Effect of protease-activated receptor (PAR)-1, -2 and -4-activating peptides, thrombin and trypsin in rat isolated airways

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1 Mechanisms of relaxation and contraction to protease-activated receptor- (PAR) tethered ligand peptides (SFLLRN/TFLLR, SLIGRL and GYPGKF (all C-terminally amidated) for PAR1, PAR2 and PAR4, respectively) and enzymes (thrombin and trypsin) were investigated in isolated segments of rat trachea, main and first order intrapulmonary bronchi.

2 In airway segments previously exposed to SLIGRL, SFLLRN caused contractions that were potentiated by indomethacin, but were independent of mast cell degranulation. Contractions to TFLLR in the intrapulmonary bronchi were similarly potentiated by indomethacin.

3 SLIGRL caused epithelium-dependent relaxations which were unaffected by N^G-nitro-L-arginine, 1-H-oxodiazol-[1,2,4]-[4,3-a]quinoxaline-1-one or zinc-protoporphyrin-IX but were abolished by haemoglobin in all three regions of the airways. Relaxations to SLIGRL were markedly attenuated by indomethacin only in the main and intrapulmonary bronchi.

4 GYPGKF caused epithelium-dependent relaxations in all three regions of the airway which were only significantly inhibited by indomethacin in the intrapulmonary bronchi.

5 In general, thrombin and trypsin failed to cause any response in the airways tested.

6 Intense PAR2-immunoreactivity was observed on airway epithelium. PAR1-immunoreactivity was faint on airway epithelium and smooth muscle, but was prevalent in mast cells.

7 These findings indicate that PAR2 and possibly PAR4 present on rat airway epithelia mediate smooth muscle relaxation *via* cyclo-oxygenase-dependent and -independent mechanisms. PAR1-mediated contractions were most likely due to activation of smooth muscle receptors. The general failure of thrombin and trypsin to cause responses which may have been due to endogenous protease inhibitors, highlights the need for caution in assessing pathophysiological roles for PARs if only enzymes are used to activate PARs.
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Abbreviations: Hb, haemoglobin; IBMX, iso-butyl-methylxanthine; L-NOARG, N^G-nitro-L-arginine; ODQ, 1-H-oxodiazol-[1,2,4]-[4,3-a]quinoxaline-1-one; PAR, protease-activated receptors; ZnPP-IX, zinc-protoporphyrin-IX

Introduction

Four members of the seven-transmembrane family of protease-activated receptors (PARs) are known (for review see Coughlin, 1994; 1999; Déry *et al.*, 1998; Hollenberg, 1999). We (Cocks *et al.*, 1999) and others (Lan *et al.*, 2000) have shown that activation of epithelial PAR1, PAR2 and PAR4 in mouse airways with either the serine proteases thrombin and trypsin, respectively, or synthetic peptide mimetics of each receptor's tethered ligand sequence, cause powerful, cyclo-oxygenase-dependent relaxation of airway smooth muscle. Also, we demonstrated that the PAR2-selective synthetic peptide, SLIGRL caused similar epithelium-dependent relaxation in rat isolated bronchi as well as inhibition of the increase in airway resistance to intravenous administration of serotonin in anaesthetized rats (Cocks *et al.*, 1999). These results together with our additional finding that PAR2 and its proteolytic activator, trypsin, are co-localized in human airways epithelium (Cocks *et al.*, 1999), have led us to hypothesize that epithelial PARs act as sensors of early inflammation (Cocks & Moffatt, 2000). Given the importance of our *in vivo* rat studies in

formulating this hypothesis, the aim of our present study was to examine the mechanisms underlying changes in reactivity to PAR activators in different regions of rat isolated airways.

Methods

Preparation of rat airways ring segments

Sprague-Dawley rats (200–300 g) of either sex were killed with a lethal dose of sodium pentobarbitone (120 mg kg^{-1}, i.p.) and immediately exsanguinated to avoid blood clot formation in the lungs. The thorax was then opened and the lung and trachea removed and immersed in Krebs solution (composition in mM: Na$^+$ 144, Cl$^-$ 128.7, HCO$_3^-$ 25, K$^+$ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, H$_2$PO$_4^-$ 1.2, SO$_4^{2-}$ 1.2 and glucose 11) continuously bubbled with 95% O$_2$, 5% CO$_2$ to maintain a high pO$_2$ and pH of 7.4. The trachea, main bronchi and the first order (intrapulmonary) bronchi from the left lobe of the lung were then dissected free from surrounding parenchyma and blood vessels.

Tracheal and bronchial rings (3–4 cartilage rings in length) were mounted in 5 or 10 ml organ baths containing

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Krebs solution (37°C) on 150 μm diameter stainless steel hooks with one hook connected to a micrometer-adjustable support rod and the other to a force transducer (Grass Instruments, Model FT03C, MA, U.S.A.). Segments of intrapulmonary bronchi 2 mm in length were mounted on 40 μm stainless steel wires in dual chamber Mulvany-Halpern myographs (JP Trading, Aarhus, Denmark) containing Krebs solution (37°C). Changes in circumferential isometric force were amplified and recorded on flat bed chart recorders (W & W Scientific Instruments, Basel, Switzerland). In some segments of trachea and bronchi the lumen was gently rubbed with a moistened tapered wooden stick. To similarly remove the epithelium from the intrapulmonary bronchi, Krebs solution containing 0.1% Triton X-100 was gently flushed through the intact lung then washed with Krebs solution before dissection (Cocks *et al.*, 1999). The successful removal of the epithelium was confirmed histologically in haemotoxylin and eosin-stained 4 μm paraffin sections.

