Presence and Bronchomotor Activity of Protease-Activated Receptor-2 in Guinea Pig Airways

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The protease activated receptor-2 (PAR-2) belongs to a family of G-protein–coupled receptors that are activated by proteolysis. Trypsin cleaves PAR-2, exposing an *N*-terminal tethered ligand (SLIGRL) that activates the receptor. Messenger RNA (mRNA) for PAR-2 was found in guinea pig airway tissue by reverse transcription–polymerase chain reaction, and PAR-2 was found by immunohistochemistry in airway epithelial and smooth-muscle cells. In anesthetized guinea pigs, trypsin and SLIGRL-NH_2 (given intratracheally or intravenously) caused a bronchoconstriction that was inhibited by the combination of tachykinin-NK_1 and -NK_2 receptor antagonists and was potentiated by inhibition of nitric oxide synthase (NOS). Trypsin and SLIGRL-NH_2 relaxed isolated trachea and main bronchi, and contracted intrapulmonary bronchi. Relaxation of main bronchi was abolished or reversed to contraction by removal of epithelium, administration of indomethacin, and NOS inhibition. PAR-1, PAR-3, and PAR-4 were not involved in the bronchomotor action of either trypsin or SLIGRL-NH_2, because ligands of these receptors were inactive either *in vitro* or *in vivo*, and because thrombin (a PAR-1 and PAR-3 agonist) did not show cross-desensitization with PAR-2 agonists *in vivo.* Thus, we have localized PAR-2 to the guinea-pig airways, and have shown that activation of PAR-2 causes multiple motor effects in these airways, including *in vivo* bronchoconstriction, which is in part mediated by a neural mechanism.

Protease-activated receptors (PARs) are a new and expanding family of G-protein–coupled receptors that are activated by proteolysis (1). Proteases cleave PARs within the extracellular *N*-terminal domain, exposing a new *N*-terminus that acts as a tethered ligand by binding to extracellular domains of the receptor and thereby activating the cleaved receptor molecule (1–3). Thrombin preferentially cleaves and triggers PAR-1, PAR-3, and PAR-4 (1, 4, 5). Trypsin cleaves PAR-2 and PAR-4 (5–7). Synthetic peptides corresponding to the tethered ligands of PAR-1, PAR-2, and PAR-4 also activate the corresponding PARs, although with much reduced potency when compared with the proteases (2, 5).

PARs are distributed in a number of organs and tissues. However, apart from a few tissues, their physiologic roles in these locations are far from understood. For example, PAR-1 and PAR-3 are present in platelets, where their activation causes aggregation (1–3). Activation of PAR-1 and PAR-2 leads to an endothelium-dependent relaxation of a large array of arterial blood vessels (2) and contraction of gastric smooth muscle (8).

Mouse PAR-2 was first cloned through a reduced-stringency hybridization search of a genomic library with a tachykinin-NK_2 receptor-derived oligonucleotide (6). Since then, human and rat PAR-2 have been cloned (8, 9). PAR-2 is coupled to $G\alpha_{q/11}$ or $G\alpha_0$; however, more information is available about PAR-1, which may be coupled to a large variety of G proteins (8). Much less is known about the transduction signaling pathways of PAR-3 and PAR-4. PAR-2 was found to be abundant in the gastrointestinal tract, pancreas, kidney, liver, lung, ovary, and eye (6–8). Mouse PAR-2 contains a putative trypsin cleavage site, $SKGR^{34} \downarrow S^{35}LIGR$, with cleavage exposing a tethered ligand that binds to and activates the receptor (6). Synthetic peptides corresponding to the tethered ligand domain (SLIGRL in the mouse and SLIGKV in humans) can activate PAR-2 without receptor cleavage. These hexapeptides in the amidated form of the COOH terminus retain maximum activity, although being about two or three orders of magnitude less active than trypsin (2, 8).

PAR-2 has been localized to the human airways (10). PAR-2 may be activated by tryptase (11, 12), an enzyme released after mast cell degranulation and considered to play an important role in airway inflammation and hyperresponsiveness (13, 14). In addition, PAR-2 is markedly upregulated after exposure to proinflammatory stimuli or cytokines (9), which have been shown to play a critical role in chronic airway diseases. We therefore investigated whether PAR-2 was present in guinea pig airways and whether it had any functional relevance in these tissues.

In this study we examined the localization of PAR-2 in the guinea pig airways through reverse transcriptase–polymerase chain reaction (RT–PCR), and through immunohistochemistry with an antiserum that recognizes PAR-2 (15). We also studied whether PAR-2 activation causes bronchomotor effects in guinea pigs *in vivo* and *in vitro*. We found that both trypsin and PAR-2–activating peptides caused bronchoconstriction *in vivo* and both bronchodilatation and bronchoconstriction *in vitro*. We also investigated the role of indirect mechanisms, including release of prostanoids, nitric oxide (NO), acetylcholine, histamine, bradykinin, and tachykinins in the bronchoconstriction produced by PAR-2 ligands. For this purpose, we used cyclooxygenase (COX) and NO synthase (NOS) inhibitors and antagonists for muscarinic, histamine H_1, bradykinin B_2 (Hoe 140) (16), and tachykinin-NK_1 (SR 140333) (17) and -NK_2 (SR 48968) (18) receptors.

METHODS

Animals

Male Hartley guinea pigs weighing 300 to 350 g were kept in a temperature-controlled environment with standard laboratory food and water freely available. Animals were anesthetized or euthanized with

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sodium pentobarbitone (60 to 100 mg/kg, intraperitoneally). Experimental procedures followed in the study were approved by the Committee on Animal Research of the University of Ferrara.

Detection of PAR-2 by RT-PCR

Total RNA was isolated from the trachea and colon of adult male guinea pigs through use of the RNAzol-reagent (Tel-Test Inc., Friendswood, TX). RNA (2.2 to 2.7 μg for trachea, 0.5 μg for colon) was reverse transcribed with random hexamers, using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Inc., Emeryville, CA). Oligonucleotide primers for guinea pig PARs were designed from the partial sequences obtained by cloning (N. W. Bunnett, unpublished data), as follows: PAR-2 forward: 5′–CATGTTCAGCTACTTCCTCTCCTT–3′, reverse: 5′–GGTTTTA-ACACTGGTG GAGCTTGA–3′), and were chosen to amplify a 472-bp fragment. The PCR reaction included 2 μl of the template complementary DNA (cDNA), 2.5 units of Taq DNA polymerase (Promega, Madison, WI), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (vol/vol) Triton X-100, and 0.2 mM each of deoxynucleotide triphosphates in 50 μl of 10 mM Tris-HCl buffer, pH 9.0. The PCR conditions consisted of denaturation for 10 min, 35 cycles of replication at 94° C for 1 min, 58° C for 1.5 min, and 72° C for 1 min, and a final elongation step at 72° C for 10 min. Taq polymerase was added after the first denaturation step (hot start). Guinea pig colon was used as a positive control for the PCR reaction. As negative controls, water was used instead of RNA, or Taq polymerase was omitted. PCR products were analyzed by electrophoresis on a 1.2% agarose gel with ethidium bromide.

