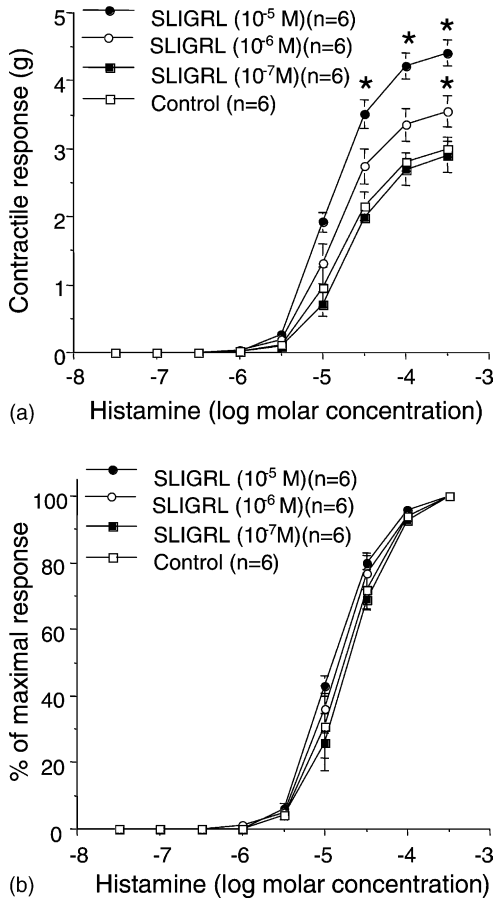

Fig. 2. Dose-dependent stimulation of histamine hyperresponsiveness by the PAR-2 agonist SLIGRL. (A) Isolated bronchi treated with SLIGRL were subsequently contracted with histamine. Bronchial response to histamine is expressed as maximum contractile response (g, mean ± SEM) ($^{*}P < 0.05$ vs. histamine response of control tissue; N = 3–6). (B) Isolated bronchi treated with SLIGRL were subsequently contracted with histamine. Bronchial response to histamine is expressed as percentage of maximum histamine contractile response (g, mean ± SEM) (N = 3–6).

30 min was required to promote hyperreactivity to histamine. In comparison, we previously showed that tryptase required a 45-min incubation with bronchial tissue for maximal promotion of hyperreactivity [10].

Differences in the time-dependence of the response may be a result of differences in specific receptor/ligand interactions provided by the use of SLIGRL compared with tryptase-mediated proteolytic cleavage/activation of PAR-2. While the tryptase-induced response could be prevented with the tryptase inhibitor APC-366 [10], these protease inhibitors had no effect on the SLIGRL-induced response (Fig. 4). These results confirm that peptide acts downstream and independent from the proteolytic response to tryptase.

2.3. Role of neurokinins in PAR-2-mediated hyperreactivity

A potential neurogenic mechanism for PAR-2-mediated hyperreactivity was assessed using specific neurokinin and neuropeptide antagonists. Isolated bronchial tissue was

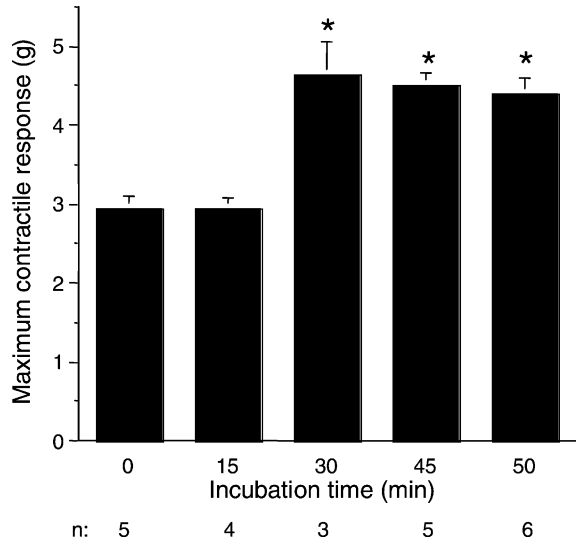

Fig. 3. Time-dependent stimulation of histamine hyperreactivity by SLIGRL. Isolated bronchi treated with SLIGRL (10^{-5} M) for various times at 37°. Tissues were subsequently contracted with histamine (3×10^{-4} M). Bronchial response to histamine is expressed as maximum contractile response (g, mean ± SEM) ($^{*}P < 0.05$ vs. histamine response of control tissue; N = 3–6).

treated with SLIGRL (10 μM) alone or in the presence of the NK_1 antagonist L-703,606, the NK_2 antagonist L-659,877, or CGRP receptor antagonist αCGRP (8–37) (Fig. 5). Each antagonist gave significant inhibition of SLIGRL-induced hyperreactivity. Combination treatment with all three antagonists resulted in complete inhibition of hyperreactivity. These results suggest that SLIGRL promotes hyperreactivity indirectly by stimulating the release of neurokinins from afferent neurons in the bronchial tissue preparation.