Rings of airways mounted in organ baths and myographs were allowed to equilibrate for 30–60 min before being stretched to a tension of 2, 1, and 0.5 g for trachea, main and intrapulmonary bronchi, respectively. When passive tension

had stabilized the maximum contraction (F_{max}) was determined for each ring with 30 μM acetylcholine (ACh). The preparations were then washed three times with Krebs solution and treated with 0.3 μM nifedipine to inhibit the development of spontaneous activity. Thirty minutes later, tissues were contracted with titrated concentrations of the stable muscarinic agonist, carbachol (0.1–1 μM), to achieve a maintained contraction of 40–60% F_{max}.

Effect of PAR agonists

Sequential, cumulative concentration responses to the synthetic PAR-tethered ligand peptide sequences, SLIGRL (PAR2) and SFLLRN (PAR1) were obtained in carbachol-contracted tissues without washing between each curve, since the tissues spontaneously recovered to their initial levels of carbachol-induced force following the maximum response to SLIGRL. Similarly, sequential cumulative concentration responses were obtained to thrombin followed by trypsin. This protocol of sequential additions of agonists without washout resulted in complete desensitization of PAR2 with SLIGRL or PAR1 with thrombin (data not shown). Therefore, specific effects of the peptides and
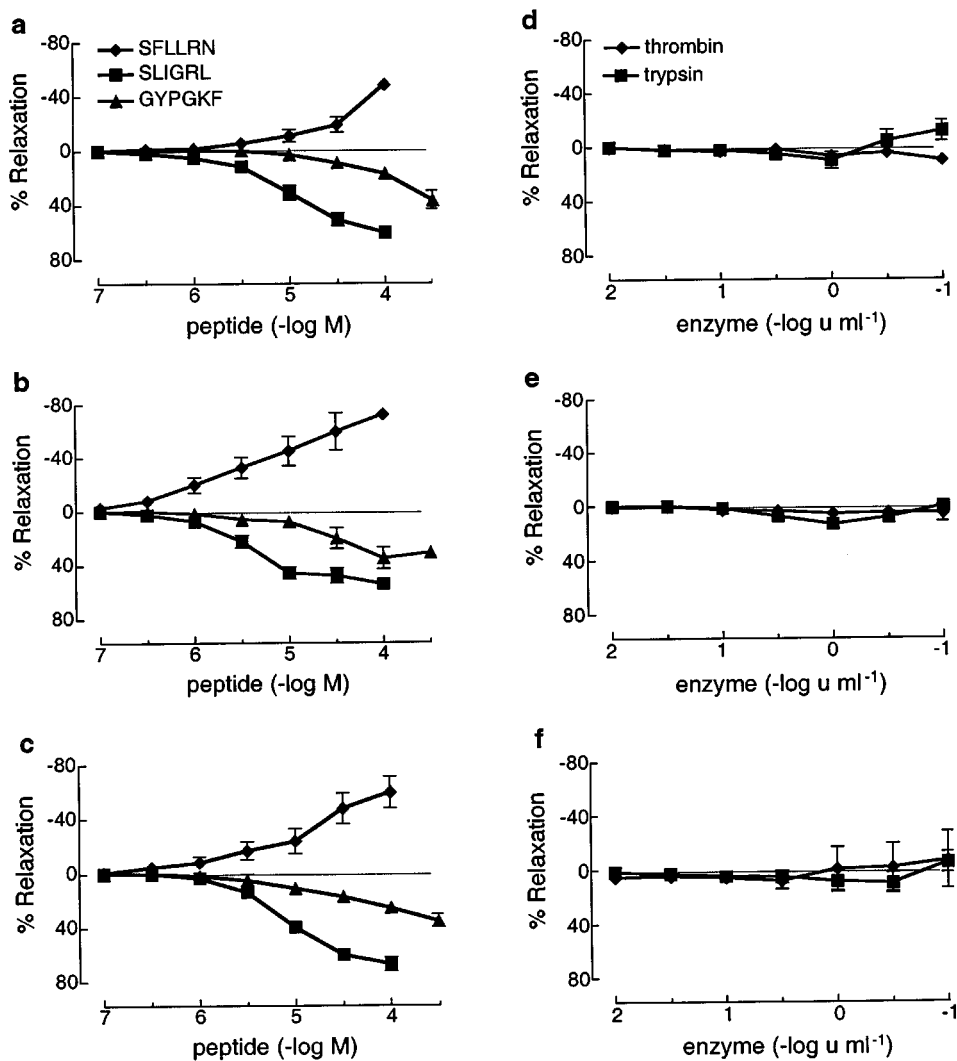

Figure 1 Cumulative concentration-response curves to the PAR1, PAR2 and PAR4-activating peptides, SFLLRN, SLIGRL and GYPGKF, respectively and the PAR-activating enzymes, thrombin and trypsin in isolated rat trachea (a,d), main bronchi (b,e) and intrapulmonary bronchi (c,f) contracted to ≈50% F_{max} with carbachol. Values are expressed as percentage (mean±s.e.mean) relaxations or contractions of the carbachol-induced contraction ($n=6–12$).

enzymes at their designated receptors could be evaluated without any potential receptor cross-over since in some tissues SFLLRN has been shown to activate PAR2 (Blackhart *et al.*, 1996; Hollenberg *et al.*, 1997) and trypsin can activate PAR1 (Vu *et al.*, 1991; Molino *et al.*, 1997). In other tissues, however, SFLLRN appears to selectively activate PAR1 (Hamilton & Cocks, 2000). There is no evidence that SLIGRL activates PAR1 or thrombin activates PAR2. Cumulative concentration responses were obtained to the PAR4 peptide, GYPGKF or the selective PAR1 peptide, TFLLR (Hollenberg *et al.*, 1997) in separate tissues contracted with carbachol. In all cases, after responses to maximum concentrations of the PAR agonists were obtained, isoprenaline (3 μM) and 3-isobutyl-1-methyl-xanthine (IBMX, 100 μM) were added to obtain each tissue's maximum relaxation.