Detection of PAR-2 by Immunohistochemistry

Antiserum B5 to PAR-2 was raised in rabbits against a peptide fragment of rat PAR-2 (^{30}GPNSKGR ↓ SLIGRLDT^{46}P-YGGC (where ↓ designates the trypsin cleavage site) (15). Guinea pigs were anesthetized with sodium pentobarbital (60 mg/ kg intravenously) and were perfused transcardially with 50 ml of phosphate-buffered saline (PBS) containing 100 U of heparin, followed by perfusion with 500 ml of 4% paraformaldehyde in 100 mM PBS, pH 7.4. Immunohistochemistry was performed as previously described (15). Briefly, trachea and bronchi were embedded in ornithyl carbamyltransferase compound (Miles, Elkhart, IN), and frozen sections (6 to 8 μm) were prepared. Slides were incubated with B5 antiserum (1:1,000 to 1:2,000), whereas for controls the primary antiserum was incubated with the peptide used for immunization (1 μM) before staining of tissues. Immunoreactivity was visualized with the unlabeled antibody–enzyme avidin–biotin complex (ABC; Vectastain, Burlingame, CA) technique. Labeled tissues were examined under an Axioplan microscope (Zeiss, Jena, Germany) with Nomarsky optics.

Measurement of Total Lung Resistance

Lung resistance (R_L) was measured according to a method reported previously (19). Briefly, guinea pigs were anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneally) and were then ventilated artificially through a tracheal cannula at a frequency of 60 breaths/min. The tidal volume (V_T) was adjusted to maintain normal arterial blood gas tensions as described previously (19). Airflow was monitored continuously with a pneumotachograph (A. Fleisch Medical, Inc., Richmond, VA) connected to a differential pressure transducer (Model DP45; Validyne Engineering Corp., Northridge, CA). Intratracheal pressure and esophageal pressure (Pes; as an approximation of pleural pressure) were measured. Transpulmonary pressure (Ptp; defined as the pressure difference between the intratracheal pressure and Pes) was measured with a differential pressure transducer (Model DP7; Validine). Output signals representing Ptp and airflow were recorded on a polygraph recorder (Model 1508 B Visicorder; Honeywell, Inc., Denver, CO). The right jugular vein and the left carotid artery were cannulated for drug administration and withdrawal of blood samples for arterial blood gas measurement, respectively.

Enzyme inhibitors were given at 5 min (captopril and phosphoramidon, 2.5 mg/kg, intravenously), 15 min [D-NMMA] or L-NMMA, 50 mg/kg, intravenously), or 30 min (indomethacin, 5 mg/kg, intraperitoneally) before the stimulus. The receptor antagonists atropine (2 mg/kg), pyrilamine (10 mg/kg), Hoe 140 (icafibant; D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin) (0.1 μmol/kg), SR 140333 (0.3 μmol/kg), and SR 48968 (0.3 μmol/kg) (all given intravenously at 15 min before the stimulus) were used at doses that have been shown to selectively block their respective receptors in guinea pigs (17, 19–21). Desensitization to thrombin or SLIGRL-NH_2 was obtained after three subsequent injections of each agonist at 20 min intervals, as reported previously for thrombin (22). SLIGRL-NH_2 was given 20 min after the last thrombin administration, and vice versa.

Preliminary experiments showed that aerosol administration (40 breaths) of the highest possible concentration (1 mM) of SLIGRL-NH_2 did not affect R_L. In another set of experiments, PAR-2 ligands were instilled locally (in 50 μl of 0.9% NaCl) into the tracheal lumen with a 30-gauge needle. Intratracheal instillation of 0.9% saline (50 μl) caused an immediate (1 to 2 s after instillation) increase in R_L (0.18 ± 0.02 cm H_2O/ml/s above baseline, $n = 8$). Intratracheal instillation of drugs caused an early increase in R_L (0.21 ± 0.03 cm H_2O/ml/s, $n = 8$) similar to that caused by saline. Active drugs (trypsin or SLIGRL-NH_2) caused a delayed (10 to 30 s after instillation) increase in R_L that was superimposed on the early increase. The effect of drugs instilled in the trachea was calculated as the increase in R_L above the early increase.

Bronchomotor Responses *In Vitro*

Trachea, main bronchi, and second-order intrapulmonary bronchi were taken from guinea pigs killed by injection of pentobarbital (100 mg/kg, intraperitoneally). Tissues were mounted in 5-ml organ baths containing a modified Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$,1 mM $NaHPO_4$, and 11.1 mM glucose) maintained at 37° C and oxygenated with a mixture of 95% O_2 and 5% CO_2 in the presence of captopril and phosphoramidon (both 1μM). An optimal tension of 1 g was applied to tissues fixed to the base of the organ bath and connected to an isometric force transducer (Unirecord; Basile, Comerio, Italy). During the initial stabilization period (30 min), tissues were washed three times. Motor responses (either contraction or relaxation) were expressed as a percentage of the contraction produced by carbachol (1 μM). Cumulative concentration–response curves for peptides (0.01 to 10 μM) or trypsin (0.1 to 100 nM) were constructed. Preliminary experiments showed that pretreatment with phosphoramidon and captopril (1 μM each) had a tendency to increase motor responses (both relaxation and contraction) to PAR-2 agonists (data not shown). However, these increases did not reach the level of significance. Nevertheless, for consistency with *in vivo* experiments, the two peptidase inhibitors were always added in *in vitro* experiments (*see* RESULTS).

The epithelial layer of the trachea and main bronchi was removed with a cotton swab. Histologic examinations of the tissues were performed as reported previously (23), and showed that the epithelial layer was completely removed, whereas no damage was observed to the lamina propria (data not shown).

Release Experiments

Trachea, main bronchi, and second-order intrapulmonary bronchi were taken from guinea pigs killed by injection of pentobarbital (100 mg/kg, intraperitoneally) and kept in a cold, oxygenated (96% O_2–4% CO_2) Krebs solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5, glucose 11. Tissue slices (0.4 mm thick) were placed into thermostated (37° C) 1-ml chambers and superfused at a rate of 0.4 ml/min with oxygenated Krebs solution containing 0.1% bovine serum albumin, 1 μM phosporamidon, and 1 μM captopril. After a 120-min stabilization period, 5-min fractions were collected (four baseline fractions and four fractions during exposure to the stimulus) into polypropylene tubes containing acetic acid (final concentration 2 N). At the end of the experiment, slices from each chamber were weighed. Fractions were freeze-dried, reconstituted with the assay buffer, and assayed for substance P-like immunoreactivity (SP-LI) content as reported previously (24). The mean baseline value was subtracted from each value obtained during exposure to the stimulus.