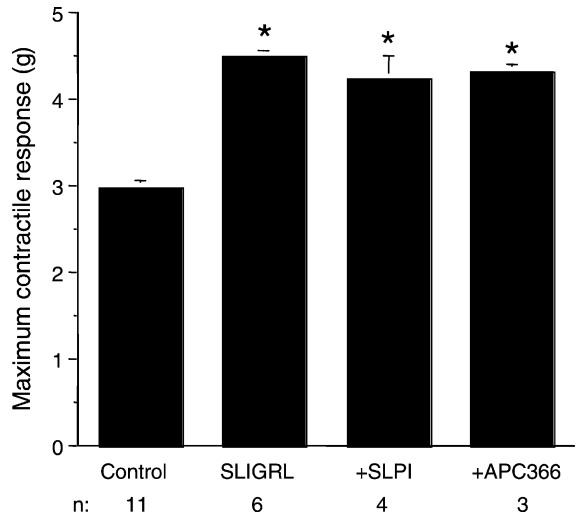

Fig. 4. Effect of SLPI and APC-366 on SLIGRL-stimulated airway hyperreactivity. Isolated bronchi were incubated with SLIGRL (10^{-5} M) alone or in the presence of SLPI (3×10^{-6} M) or APC-366 (3×10^{-6} M) at 37°. Tissues were subsequently contracted with histamine (3×10^{-4} M). Bronchial response to histamine is expressed as maximum contractile response (g, mean ± SEM) ($^{*}P < 0.05$ vs. histamine response of control tissue; N = 3–11).

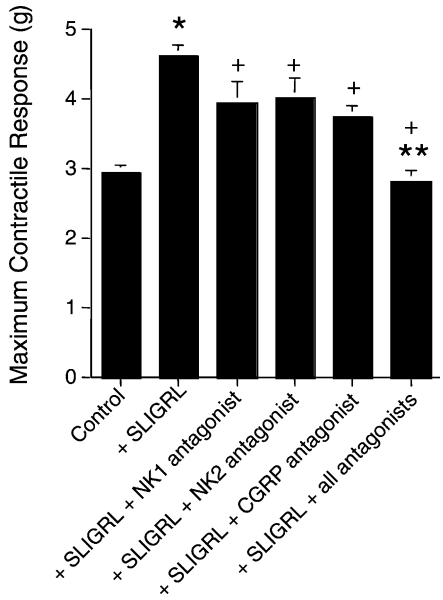

Fig. 5. Effect of neuropeptide antagonists on SLIGRL-induced airway hyperreactivity. Isolated bronchi were incubated with SLIGRL (10^{-5} M) for 30 min at 37° in the absence or presence of neuropeptide antagonists (3×10^{-4} M). The NK1 antagonist L-703,606, the NK_2 antagonist L-659,877, or the CGRP receptor antagonist αCGRP (8–37) were incubated individually or together. Bronchial response to histamine is expressed as maximum contractile response (g, mean ± SEM) ($^{*}P < 0.05$ vs. histamine response of control tissue; $^{+}P < 0.05$ vs. SLIGRL-stimulated response; $^{**}P < 0.05$ vs. effect of individual antagonists; N = 4–17).

To confirm this result, the effect of SLIGRL was assessed in tachykinin-depleted tissue (Fig. 6). C fiber nerve terminals were depleted of tachykinins by pretreatment with

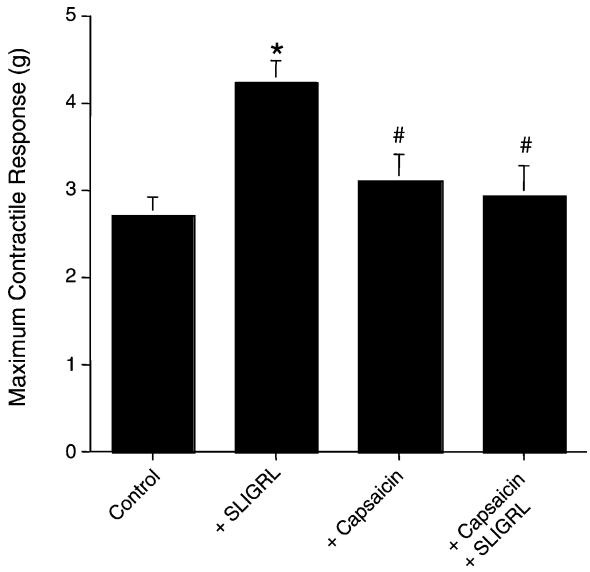

Fig. 6. Effect of capsaicin pretreatment on SLIGRL-induced airway hyperreactivity. Isolated bronchi were treated with capsaicin (10^{-6} M) for 15 min at 37°. After several washes, tissues were incubated with SLIGRL (10^{-5} M) for 30 min. The tissues were subsequently contracted with histamine (3×10^{-4} M). Bronchial response to histamine is expressed as maximum contractile response (g, mean ± SEM) ($^{*}P < 0.05$ vs. histamine response of control tissue; $^{\#}P < 0.05$ vs. SLIGRL-stimulated response; N = 7–17).