Mechanisms of responses to PAR-activating peptides

Prior to contracting with carbachol, some tissues were treated with various inhibitors for 30 min and run in parallel with untreated (control) tissues from the same animal. Inhibitors tested were indomethacin (3 μM), N^G-nitro-L-arginine (L-NOARG, 100 μM), oxyhaemoglobin (oxyHb, 20 μM), metHb (20 μM), 1-H-oxodiazol-[1,2,4]-[4,3-a]quinoxaline-1-one (ODQ, 10 μM) and zinc-protoporphyrin-IX (ZnPP-IX, 10 μM). Also, the effect of mast cell degranulation on SFLLRN-induced contractions in rat bronchi was examined. These preparations were treated with indomethacin (3 μM) and compound 48/80 (3 μM) for 30 min prior to contraction with carbachol to 40 – 60% F_{max}. When a stable level of contraction was achieved, SLIGRL (10 μM) was added before cumulative additions of SFLLRN, again to ensure that SFLLRN activated only PAR1.

Immunohistochemistry

Similar Sprague-Dawley rats as used in the functional studies were killed with an overdose of sodium pentobarbitone (120 mg kg^{-1}, i.p.) and the lung perfused with phosphate-buffered 4% (w v^{-1}) paraformaldehyde (pH 7.3). The lungs were removed and immersed overnight in the same fixative before being thoroughly washed in phosphate-buffered saline (PBS). Segments of trachea, main bronchi and intrapulmonary bronchi were then cryoprotected in a 15% (w v^{-1}) sucrose solution in PBS for up to a week before being snap frozen in Tissue-Tek® OCT compound (Sakura Finetek, U.S.A.) and stored at -70°C until sectioned. Frozen sections (14 μm) were collected on 1% gelatin-coated slides.

PAR2 was localized using two different antibodies. Tissue sections were first exposed to a polyclonal rabbit antiserum raised against the C-terminus of the mouse PAR2 (CSVKTSY, PAR2-C, 1 : 500 dilution; gift from N. Bunnett, San Francisco, U.S.A.) for 48 h. Use of the PAR2-C antiserum was compared to controls where the antiserum was incubated (4°C, 24 h) with the immunizing peptide (10 μM). The PAR2 antiserum labelled tissues were then sequentially labelled with a biotinylated donkey anti-rabbit antiserum (1 : 100 dilution, 1 h; Amersham, U.K.) and fluorescein isothiocynate (FITC)-conjugated streptavidin (1 : 20 dilution, 1 h; Amersham, U.K.). The same sections were then exposed to a monoclonal mouse antibody raised against the N-terminus of human PAR2 (SLIGKVDGTSHVTG amino acid sequence, PAR2-N, 1 : 20 dilution; gift from L. Brass, Philadelphia, U.S.A.) for 24 h and then labelled with horse anti-mouse texas red (1 : 200 dilution, 1 h; Vector Lab, CA, U.S.A.). PAR1 was localized with a monoclonal mouse antiserum raised against the N-terminus (SFLLRNPNDKYEPF, 1 : 100 dilution, 48 h;
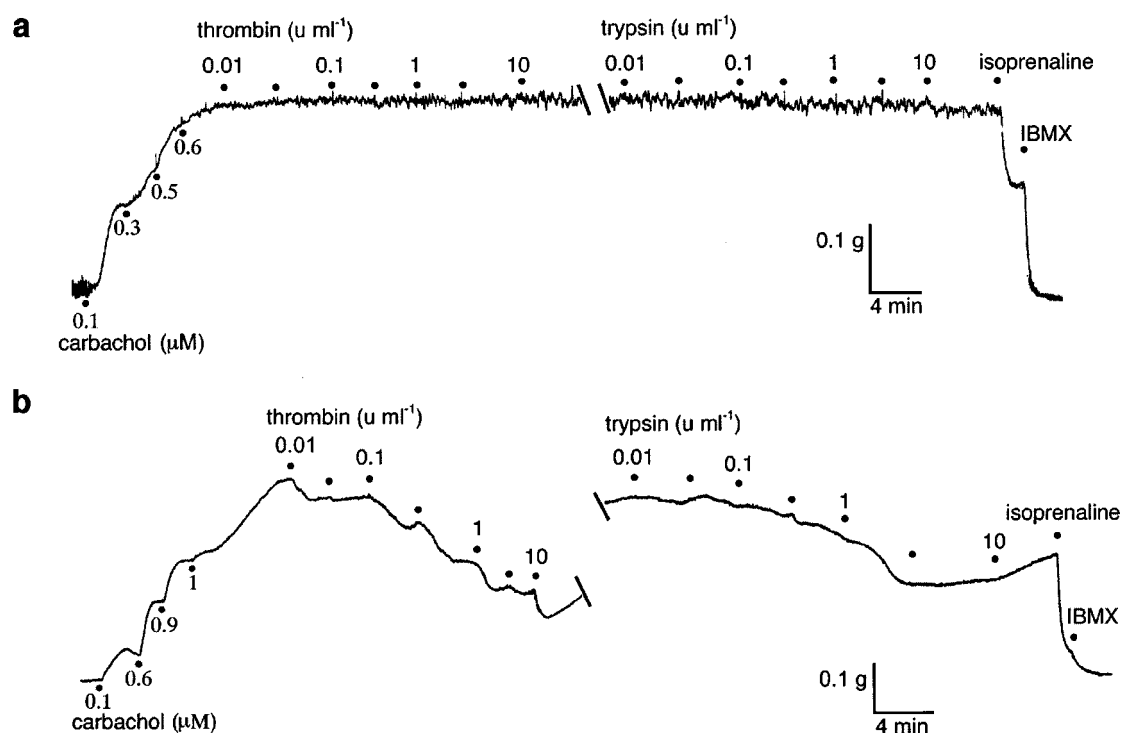