Reagents

Mouse and human PAR-2–activating peptides (SLIGRL-NH_2 and SLIGKV-NH_2, respectively), mouse and human PAR-2 reverse peptides (LRGILS-NH_2 and VKGILS-NH_2, respectively), and PAR-4 (GYPGQV-NH_2)–activating peptide were synthesized at the Laboratory of Pharmaceutical Chemistry of the University of Ferrara. Trypsin

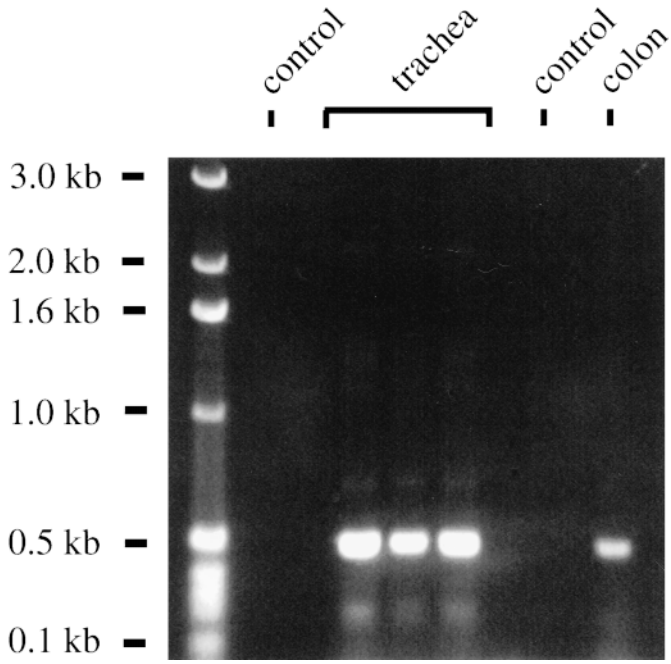

Figure 1. Detection of PAR-2 RNA in tissues of guinea pig trachea through RT–PCR. A PAR-2 product of the predicted size of 472 bp was amplified after 35 cycles from guinea pig trachea (each *lane* is RNA prepared from a different animal) and colon (positive control). No specific band for PAR-2 RNA was detected in negative controls (*lane 2*: without reverse transcriptase; *lane 6*: without Taq polymerase).

was purchased from Worthington Biochemical Co. (Freehold, NJ). Phosphoramidon, captopril, indomethacin, thrombin, pyrilamine, atropine, L-NMMA, D-NMMA, capsaicin, and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). Hoe 140 was a gift of Dr. K. Wirth (Hoechst GmbH, Frankfurt, Germany) and SR 140333 and SR 48968 were gifts of Dr. X. Emonds-Alt (Sanofi, Montpellier, France). All drugs were dissolved in 0.9% saline except indomethacin, which was dissolved in ethanol and 0.9% saline (1:5 ratio [vol/vol]), and SR 48968 and SR 140333 (both 10 mM), which were dissolved in dimethylsulfoxide. The maximum amount of ethanol or dimethylsulfoxide injected did not modify the respiratory responses of guinea pigs. All drugs were freshly prepared for each experiment.

Statistical Analysis

Values are mean ± SEM. Changes in R_L are given as differences from baseline. R_L values after intratracheal instillation are given as the difference between the delayed increase and the early increase in R_L. Statistical analysis was done through one-way analysis of variance, and with Dunnett's test for multiple comparisons or with Student's *t* test for unpaired data. In all cases, a value of $p < 0.05$ was considered significant.

RESULTS

Detection of PAR-2 RNA in Tissues of Guinea Pig Trachea by RT–PCR

For RT–PCR, we used primers based on the partial cloning of guinea pig PAR-2 to determine whether PAR-2 messenger RNA (mRNA) was expressed in guinea pig airway tissue. A PAR-2 product of the predicted size of 472 bp was amplified after 35 cycles from this tissue (Figure 1). This band migrated identically to a PCR product from guinea pig colon, which was used as a positive control (Figure 1). The lower signal of PAR-2 in the colon as compared with the trachea can be explained by a lower concentration of colon RNA used for reverse transcription. PAR-2 RNA was not detected in negative controls.

Detection of PAR-2 by Immunohistochemistry

Immunoreactive PAR-2 was readily detected in several cell types in the trachea and bronchi of the guinea pig. In both the trachea and the bronchi, PAR-2 immunoreactivity was detected in epithelial cells (Figures 2A, 2D, and 2E); smooth muscle surrounding the trachea, bronchi, and blood vessels (Figures 2A and 2C through 2F); and endothelial cells (Figures 2D and 2F). Examination under high magnification revealed that PAR-2 immunoreactivity was prominent at the apical surface of epithelial cells (Figures 2A and 2E), and was also present at the basolateral membrane (Figure 2E). PAR-2 was also detected in intracellular locations in epithelial cells. Staining was abolished when the PAR-2 antibody was preabsorbed with the receptor fragment that was used for immunization (Figure 2B).

Bronchoconstriction *In Vivo*

After a stabilization period of 60 min, baseline R_L remained stable for at least 2 h. In naive guinea pigs, the baseline value of R_L was 0.19 ± 0.02 cm H_2O/ml/s (n = 6). Intravenous injection (1 ml/kg) of 0.9% NaCl did not change the baseline value of R_L (data not shown). Injection of trypsin, SLIGRL-NH_2, or SLIGKV-NH_2 caused a significant increase in R_L (Figure 3 and Table 1). Bronchoconstrictor responses to SLIGRL-NH_2 (1 μmol/kg, intravenously) (0.33 ± 0.06 cm H_2O/ml/s, n = 6) and to trypsin (50 nmol/kg, intravenously) (0.28 ± 0.05 H_2O/ml/s, n = 5) were significantly increased by pretreatment with phosphoramidon and captopril (0.51 ± 0.08 cm H_2O/ml/s, n = 5 [$p < 0.05$], and 0.48 ± 0.04 H_2O/ml/s, n = 5 [$p < 0.05$], respectively). For this reason, all experiments were performed in the presence of the two peptidase inhibitors. Injection of PAR-4–activating peptide (data not shown) and PAR-2 reverse peptide (Figure 3 and Table 1) (all 10 μmol/kg, intravenously) did not cause any change in baseline R_L, whereas thrombin injection increased R_L (Table 2). At the third injection, response to the thrombin was reduced by more than 80%. After desensitization to thrombin, bronchoconstriction in response to SLIGRL-NH_2 (Table 2) or trypsin (50 nmol/kg, data not shown) was similar to that obtained after three injections of the vehicle. After desensitization to SLIGRL-NH_2, obtained with three closely spaced administrations of this peptide, thrombin was still able to cause bronchoconstriction (Table 2).

