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Role of PGE_2 in protease-activated receptor-1, -2 and -4 mediated relaxation in the mouse isolated trachea

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1 The potential mediator role of the prostanoid PGE_2 in airway smooth muscle relaxations induced by peptidic and proteolytic activators of PAR-1, PAR-2, PAR-3 and PAR-4 was investigated in carbachol-precontracted mouse isolated tracheal segments.

2 The tethered ligand domain sequences of murine PAR-1 (SFFLRN-NH_2), PAR-2 (SLIGRL-NH_2) and PAR-4 (GYPGKF-NH_2), but not PAR-3 (SFNGGP-NH_2), induced smooth muscle relaxation that was abolished by the non-selective cyclo-oxygenase (COX) inhibitor, indomethacin. The relative order for mean peak relaxation was SLIGRL-NH_2 > GYPGKF-NH_2 $\approx$ SFFLRN-NH_2 > SFNGGP-NH_2.

3 SFFLRN-NH_2, SLIGRL-NH_2 and GYPGKF-NH_2, but not SFNGGP-NH_2, induced significant PGE_2 release that was abolished by indomethacin. Like that for relaxation, the relative order for mean PGE_2 release was SLIGRL-NH_2 > GYPGKF-NH_2 > SFFLRN-NH_2 > SFNGGP-NH_2.

4 In dose-response studies, SLIGRL-NH_2 induced concentration-dependent increases in PGE_2 release (EC_{50} = 20.4 μM) and smooth muscle relaxation (EC_{50} = 15.8 μM).

5 The selective COX-2 inhibitor, nimesulide, but not the COX-1 inhibitor valeryl salicylate, significantly attenuated SLIGRL-NH_2-induced smooth muscle relaxation and PGE_2 release.

6 Exogenously applied PGE_2 induced potent smooth muscle relaxation (EC_{50} = 60.3 nM) that was inhibited by the mixed $DP/EP_1/EP_2$ prostanoid receptor antagonist, AH6809. SLIGRL-NH_2-induced relaxation was also significantly inhibited by AH6809.

7 In summary, the results of this study strongly suggest that PAR-mediated relaxation in murine tracheal smooth muscle is dependent on the generation of the spasmolytic prostanoid, PGE_2. PAR-stimulated PGE_2 release appears to be generated preferentially by COX-2 rather than COX-1, and induces relaxation *via* activation of the EP_2 receptor.
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Abbreviations: ANOVA, analysis of variance; c.l., confidence limits; C_{max}, contractile response to 10 μM carbachol; COX, cyclo-oxygenase; DP, prostaglandin D_2 receptor; EP, prostaglandin E_2 receptor; PAR, protease activated receptor; PGE_2, prostaglandin E_2; %R, percentage relaxation, expressed as a percentage of carbachol-induced pre-contraction

Introduction

Protease activated receptors (PARs) are a novel group of seven transmembane-spanning, G-protein coupled receptors that can respond to extracellular proteolytic activity (Hollenberg, 1999). PAR activation is dependent on the proteolytic cleavage of the amino-terminus of the receptor, which reveals a new amino-terminus that serves to act as a 'tethered ligand' to self-activate the receptor. The first cloned PAR (PAR-1) was activated by the coagulation enzyme thrombin, as well as a peptide sequence that mimicked the tethered ligand sequence (SFFLRN-NH_2 for murine PAR-1; Connolly *et al*., 1996). Subsequent PARs were shown to possess a similar activation mechanism (SLIGRL-NH_2, SFNGGP-NH_2 and GYPGKF-NH_2 for PAR-2, PAR-3 and PAR-4 respectively; Kahn *et al*., 1998; Nystedt *et al*., 1995). Thrombin (PAR-1, PAR-3 and PAR-4) and trypsin (PAR-2 and PAR-4) are the well-characterized enzymatic activators of PARs, although other potential PAR activators include mast cell tryptase (Steinhoff *et al*., 1999), Coagulation Factor Xa (Fox *et al*., 1997), Granzyme A (Suidan *et al*., 1994), as well as Cathepsin G (Sambrano *et al*., 2000). These latter proteases may play a role in PAR activation at sites where trypsin and thrombin are less abundant.

PAR activation evokes a wide array of responses in biological tissues, including platelet aggregation (Kahn *et al*., 1999), neuronal apoptosis (Sarker *et al*., 1999; Turgeon *et al*., 1999), smooth muscle and fibroblast mitogenesis (Bretschneider *et al*., 1999; McNamara *et al*., 1993; Akers *et al*., 2000), as well as regulating smooth muscle tone either directly or *via* the release of mediators such as cytokines, nitric oxide (NO) or prostanoids. For example, stimulation of

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PAR-1 and PAR-2 induces vascular smooth muscle relaxation, *via* the synthesis of endothelium-derived NO (Magazine & Srivastava, 1996). In contrast, in the presence of an NO synthase inhibitor or following endothelium denudation, stimulation of PAR-1 (but not PAR-2) induced vascular smooth muscle contraction (Hwa *et al*., 1996; Magazine & Srivastava, 1996).