Figure 2 Digitized traces of original chart recordings showing responses to cumulative additions of thrombin and trypsin in two rings of rat intrapulmonary bronchi from different rats contracted to $\approx$50% F_{max} with carbachol. Maximum relaxations were obtained with isoprenaline (3 μM) and IBMX (100 μM). (a) A typical preparation that failed to respond to either trypsin or thrombin. (b) One of six preparations in which concentration-dependent relaxations to both enzymes could be obtained. The breaks in the traces represent 20 min without washout of thrombin.

gift from L. Brass) and sequentially labelled with biotinylated sheep anti-mouse antiserum (1 : 100 dilution, 1 h; Amersham, U.K.) and FITC-conjugated streptavidin. In between exposure to different reagents, the sections were washed repeatedly in PBS for 45 min. Sections were then mounted in filtered buffered glycerol and fluorescence observed under a confocal microscope (Biorad MRC 1000). Each image represents 3 – 5 z-sections of 0.5 μm thickness.

Analyses and statistics

All responses were measured as percentages of the carbachol-induced contractions. Where complete concentration response curves could be obtained, the sensitivity (pEC_{50}) was determined by regression analysis (Kemp & Cocks, 1997). Unpaired *t*-tests were used to compare responses between two groups and a one-way analysis of variance (ANOVA) with a Tukey-Kramer *post hoc* test was used to compare maximum responses (R_{max}) between multiple treatment groups within each airway branch. All results were expressed as mean $\pm$ the standard error of the mean (s.e.mean). Responses were considered significantly different if $P < 0.05$.

Drugs

Stock solutions of the PAR-activating peptides (10 mM), SFLLRN, TFLLR, SLIGRL and GYPGKF (single letter coded amino-acid sequence; Auspep, Melbourne, Australia; all C-terminally amidated) and the serine proteases, α-thrombin (1000 u ml^{-1}, bovine serum; Sigma, U.S.A.), trypsin (1000 u ml^{-1}, bovine pancreas; Worthington Biochem, U.S.A.), were dissolved in distilled water, aliquoted and stored at $-20°$C. Acetylcholine chloride, carbachol, ($-$)-isoprenaline (Sigma, U.S.A.), IBMX (Aldrich-Chemie, West Germany) and compound 48/80 (Sapphire Bioscience, Sydney, Australia) were dissolved in distilled water as 10 mM stock solutions. Stock solutions of L-NOARG (100 mM) and indomethacin (10 mM; both Sigma, U.S.A.) were prepared in 1 M NaHCO$_3$ and 1 M Na$_2$CO$_3$, respectively whilst nifedipine (Biomol, U.S.A.) was prepared as a 1 mM stock solution in absolute ethanol. MetHb (bovine serum; Sigma, U.S.A.) was dissolved in 0.9% (w v^{-1}) saline as a 1 mM solution and reduced with sodium dithionite (BDH Chemicals Ltd., U.K.). The reduced Hb (oxyHb) was then separated from excess dithionite and oxidized Hb (metHb) by gel filtration through a Sephadex PD-10 column (Pharmacia Biotech, Sweden). ODQ (Biomol, U.S.A.) and ZnPP-IX (Biomol, U.S.A.) were prepared as 10 mM stock solutions in dimethyl sulphoxide. All further dilutions of drugs were in distilled water.

Results

Responses to PAR-activating peptides

The PAR2-activating peptide, SLIGRL (0.1 – 100 μM), caused concentration-dependent relaxations with pEC_{50} and maxima of 5.1 $\pm$ 0.1, 60.3 $\pm$ 2.8%; 5.5 $\pm$ 0.1, 47.8 $\pm$ 5.6% and 5.1 $\pm$ 0.2, 66.8 $\pm$ 5.2% in the rat trachea, main and intrapulmonary bronchi, respectively (Figure 1), which in each region were abolished by the removal of the epithelium (data not shown). Similar concentration- and epithelium-dependent relaxations were observed in response to the PAR4-activating peptide, GYPGKF (1 – 300 μM) in the trachea, main and intrapul-

monary bronchi with responses at 300 μM of 41.2 $\pm$ 5.9, 32.5 $\pm$ 1.6 and 36.4 $\pm$ 3.0%, respectively (Figure 1). By contrast, the PAR1-activating peptide, SFLLRN (0.1 – 30 μM) caused only concentration-dependent contractions in the trachea, main and intrapulmonary bronchi with responses at 30 μM of 19.2 $\pm$ 5.6, 59.5 $\pm$ 14.1 and 49.3 $\pm$ 12.9%, respectively (Figure 1). The more selective PAR1-activating peptide, TFLLR (30 μM) produced no responses in the intrapulmonary bronchi ($n = 5$) but caused 19.5 $\pm$ 5.5% relaxation ($n = 8$) in the trachea (data not shown).