Pretreatment with the NOS inhibitor L-NMMA significantly increased the bronchoconstriction caused by intravenous trypsin and SLIGRL-NH_2, as compared with the effects observed after pretreatment with the inactive enantiomer, D-NMMA (Table 1). Pretreatment with indomethacin caused a significant decrease in the effect of trypsin and SLIGRL-NH_2 (Table 1). Pretreatment with atropine, pyrilamine, or Hoe 140 did not affect bronchoconstriction caused by trypsin or SLIGRL-NH_2 (data not shown). The combination of the tachykinin-NK_1 receptor antagonist SR 140333 and the tachykinin-NK_2 receptor antagonist SR 48968 markedly inhibited bronchoconstriction induced by intravenous trypsin or SLIGRL-NH_2, without affecting bronchoconstriction induced by histamine (Table 1).

Intratracheal instillation of trypsin (10 nmol) or SLIGRL-NH_2 (0.5 μmol) caused a delayed increase in R_L (0.66 ± 0.06 cm H_2O/ml/s [n = 5] and 0.48 ± 0.07 cm H_2O/ml/s [n = 4], respectively) that was abolished by the combination of SR 140333 and SR 48968 (0.06 ± 0.03 cm H_2O/ml/s, n = 5 [$p < 0.01$], and 0.08 ± 0.05 cm H_2O/ml/s, n = 5 [$p < 0.01$], respectively) but was not affected by atropine, pyrilamine, indomethacin, or Hoe 140 (data not shown). The early increase in R_L produced by intratracheal trypsin (10 nmol) or SLIGRL-NH_2 (0.5 μmol) was not affected by any of the antagonists (data not shown).

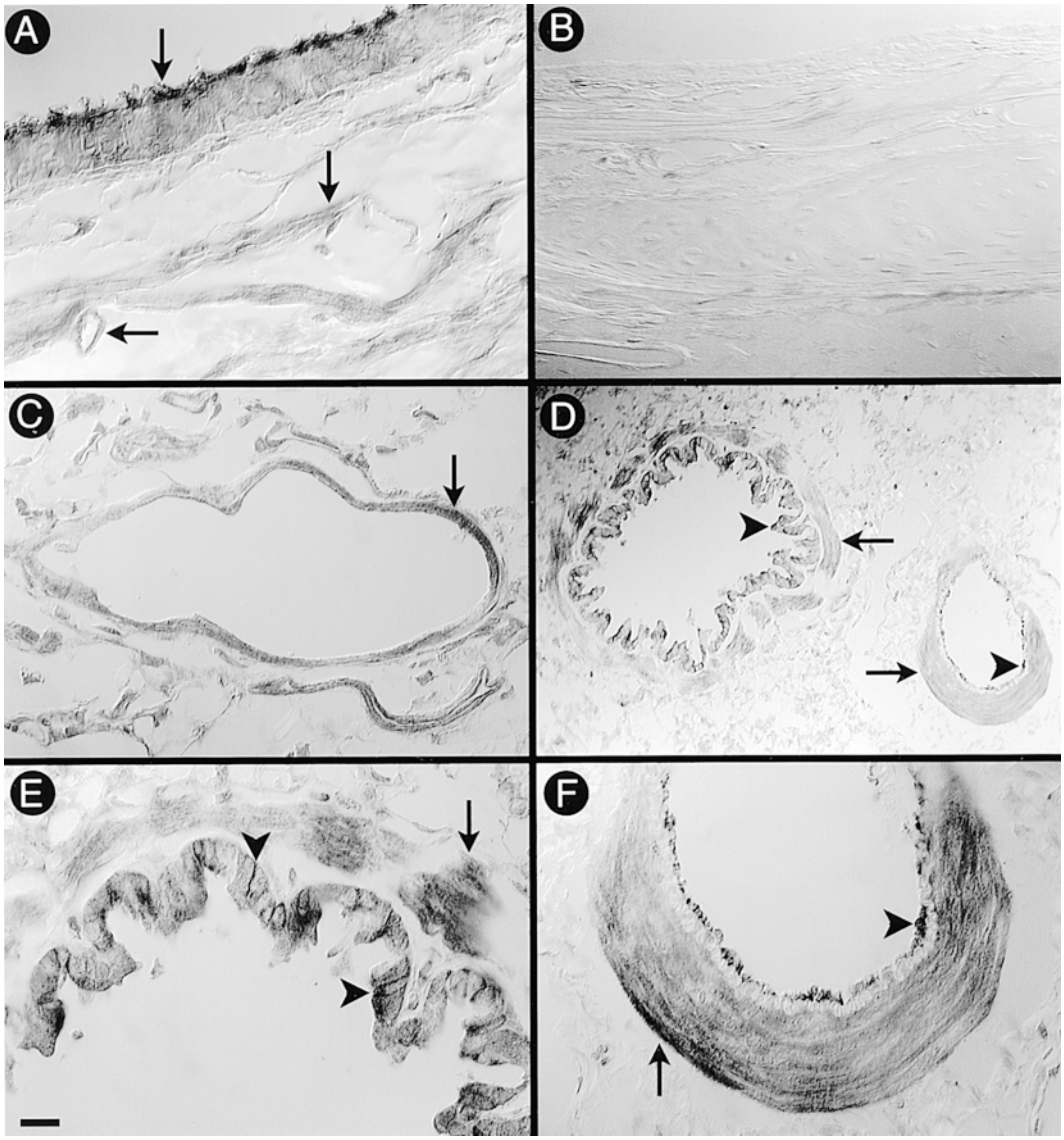

Figure 2. PAR-2 immunoreactivity in the guinea-pig trachea (*A* to *C*) and lung (*D* to *F*). (*A*) PAR-2 immunoreactivity was detected in the tracheal epithelium and smooth muscle, and in smooth-muscle cells around blood vessels (*arrows*, bar = 20 μm). (*B*) Preabsorption control, in which preincubation of the primary antiserum with the receptor fragment abolished specific staining for PAR-2 in the trachea (bar = 20 μm). (*C*) Higher magnification view shows intensive staining for PAR-2 in blood vessel smooth muscle (*arrow*, bar = 14 μm). (*D*) PAR-2 immunoreactivity in smooth-muscle cells (*arrow*) and epithelial cells (*arrowhead*) of bronchiole (left), and smooth muscle cells (*arrow*) and endothelial cells (*arrowhead*) of artery (right) (bar = 20 μm). (*E*) Higher magnification view of bronchiole in *D* reveals PAR-2 immunoreactivity at the plasma membrane, in intracellular stores of airway epithelium (*arrowheads*), and in surrounding smooth-muscle cells (*arrow*) (bar = 14 μm). (*F*) Higher magnification view of artery in *D* shows PAR-2 immunoreactivity in endothelial cells (*arrowhead*) and in the smooth muscle layer (*arrow*) (bar = 12 μm).