capsaicin (0.8 μM) prior to exposure to SLIGRL. SLIGRL had no effect on capsaicin-treated tissues. These results suggest that the SLIGRL-mediated induction of hyperreactivity is a neurogenic response involving the stimulated release of neurokinins.

3. Discussion

The present studies show that exposure of isolated bronchi to the peptidic PAR-2 agonists results in increased responsiveness of bronchial smooth muscle contractility to histamine. In contrast, the PAR-2-inactive analogue LSIGRL and the PAR-1 agonist SFLLRN have no effect on bronchial contractility in response to histamine. The results are consistent with our hypothesis that tryptase promotes airway hyperresponsiveness through cleavage and activation of PAR-2 [10]. As previously reported, the response following tryptase treatment was greater than the response observed following treatment with peptidic PAR-2 agonists. This increase may be related to the efficiency of tryptase-mediated catalytic activation of PAR-2 or to differences in agonist activities of tethered/ soluble PAR-2 agonists.

Several factors led us to evaluate the potential role of neuropeptides in PAR-2-induced airway hyperreactivity. While tryptase/PAR-2 has been shown to promote airway remodeling *in vivo* and smooth muscle cell proliferation *in vitro*, the rapid onset of the PAR-2 response precluded such a mechanism. This, however, does not preclude the potential impact of chronic *in vivo* exposure to tryptase on asthma pathophysiology. The potential for *de novo* synthesis of a PAR-2-stimulated mediator also appeared unlikely. In addition, the inability of PAR-2 to impact histamine responsiveness when measured as percentage of maximal response suggested that the smooth muscle response mediated through histamine was not directly affected by PAR-2 agonists. The expression of PAR-2 on neurons in the airway and their ability to release preformed mediators capable of promoting hyperreactivity prompted us to examine a potential neurogenic mechanism to explain the PAR-2 response.

In order to demonstrate a PAR-2-induced neurogenic mechanism for airway hyperreactivity, we first evaluated the effects of NK_1, NK_2, and CGRP antagonists. Each antagonist alone provided significant inhibition. Complete inhibition was achieved when the antagonists were used in combination. These results supported the hypothesis that activation of PAR-2 receptor in the airways may stimulate release of neuropeptides such as substance P, neurokinins and CGRP from afferent C fibers. This observation was further supported by results demonstrating that depletion of neurokinins from sensory nerves by capsaicin pretreatment prevented SLIGRL-induced hyperresponsiveness of isolated bronchi. These results are consistent with previous work demonstrating the involvement of the NK_1 receptor on the development of hyperreactivity and airway inflammation in conscious guinea pigs [40]. These results are supported by recent work demonstrating that tryptase and SLIGRL directly signals neurons to release substance P and CGRP, resulting in a neurogenic inflammatory response [41]. In addition, trypsin and PAR-2 agonists have been reported to induce neurokinin-mediated contraction of guinea pig bronchi [42,43].

At first, these observations may appear to be inconsistent with the report of Cocks *et al.* [44] that activation of PAR-2 receptors results in bronchodilation as a function of stimulated production epithelium-derived prostaglandins. However, in our studies with isolated bronchial tissue, the cyclooxygenase inhibitor indomethacin is included in the incubation buffer. In the absence of indomethacin, SLIGRL-induced hyperreactivity is not observed unless the epithelial cell layer is removed from the bronchial preparations (data not shown). These results suggest that disruption of the epithelial layer as a consequence of airway inflammation may result in increased susceptibility to PAR-2 activation. This is supported by the observation in asthma patients that damage to airway epithelium increases bronchial reactivity [45].

The results of this study support a model of asthma consistent with the anatomical [46] and physiological [47] interactions of mast cells and sensory nerves. Tryptase released from antigen-stimulated mast cells may contribute to asthma pathophysiology through proteolytic cleavage and activation of PAR-2 receptors on afferent C fibers. The subsequent release of neurokinins would lead to induction of airway hyperresponsiveness. Tryptase and PAR-2-mediated responses may also contribute to airway inflammation and remodeling associated with asthma. These results suggest that inhibitors of tryptase, as well as antagonists of PAR-2 auto-activation following tryptase cleavage may be effective modes of therapeutic intervention in asthma.

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