Responses to enzymes

The PAR-activating serine proteases, trypsin and thrombin (0.01 – 10 u ml^{-1}) failed to produce any response in all three regions of the airways examined from five animals (Figures 1 and 2). In preparations from one rat, however, concentration-dependent relaxations to both thrombin and trypsin were observed but only in the intrapulmonary bronchi (Figure 2). In two further animals the effects of higher concentrations of trypsin and thrombin were examined in the trachea and main bronchi. In both cases,
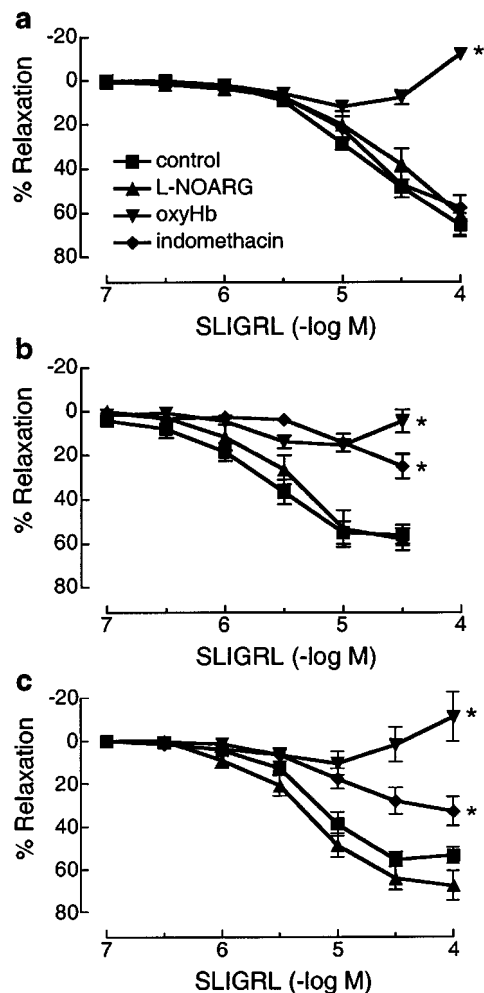

Figure 3 Cumulative concentration-relaxation curves to the PAR2-activating peptide, SLIGRL in the presence of either N^G-nitro-L-arginine (L-NOARG, 100 μM), oxyhaemoglobin (oxyHb, 20 μM) or indomethacin (3 μM) in rat isolated trachea (a), main bronchi (b) and intrapulmonary bronchi (c) contracted to $\approx 50\%$ F$_{max}$ with carbachol. Values are expressed as percentage (mean $\pm$ s.e.mean) relaxations of the carbachol-induced contraction ($n = 6 – 12$). *Denotes maximum values significantly different from controls ($P < 0.05$).

concentrations of trypsin $\geqslant 30$ u ml^{-1} caused slowly developing relaxations that were at least partly the result of tissue damage, since active tension fell below the level of passive tension in the preparations (data not shown). Similar high concentrations of thrombin had either no effect or caused weak contractions (data not shown).
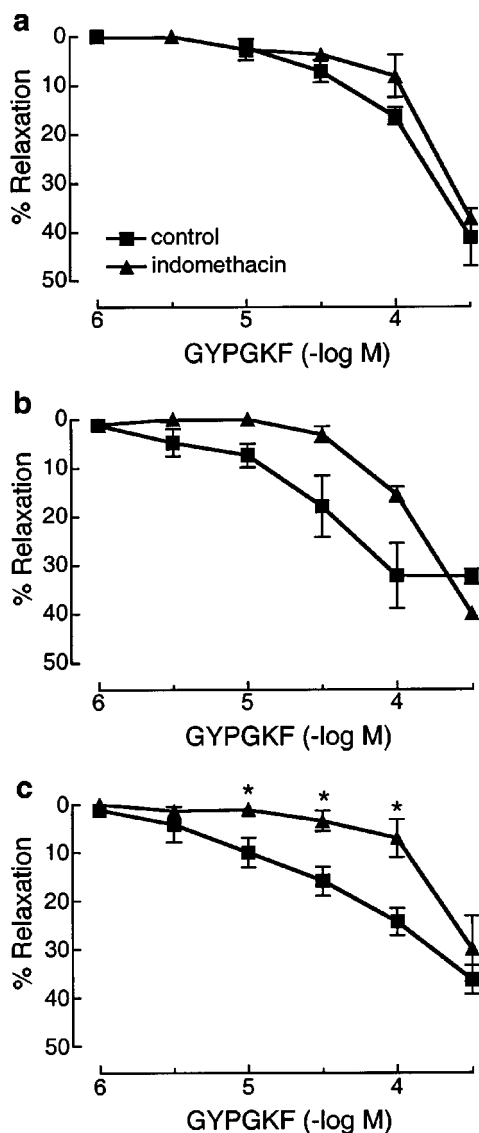

Figure 4 Cumulative concentration relaxation curves to the PAR4-activating peptide, GYPGKF in the rat trachea (a), main bronchi (b) and intrapulmonary bronchi (c) in the absence and presence of indomethacin (3 μM). Values are expressed as percentage (mean $\pm$ s.e.mean) relaxations of the carbachol-induced contraction ($n=3-6$). Note that the control curves are reproduced from Figure 1. *Denotes significant differences ($P<0.05$) from control responses.

Table 1 Effect of indomethacin (3 μM) on contractile responses (mean $\pm$ s.e.mean % of the carbachol-induced contraction; $n=5-8$) to SFLLRN (30 μM) in rat airway preparations

Branch of airway	Control (%)	+ Indomethacin (%)
Trachea	19.2 ± 5.6	46.3 ± 2.4*
Main bronchi	59.5 ± 14.1	119.9 ± 33.3*
Intrapulmonary bronchi	49.3 ± 12.9	142.4 ± 24.0*

*Significantly different from control ($P<0.05$).