Intratracheal instillation of thrombin (20 U), or mouse PAR-2 reverse peptide (0.5 μmol) did not cause any delayed increase in R_L (data not shown).

Bronchomotor Effects *In Vitro*

After a stabilization period of 60 min, carbachol (1 μM) contracted isolated guinea pig bronchi (2.15 ± 0.23 g, n = 9). In nonprecontracted bronchi, SLIGRL-NH_2, SLIGKV-NH_2 (both at 0.1 to 10 μM), or trypsin (0.1 to 100 nM) caused inconsistent changes in tone that were not investigated further. SLIGRL-NH_2, SLIGKV-NH_2 (both at 0.1 to 10 μM), or trypsin (0.1 to 100 nM) caused a concentration-dependent relaxation in carbachol-precontracted bronchi (Figures 3 and 4). In about 50% of the tissues examined, trypsin (1 μM) or SLIGKV-NH_2 (100 μM) caused a contraction (15 to 22% of that with carbachol) after a first phase of relaxation (data not shown). In epithelium-denuded preparations, the relaxation produced by PAR-2–activating peptides or trypsin was either abolished or converted into a contraction after indomethacin or L-NMMA (Figure 4). Atropine, pyrilamine, Hoe 140, or the combination of SR 14033 and SR 48968 (all 1 μM, n = 4 for each condition) did not affect either bronchorelaxation or bronchoconstriction induced by trypsin, SLIGRL-NH_2, or SLIGKV-NH_2 (data not shown).

In isolated tracheal rings precontracted with carbachol (1 μM), both trypsin (0.1 to 100 nM) and SLIGRL-NH_2 (0.1 to 10 μM) caused a concentration-dependent relaxation (Figures 3 and 5). The relaxation was inhibited by removal of the epithelial layer, by indomethacin, and by L-NMMA (Figure 5). In nonprecontracted tracheal rings, we obtained inconsistent results that were not further investigated. Thrombin and PAR-4–activating peptide did not affect the tone of either precontracted or nonprecontracted trachea or main bronchi (data not shown).

In second-order intrapulmonary bronchi precontracted with carbachol (1 μM), both trypsin (0.1 to 100 nM) and SLIGRL-NH_2 (0.01 to 10 μM) caused a moderate contraction that was not further investigated. In nonprecontracted intrapulmonary bronchi, trypsin (0.1 to 100 nM) and SLIGRL-NH_2 (0.01 to 10 μM) caused a concentration-dependent contraction (Figures 3 and 5). Contraction by trypsin and SLIGRL-NH_2 was not affected by indomethacin (5 μM), L-NMMA, atropine, pyrilamine, Hoe 140, or the combination of SR 140333 and SR 48968 (all at 1 μM) (data not shown). Thrombin or PAR-4–activating peptide did not affect the tone of intrapulmonary

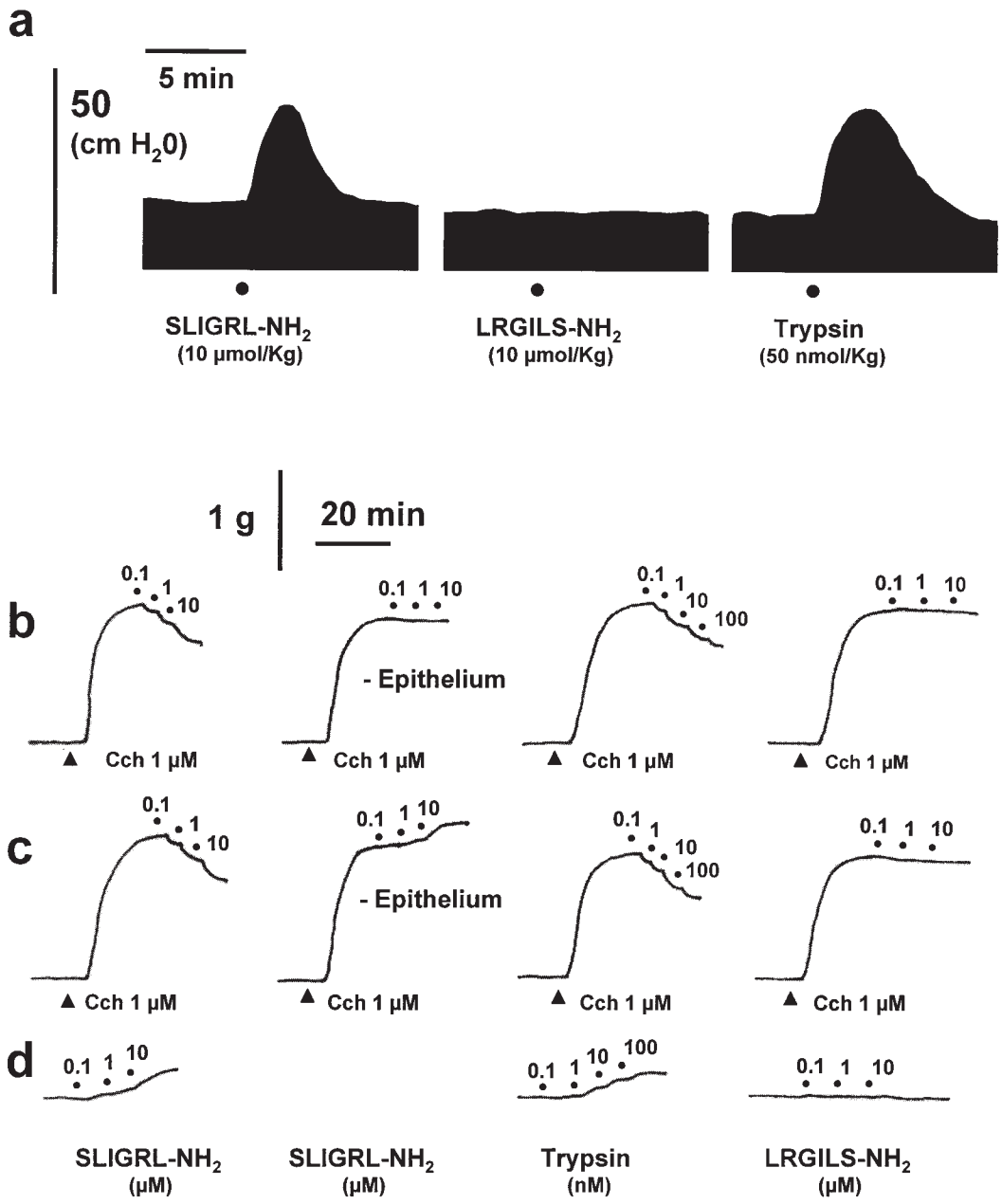

Figure 3. Typical tracings of the bronchocomotor response after intravenous injection of (*a*) PAR-2–activating peptide (SLIGRL-NH_2) and reverse peptide (LRGILS-NH_2) or trypsin in guinea pigs *in vivo.* Typical tracings of the motor response to the same agonists in isolated rings of guinea pig trachea (*b*), main bronchi (*c*), and intrapulmonary bronchi (*d*).

bronchi (data not shown). Trypsin (100 nM) treated with soybean trypsin inhibitor (10 mg/ml for 60 min) failed to affect the tone of tracheal and bronchial preparations (data not shown), indicating a requirement for enzymatic activity in activation.