Effect of inhibitors of cyclo-oxygenase, nitric oxide (NO) and haem oxygenase on responses to PAR-activating peptides

Indomethacin (3 μM) significantly inhibited the relaxations to SLIGRL in the main (R_{max}: 24.9 ± 5.7%) and intrapulmonary bronchi (R_{max}: 32.8 ± 6.8%) but had no effect on relaxations to SLIGRL in the trachea (R_{max}: 47.1 ± 6.0%; Figure 3). Relaxations to SLIGRL in all segments of the airways were abolished by either oxyHb (Figure 3) or metHb (data not shown) yet were unaffected by either the NO synthase inhibitor, L-NOARG (Figure 3), the guanylyl cyclase inhibitor, ODQ ($n=4$, data not shown) or the haem oxygenase inhibitor, ZnPP-IX (10 μM; $n=4$, data not shown). Like SLIGRL, epithelium-dependent relaxations to GYPGKF were unaffected by indomethacin in the trachea, but were inhibited in the intrapulmonary bronchi (Figure 4). Also, there was a trend for indomethacin to inhibit relaxations to GYPGKF in the main bronchi (Figure 4). By contrast, contractions to SFLLRN were significantly potentiated by indomethacin in each region of the airways (Table 1), but were unaffected following treatment of bronchi with 3 μM compound 48/80 (Figure 5). Indomethacin unmasked a 32.8 ± 8.9% ($n=6$) contraction to TFLLR in the intrapulmonary bronchi and abolished the relaxation to TFLLR in the trachea (7.2 ± 5.6%, $n=8$).
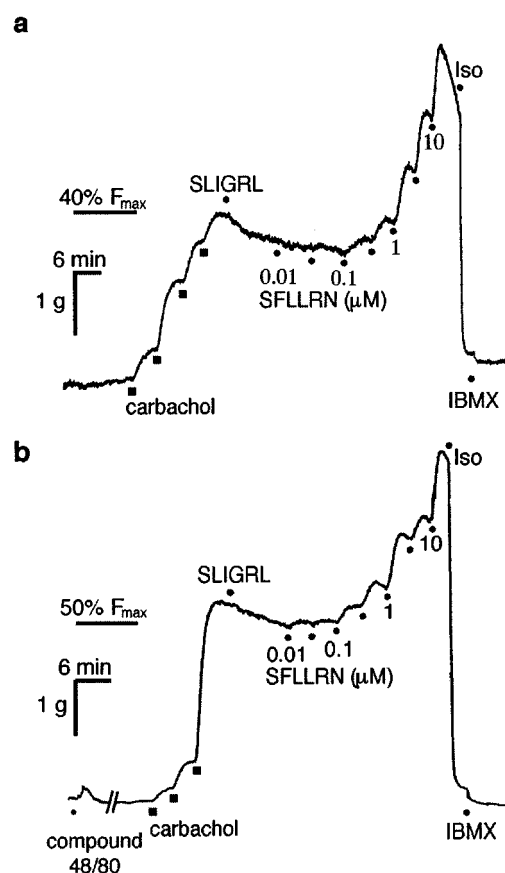

Figure 5 Digitized traces of original chart recordings showing concentration-dependent contractions to SFLLRN in the absence (a) and presence (b) of compound 48/80 (3 μM). Both tissues were treated with indomethacin (3 μM) and nifedipine (0.3 μM) prior to contracting to 40–50% F_{max} with carbachol and subsequently treated with SLIGRL (10 μM) before cumulative additions of SFLLRN. Maximum relaxations were obtained with isoprenaline (Iso, 3 μM) and IBMX (100 μM). The break in (b) represents 30 min. Representative of three similar experiments.

Immunohistochemical localization of PAR2 and PAR1

Immunoreactivities to PAR2-C and PAR2-N antibodies co-localized and were most commonly observed on the apical surface of ciliated columnar epithelial cells in the rat trachea, bronchi and its first branch (Figure 6). In some preparations PAR2 immunofluorescence was observed on the plasma membrane and cytoplasm of non-ciliated epithelial cells and on airways smooth muscle. Immunofluorescence in sections labelled with PAR2-C-antiserum preabsorbed with the immunizing peptide was abolished (data not shown). Very weak PAR1 immunoreactivity was observed on epithelial and smooth muscle cells of the trachea, main bronchi and intrapulmonary bronchi, but the cytoplasm of mast cells was intensely immunofluorescent (Figure 7).