SP-LI Release

Exposure of slices of guinea pig airways to SLIGRL-NH_2 (10 μM) caused an increase in SP-LI outflow of 1.72 ± 0.26 fmol/g/20 min above baseline. After preexposure to capsaicin (10 μM for 20 min, at 60 min before the stimulus), a treatment that has been shown to desensitize primary sensory neurons (25), the increase in SP-LI outflow induced by SLIGRL-NH_2 (10 μM) was significantly lower (0.38 ± 0.11 fomol/g/20 min, $n = 6$, $p < 0.05$). The increase in SP-LI outflow induced by LRGILS-NH_2 (10 μM) was negligible and significantly different (0.13 ± 0.09 fomol/g/20 min, $n = 6$, $p < 0.05$) from that induced by SLIGRL-NH_2.

DISCUSSION

In Vivo Bronchoconstriction by PAR-2 Ligands

In the present study, intravenous or intratracheal administration of trypsin and two different PAR-2–activating peptides caused bronchoconstriction in guinea pigs *in vivo*, whereas PAR-2 reverse peptides were inactive. PAR-4–activating peptide was also inactive, therefore excluding a role for this receptor in bronchoconstriction. Thrombin was recently shown to cause bronchoconstriction in guinea pigs via a platelet-dependent mechanism (22). This effect of thrombin underwent desensitization after repeated thrombin administration (22). In the current study, desensitization to thrombin did not reduce the ability of SLIGRL-NH_2 or trypsin to cause bronchoconstriction. Conversely, after desensitization to SLIGRL-NH_2, the ability of thrombin to cause bronchoconstriction was unaffected. These findings exclude a role of PAR-1 and PAR-3 (both activated by thrombin) in bronchoconstriction induced by PAR-2 ligands. Our results therefore indicate that bron-

TABLE 1

INCREASE IN TOTAL LUNG RESISTANCE ABOVE BASELINE INDUCED BY INTRAVENOUS HISTAMINE, TRYPSIN AND PROTEASE-ACTIVATED RECEPTOR-ACTIVATING PEPTIDES AND REVERSE PEPTIDES UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Peptide	Increase (*cm H_2O/ml/s*)	Peptide	Increase (*cm H_2O/ml/s*)
Trypsin, 50 nmol/kg	0.42 ± 0.05	Trypsin, 100 nmol/kg	1.19 ± 0.14†
SLIGRL-NH_2, 1 μmol/kg	0.48 ± 0.04	SLIGRL-NH_2, 10 μmol/kg	1.15 ± 0.13†
SLIGKV-NH_2, 1 μmol/kg	0.16 ± 0.02	SLIGKV-NH_2, 10 μmol/kg	0.76 ± 0.08†
LRGILS-NH_2, 10 μmol/kg	0.04 ± 0.03	VKGILS-NH_2, 10 μmol/kg	N.D.
Trypsin, 50 nmol/kg D-NMMA, 50 mg/kg, intravenously	0.39 ± 0.06	Trypsin, 50 nmol/kg L-NMMA, 50 mg/kg, intravenously	0.80 ± 0.09†
SLIGRL-NH_2, 1 μmol/kg D-NMMA, 50 mg/kg, intravenously	0.46 ± 0.05	SLIGRL-NH_2, 1 μmol/kg L-NMMA, 50 mg/kg, intravenously	1.25 ± 0.14†
Trypsin, 100 nmol/kg		Trypsin, 100 nmol/kg	
Vehicle	1.07 ± 0.15	Indomethacin, 5 mg/kg, intravenously	0.47 ± 0.06†
Vehicle	1.12 ± 0.19	SR 48968 + SR 140333, 0.3 μmol/kg	0.63 ± 0.08*
SLIGRL-NH_2, 10 μmol/kg		SLIGRL-NH_2, 10 μmol/kg	
Vehicle	1.30 ± 0.12	Indomethacin, 5 mg/kg, intravenously	0.85 ± 0.10*
Vehicle	1.19 ± 0.14	SR 48968 + SR 140333, 0.3 μmol/kg	0.61 ± 0.09†
Histamine, 50 nmol/kg		Histamine, 50 nmol/kg	
Vehicle	0.93 ± 0.16	SR 48968 + SR 140333, 0.3 μmol/kg	0.76 ± 0.12

Definition of abbreviations: D-NMMA = N^G-monomethyl-D-arginine; L-NMMA = N^G-monomethyl-L-arginine; N.D. = not detectable.
Each entry is the mean ± SEM of at least 5 experiments.
* $p < 0.05$ versus respective lowest dose.
† $p < 0.01$ versus the respective lowest dose, D-NAME, or vehicle.

choconstriction induced *in vivo* by trypsin, SLIGRL-NH_2, and SLIGKV-NH_2 is mediated by PAR-2 or by an unidentified receptor with similar sensitivity to trypsin and PAR-2–activating peptides.

Studies with COX and NOS inhibitors showed that bronchoconstriction induced by trypsin and SLIGRL-NH_2 was in part mediated by prostanoids and could be inhibited by NO. Studies with receptor antagonists indicated that this effect of trypsin and SLIGRL-NH_2 was independent of the generation of kinins, the release of histamine from mast cells, and the release of acetylcholine from postganglionic cholinergic nerves. In contrast, the marked reduction by the combination of tachykinin-NK_1 and -NK_2 receptor antagonists, of the bronchoconstriction caused by PAR-2 ligands indicated that tachykinin release from sensory nerves was involved in this bronchoconstriction. This hypothesis is strongly supported by the observation that SLIGRL-NH_2, but not the reverse peptide LRGILS-NH_2, caused a capsaicin-sensitive release of substance P (SP) from slices of guinea-pig airways. Tachykinins released from sensory nerve terminals are powerful bronchoconstrictors, and tachykinin-induced bronchoconstriction in guinea pigs is mediated by both NK_1 and NK_2 receptors (19). Previous studies have shown that a variety of mediators cause the release of tachykinins from sensory nerves (25). Data from the present study indicate PAR-2 activation is an additional mechanism that promotes bronchoconstriction via tachykinin release from sensory nerve endings.