Discussion

The main finding of this study was that although the PAR1-, PAR2- and PAR4-activating peptides, SFLLRN, SLIGRL and GYPGKF, respectively caused contraction (PAR1) and epithelium-dependent relaxation (PAR2 and PAR4) in three discrete regions of the rat isolated airways, the enzyme
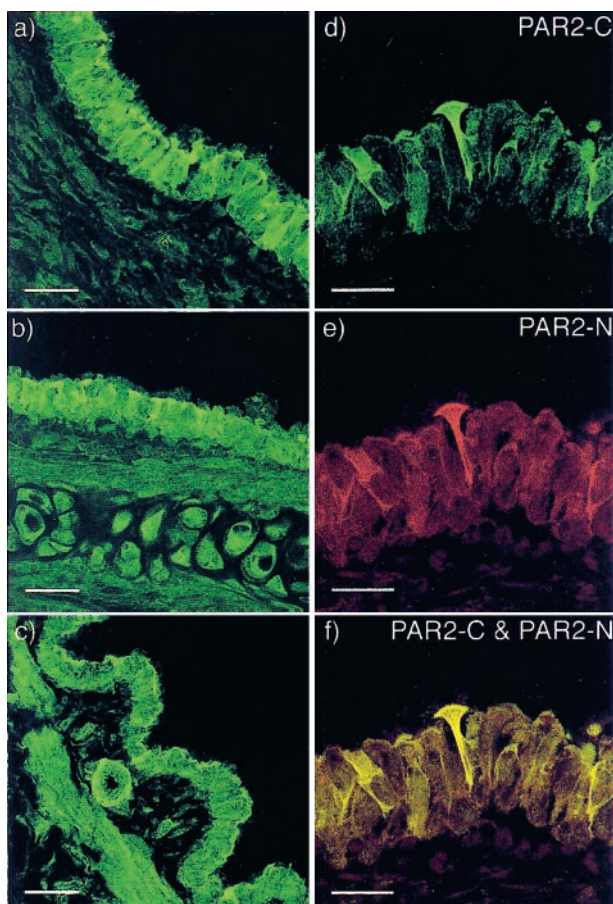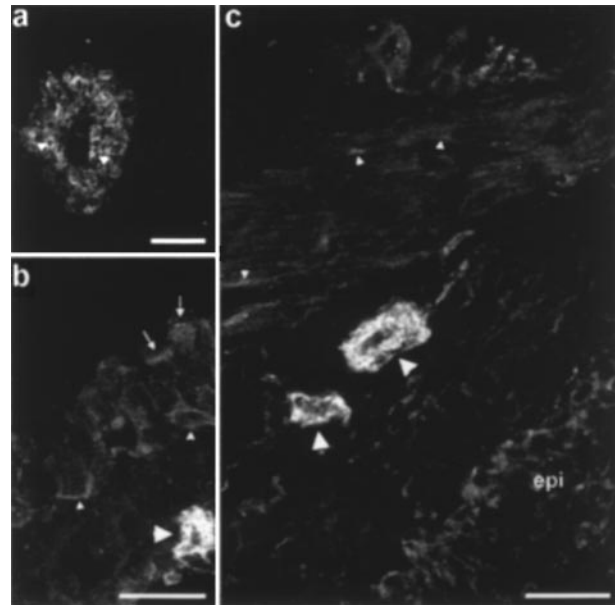

Figure 7 Confocal microscopic images showing PAR1 immuno-fluorescence in rat bronchi, which is representative of the other two airway regions used in the functional studies. (a) A mast cell showing intense cytoplasmic immunofluorescence with distinct granules (arrowheads) showing no staining. (b) Faint staining of ciliated (arrows) and basal (small arrowheads) epithelial cells. A mast cell (large arrowhead) is shown to illustrate the low level of epithelial fluorescence. (c) Comparison of PAR-1 immunofluorescence on smooth muscle (small arrowheads), mast cells (large arrowheads) and the epithelium (epi). Only a few smooth muscle cells show patchy surface staining while most cells appear to show very weak cytoplasmic staining. The scale bar in (a) represents 10 μm, whilst those in (b) and (c) represent 25 μm.

Figure 6 Confocal microscopic images showing immunohistochemical localization of PAR2 in rat trachea (a), main bronchi (b) and intrapulmonary bronchi (c) with the PAR2-C antibody (green). Higher magnification images (d,e,f) of a single section of the main bronchus to show co-localization of PAR2-like immunofluorescence with two separate PAR2 antibodies; (d) PAR2-C antibody (green), (e) PAR2-N antibody (red) and (f) superimposed images of (d) and (e) showing co-localization as yellow. Scale bars for left panel (a–c) represent 30 μm; whilst the right panel (d–f) scale bars represent 15 μm.

activators of these receptors, thrombin and trypsin (Nystedt *et al.*, 1994; Saifeddine *et al.*, 1996; Molino *et al.*, 1997; Xu *et al.*, 1998) were mostly ineffective. Also, another novel and puzzling result was that SLIGRL-induced relaxations were inhibited by haemoglobin regardless of the redox state of its haem prosthetic group (i.e. Fe^{2+}/Fe^{3+}) but not by other conventional NO inhibitors. Thus, the inhibition by Hb most likely occurred *via* a mechanism independent of its NO- or CO-scavenging properties (Bowman *et al.*, 1982; Martin *et al.*, 1985).

One explanation for the failure of trypsin and thrombin to mimic the responses observed to the PAR-activating peptides could be that the ligands were acting independent of the cloned PARs. Activation of PAR1 and PAR2 by the activating peptides is supported by immunohistochemical localization of these receptors on rat airway epithelia (and smooth muscle). Moreover, precise co-localization of immunofluorescence with two distinct PAR2 antibodies validates labelling of the cloned PAR2. Although similar immunohistochemical techniques were not used in this study to localize PAR4, evidence currently available suggests that GYPGKF selectively activates PAR4 (Hollenberg *et al.*, 1999; Lan *et al.*, 2000). Therefore, responses observed to this peptide in the rat airways were most likely mediated by PAR4. One of the PAR2 antibodies used was raised against part of the N-terminal of the human PAR2 which includes ser[37] of the arg[36]-ser[37] cleavage site (D'Andrea *et al.*, 1998) and the other raised against the C-terminal of the mouse PAR2 (Cocks *et al.*, 1999; Corvera *et al.*, 1999). Thus, unless the rat airway epithelial PAR2 contains a mutation at the specific cleavage site for trypsin, it is difficult to explain why trypsin failed to cleave the receptor and cause