In Vitro Bronchomotor Effects of PAR-2 Ligands

The motor action of PAR-2 in guinea pig airways results from the activation of multiple pathways. The prominent *in vitro* effect of trypsin, SLIGRL-NH_2, and SLIGKV-NH_2 observed in our study was an epithelium-, NO-, and prostanoid-dependent relaxation of trachea and main bronchi. After removal of the relaxing factors (epithelium, NO, and prostanoids), a moderate contraction was produced in main bronchi. In smaller intrapulmonary bronchi, the sole effect of trypsin and SLIGRL-NH_2 was airway smooth-muscle contraction, which was independent of tachykinin, histamine, acetylcholine, or ki-

TABLE 2

INCREASE IN TOTAL LUNG RESISTANCE ABOVE BASELINE INDUCED BY INTRAVENOUS INJECTION OF THROMBIN OR SLIGRL-NH_2 UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Thrombin/SLIGRL-NH_2	Increase (*cm H_2O/ml/s*)	Vehicle/Thrombin/SLIGRL-NH_2	Increase (*cm H_2O/ml/s*)
I-Thrombin, 100 U/kg, intravenously	0.93 ± 0.14	I-Vehicle	N.D.
II-Thrombin, 100 U/kg, intravenously	0.38 ± 0.11	II-Vehicle	N.D.
III-Thrombin, 100 U/kg, intravenously	0.13 ± 0.05	III-Vehicle	N.D.
SLIGRL-NH_2, 10 μmol, intravenously	1.11 ± 0.21	SLIGRL-NH_2, 10 μmol, intravenously	1.23 ± 0.24
I-SLIGRL-NH_2, 10 μmol, intravenously	1.08 ± 0.13	I-Vehicle	N.D.
II-SLIGRL-NH_2, 10 μmol, intravenously	0.56 ± 0.09	II-Vehicle	N.D.
III-SLIGRL-NH_2, 10 μmol, intravenously	0.16 ± 0.05	III-Vehicle	N.D.
Thrombin, 100 U/kg, intravenously	1.05 ± 0.21	Thrombin, 100 U/kg, intravenously	1.13 ± 0.12

Definition of abbreviations: D-NMMA = N^G-monomethyl-D-arginine; L-NMMA = N^G-monomethyl-L-arginine; N.D. = not detectable.
Each entry is the mean ± SEM of at least 5 experiments.
* $p < 0.05$ versus vehicle or D-NMMA.
† $p < 0.01$ versus vehicle or D-NMMA. Thrombin, SLIGRL-NH_2 or vehicle were given three times (I, II, and III) at 20-min intervals each.
N.D., not detectable.

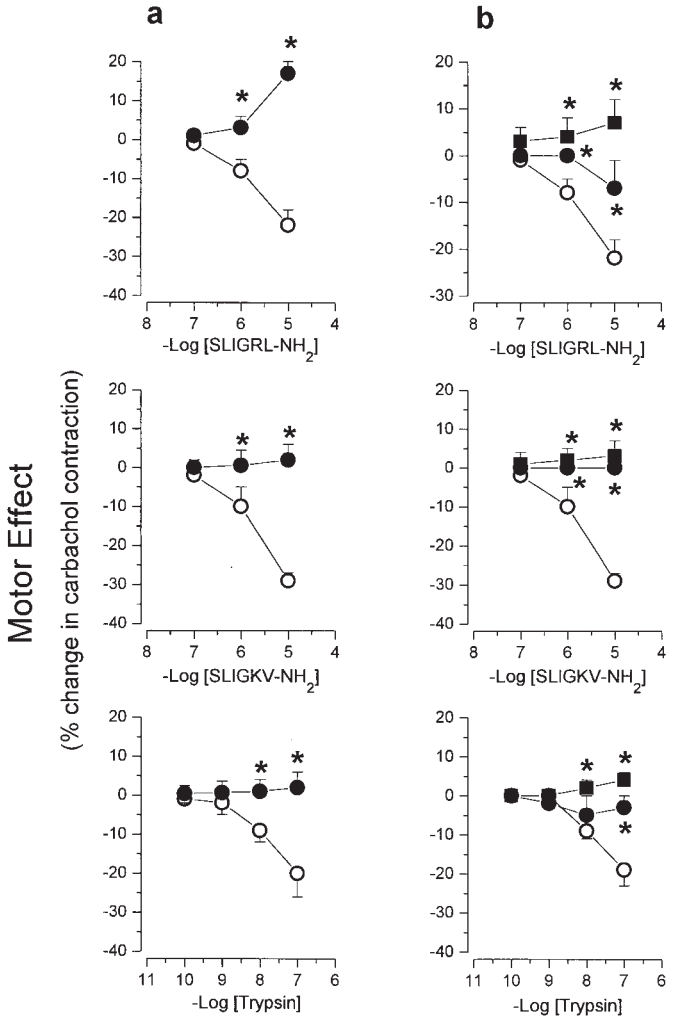

Figure 4. Motor effect of mouse (SLIGRL-NH_2) and human (SLIGKV-NH_2) PAR-2–activating peptides or trypsin in isolated guinea pig main bronchi with: (*a*) intact epithelium (*open circles*) or denuded of epithelium (*closed circles*); and (*b*) pretreated with D-NMMA (*open circles*), L-NMMA (*closed circles*), or indomethacin (*closed squares*). Entries are mean ± SEM of at least 5 experiments (*$p < 0.05$ versus bronchi with intact epithelium).

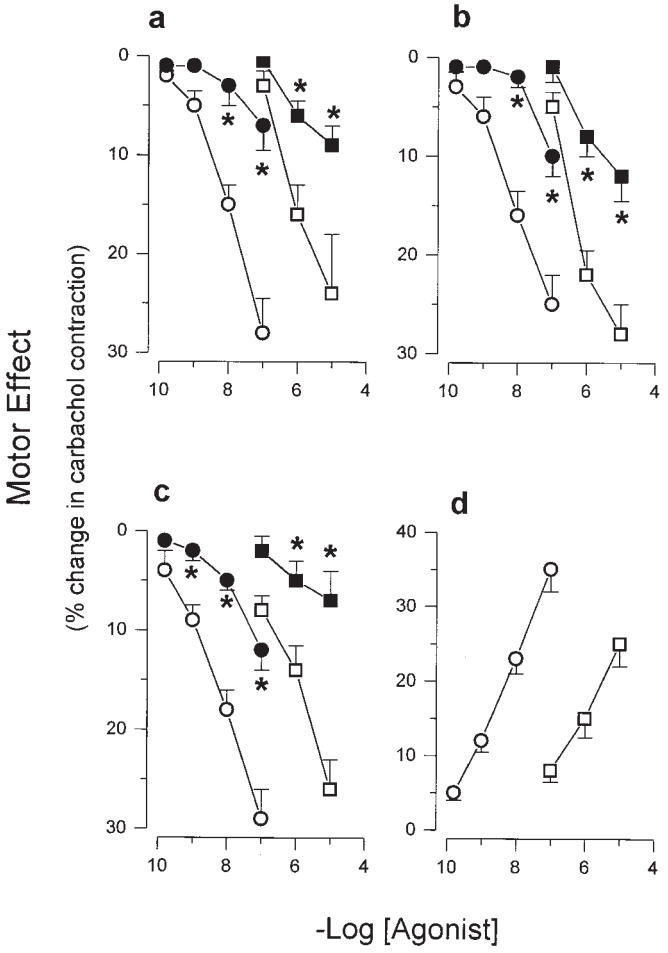

Figure 5. Motor effect of trypsin (*circles*) or SLIGRL-NH_2 (*squares*) in isolated tracheal rings with: (*a*) intact epithelium (*open symbols*) and epithelium denuded (*closed symbols*); (*b*) indomethacin (5 μM, *closed symbols*) and its vehicle (*open symbol*); (*c*) D-NMMA (*open symbols*) and L-NMMA (*closed symbols*) (both 100 μM). (*d*) Effect of trypsin (*circles*) or SLIGRL-NH_2 (*squares*) in isolated intrapulmonary bronchial rings. Entries are mean ± SEM of at least 5 experiments (*$p < 0.05$ versus respective open symbols).

nin release. RT–PCR showed the presence of PAR-2 in the airway tissue. More importantly, immunohistochemistry showed localization of PAR-2 to airway epithelial and smooth-muscle cells. Thus, it is possible that direct activation of PAR-2 on smooth muscle causes contraction of isolated bronchi. Small intrapulmonary airways are likely to contribute more than larger airways to resistance to flow in the lungs. *In vivo* and *in vitro* experiments suggest that modulation of bronchomotor tone *in vivo* by PAR-2 depends more strongly on indirect bronchoconstrictor mechanisms (sensory nerve activation and release of prostanoids) and direct smooth-muscle contraction of intrapulmonary bronchi than on bronchodilatation produced in the trachea and main bronchi.

Protective or Detrimental Role of PAR-2

During completion of the present study, Cocks and colleagues reported (26) that PAR-2 activation mediates an epithelium- and prostanoid-dependent relaxation in mouse isolated bronchi, and that both trypsin and PAR-2 peptidomimetics produced a moderate relaxation in isolated guinea pig bronchi (26). PAR-2 activation also inhibited serotonin-induced bronchoconstriction in rats *in vivo* (26). On the basis of these data, Cocks and colleagues (26) proposed that PAR-2 had a prostanoid (prostaglandin E_2)-dependent bronchoprotective role in the airways. We also observed a pronounced, indomethacin-sensitive relaxation of mouse trachea by trypsin and mouse PAR-2–activating peptide (F. Van Noouten and P. Geppetti, unpublished observations). However, in mouse and rat airways, a number of mediators, including SP (via NK_1 receptors) (27) and bradykinin (via B_2 receptors) (28) produce epithelial- and prostanoid-dependent bronchorelaxation. However, SP and bradykinin are powerful bronchoconstrictors in guinea pig and human airways (25, 29). Additionally, tachykinin NK_1 receptor stimulation causes a prostanoid-dependent relaxation of the isolated mouse bronchus (27) and a prostanoid-dependent contraction of the isolated small human bronchus (30). Thus, the guinea pig model, rather than the mouse model, seems to better reflect the role of mediators like bradykinin and tachykinins in the human airways.

We routinely used peptidase inhibitors, whereas these drugs were not used in the experiments done by Cocks and colleagues (26). However, this factor cannot be the reason for

the different results obtained, because peptidase inhibitors did not qualitatively change the motor responses to PAR-2 agonists in guinea pigs but affected only the quantitative magnitude of *in vivo* bronchoconstriction. The present data from guinea pigs show that PAR-2 activates different bronchomotor mechanisms that may or may not be protective. PAR-2 activation stimulates bronchoconstriction that is in part neurally mediated and in part likely to be caused by direct airway smooth-muscle contraction. PAR-2 also activates an epithelial-, NO-, and prostanoid-dependent bronchodilator pathway. These multiple roles of PAR-2 bear some similarity to the different effects produced by bradykinin. Bradykinin, via B_2-receptor activation, causes an epithelial-, NO-, and prostanoid-dependent bronchodilatation (23, 31) as well as an indirect, neurally mediated bronchoconstriction (32). In addition, as observed in the present study with PAR-2 agonists, indomethacin inhibits about 50% of the bronchoconstriction induced by intravenous bradykinin, but not that induced by intratracheal bradykinin (25). A possible explanation for this differential response is that bradykinin injected into the blood stream releases bronchoconstrictor prostanoids, whereas with bradykinin applied locally in the airways, the bronchomotor role of prostanoids, if any, is negligible (25). The contractile action of PAR-2 agonists *in vitro* is also similar to that of bradykinin, which contracts isolated guinea-pig bronchi in a manner that is independent of sensory nerve activation and which apparently occurs through a direct effect on smooth-muscle cells (25). These interpretations may also apply to the bronchomotor effects produced by PAR-2 agonists.

The bronchocomotor effects of bradykinin in humans resemble those described in guinea pigs. In humans, bradykinin-induced bronchoconstriction is mediated indirectly by neural mechanisms (25, 33) and is limited by an NO-dependent bronchodilator pathway (34). If the PAR-2–mediated responses found in guinea pigs are present in human airways, PAR-2 stimulation in severe asthma, a condition associated with epithelial damage, might promote bronchoconstriction rather than protective bronchodilatation.

The observation that trypsinogen and PAR-2 colocalize to the human airway epithelium has suggested the interesting hypothesis that PAR-2 acts in a paracrine manner to promote protective bronchodilatation in the airway mucosa (26). Localization of PAR-2 to the airway smooth muscle, and its ability to stimulate sensory nerves, explain its bronchoconstrictor effect in guinea pigs. In addition to trypsin released from the epithelium, other agonists may stimulate PAR-2 in the airways. Tryptase released from mast cells during anaphylaxis or other inflammatory processes (13, 14) might stimulate epithelial and extraepithelial PAR-2. Protease activity is present in house dust mite extracts. In particular, the allergen contained in the house dust mite *Dermatophagoides pteronyssinus* is a trypsinlike enzyme (35). Thus, the hypothesis may be considered that inhaled allergens having protease activity cause PAR-2–mediated bronchocomotor effects in allergic asthma.

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