relaxation. The inability of enzymes to activate both epithelial and smooth muscle PARs in rat airways may be related to the presence of endogenous inhibitors of serine proteases (e.g. serpins: Potempa *et al*., 1994; Wright, 1996; Whisstock *et al*., 1998). For example, the main regulator of extracellular trypsin-like activity in both the airways (Cichy *et al*., 1997) and gut (Potempa *et al*., 1994) is α_1-antitrypsin (α_1-AT) which is also able to inhibit thrombin (Bonniec *et al*., 1995). Relative high concentrations of trypsin were required to stimulate ion transport across rat intestinal epithelium compared to a cloned PAR2 assay (Vergnolle *et al*., 1998). These authors, however, argued that both the high concentrations of trypsin and the reduced potency of peptide PAR2 activators required to stimulate ion transport in the rat intestinal assay indicated that a separate PAR2-like, but relatively trypsin-insensitive receptor, was present in the rat jejunum (Vergnolle *et al*., 1998). However, in the present study SLIGRL and SFLLRN had similar potencies as reported previously for mouse airways (Cocks *et al*., 1999; Lan *et al*., 2000). Therefore, we suggest that the PARs present in rat airways are similar to the cloned PARs and that the inability of both trypsin and thrombin to activate PAR1, PAR2 and PAR4 is most likely due to the presence of broad-spectrum anti-proteases, like α_1-AT, in levels sufficient to block the activity of exogenously applied enzymes.

Although PAR1 immunoreactivity was weak in the epithelium and smooth muscle of rat airways, mast cells were strongly PAR1 immunoreactive. It is unlikely, however, that mast cell degranulation products such as histamine and serotonin accounted for the contractions to SFLLRN observed since degranulation of mast cells with compound 48/80 (Ennis *et al*., 1984; Levi-Schaffer & Riesel-Yaron, 1990) failed to affect the contractions to SFLLRN. Furthermore, compound 48/80 caused much weaker contractions than SFLLRN (see Figure 5). The finding that the contractions to SFLLRN were potentiated by indomethacin in all sections of the rat airway at first suggested the involvement of a basally-released inhibitory prostanoid. However, this is unlikely since contractions to serotonin were not similarly potentiated by indomethacin (Chow & Cocks, unpublished data). Also, the enhanced contractile responses to SFLLRN after treatment with indomethacin was not due to the inhibition of prostanoid-mediated relaxations to this ligand acting non-specifically on PAR2 (Blackhart *et al*., 1996; Hollenberg *et al*., 1997; Moffatt & Cocks, 1998; Al-Ani *et al*., 1999; Kawabata *et al*., 1999) since PAR2 were desensitized with SLIGRL prior to the addition of SFLLRN. The contractions unmasked by indomethacin in the intrapulmonary bronchi by the more selective but less potent PAR1-activating peptide, TFLLR (Hollenberg *et al*., 1997) support the conclusion here that similar contractions to SFLLRN in the rat airways were PAR1-mediated. Any role for PAR2-induced release of inhibitory prostanoids in the response to SFLLRN is further unlikely as indomethacin failed to affect the relaxations to SLIGRL in the trachea, yet the contractions to SFLLRN in this preparation were potentiated as in the other regions of the airways. Therefore, although PAR1 immunofluorescence was only weakly detected on epithelial and smooth muscle cells, we suggest that epithelial PAR1 were activated by

SFLLRN to release inhibitory prostanoids in sufficient amounts to blunt the contractile response to simultaneous activation of smooth muscle PAR1. This is further supported by concentration-dependent relaxations observed to TFLLR in the trachea which were inhibited by indomethacin. The failure of the cyclo-oxygenase inhibitor to have any effect on the epithelium-dependent relaxations to SLIGRL and GYPGKF in the trachea suggests that unlike epithelial PAR1, epithelial PAR2 and PAR4 are not coupled to cyclo-oxygenase in this region of the rat airways.

The dependence of the relaxant responses to SLIGRL and GYPGKF on cyclo-oxygenase products appeared to increase with decreasing airway calibre, since indomethacin caused greater inhibition of responses to each peptide in the bronchi but had no effect in the trachea. We considered it likely that the non-prostanoid components of these responses were mediated by NO, since similar epithelium-dependent relaxations to substance P in rat airways have been shown to be mediated by both prostaglandin E_2 (PGE_2) and NO (Szarek *et al*., 1998; Mhanna *et al*., 1999). Whilst relaxations to SLIGRL were abolished by either oxyHb or metHb, this was unlikely due to removal of NO or CO since inhibitors of NO synthase (L-NOARG: Moore *et al*., 1990; Rees *et al*., 1990), guanylyl cyclase (ODQ: Garthwaite *et al*., 1995) and haem oxygenase (ZnPP-IX: Ny *et al*., 1996) had no effect on the responses to SLIGRL in any region of the airways studied. In addition, any direct interaction between Hb and SLIGRL or PGE_2 seems unlikely for two reasons. First, as we have previously reported, oxyHb had no effect on similar epithelium- and PGE_2-dependent relaxations to SLIGRL in the mouse isolated bronchi (Cocks *et al*., 1999) and second, oxyHb did not affect the relaxations to exogenously applied PGE_2 in both the mouse and rat bronchi (Chow & Cocks, unpublished observations). Therefore, although we have not determined the nature and mechanism of the mediator inducing indomethacin-insensitive relaxations to the PAR-activating peptides, SLIGRL (and GYPGKF), the most likely site of action for Hb is proximal to the production of mediator(s) from the epithelium, since cyclo-oxygenase-derived products contributed differentially to relaxations in each region of the airway despite universal inhibition of responses with Hb.

In conclusion, this study indicates that rat airway epithelial PAR2 and PAR4 as well as smooth muscle PAR1 appear to be activated by their respective synthetic tethered ligands but not enzymatically by either trypsin or thrombin. If this inactivity of trypsin and thrombin is due to endogenous anti-proteases then our study highlights the need to consider species differences in the environments in which PARs are expressed when assessing physiological roles for these receptors.

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