In airway tissues, PAR-1, -2 and -4-stimulated relaxations also appear to be mediated *via* the generation of an intermediary relaxant factor(s). However, in contrast to vascular tissues, PAR-mediated relaxations were inhibited by indomethacin, suggesting the relaxant factor was a cyclo-oxygenase (COX), rather than a nitric oxide synthase, product (Cocks *et al*., 1999; Lan *et al*., 2000). These findings suggest a role of bronchorelaxant prostanoids in PAR-induced airway smooth muscle relaxation (Lan *et al*., 2000). To investigate the potential role of cyclo-oxygenase-derived PGE_2 as a mediator of PAR-induced relaxations, simultaneous measurements of PGE_2 release and smooth muscle relaxation were obtained in mouse isolated tracheal preparations in response to the selective PAR-1, -2, -3 and -4 ligands, as well as to the enzymatic activators, namely, trypsin and thrombin. PAR-2-mediated relaxations were further characterized by the use of valeryl salicylate (a selective COX-1 enzyme inhibitor, Bhattacharyya *et al*., 1995; Johnson *et al*., 1995), nimesulide (a selective COX-2 inhibitor, Pang & Knox, 1997; Range *et al*., 2000), indomethacin (a non-selective COX-1 and COX-2 inhibitor, Vane *et al*., 1998), and a mixed $DP/EP_1/EP_2$ prostanoid receptor antagonist, AH6809 (Funk *et al*., 1993; Keery & Lumley, 1988; Woodward *et al*., 1995).

Methods

Tissue preparation

Male CBA/CaH mice (8–10 weeks of age) were killed with an overdose of pentobarbitone sodium (250 mg kg^{-1} i.p.). The upper respiratory tract and associated alimentary tissue were rapidly excised and placed in a petri dish where the trachea was dissected free from surrounding tissue. Entire tracheal segments (4 mm long) were mounted between two stainless steel supports under a resting tension of 0.5 g in organ baths containing Krebs bicarbonate solution (composition in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4.7H_2O$ 0.57, $CaCl_2$ 2.5, D-glucose 11.1) that was bubbled with 5% CO_2 in O_2 (carbogen) and maintained at 37°C. Changes in isometric tension were detected using FTO3 Grass force displacement transducers coupled to a custom-built preamplifier and a data acquisition system. After a 45 min period of equilibration, during which time the bath fluid was changed every 15 min and the tension readjusted to 0.5 g, the tracheal segments were exposed to the cumulative additions of a submaximal (0.2 μM) and a supramaximal (10 μM) concentration of carbachol. The response to 10 μM carbachol was termed C_{max}.

Effects of PAR activators on airway smooth muscle tone

Following a 20 min washout and rest period, tracheal preparations were precontracted to approximately 60% C_{max} with carbachol, after which 100 μM of PAR-1, PAR-2, PAR-3 or PAR-4 scrambled peptide (FSFLRN-NH_2, LSIGRL-NH_2, FSNGGP-NH_2 or GYPGFK-NH_2 respectively) was added to the bath for 10 min. The entire bath fluid was collected and stored at −85°C prior to PGE_2 analysis. The maximal relaxation response (%R, expressed as a percentage of the precontraction, i.e.: 100%R represented a total reversal of carbachol-induced contraction) was also recorded. The preparation was washed and rested for a further 15 min, and precontacted to 60% C_{max} with carbachol. One hundred μM of the corresponding active peptide (SFFLRN-NH_2, SLIGRL-NH_2, SFNGGP-NH_2 or GYPGKF-NH_2 respectively) was then added for 10 min, and the bath fluid collected and relaxation response measured as described above. A similar protocol was used to measure the effects of the proteolytic activators, trypsin and thrombin. Responses evoked by PAR peptidic and enzymatic activators were compared to an appropriate time control (Figure 1). In some experiments, the role of cyclo-oxygenase (COX) and its isoforms was investigated by including 1 mM valeryl salicylate (COX-1-selective inhibitor), 1 μM nimesulide (COX-2-selective) or 3 μM indomethacin (non-selective

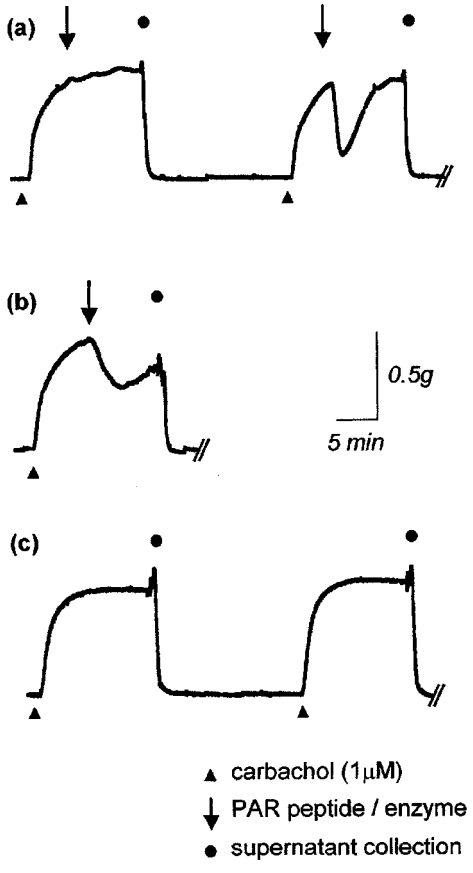

Figure 1 Representative tension-recording traces of PAR-induced relaxation on the mouse isolated trachea precontracted to 60% C_{max} with carbachol (1 μM). (a) Relaxations induced by a scrambled PAR peptide (in this case, 100 μM LSIGRL-NH_2), and then by the corresponding active PAR peptide (100 μM SLIGRL-NH_2). Supernatants were collected for PGE_2 quantification 10 min after addition of each peptide. (b) Relaxations induced by an enzymatic PAR activator (in this example, 100 U ml^{-1} trypsin). (c) Unstimulated preparations were used as appropriate time controls to determine basal PGE_2 release.

COX-1 and COX-2 inhibitor) in the Krebs bicarbonate solution for the duration of the experiment. At the completion of all the experiments, tracheal segments were blotted dry and weighed.

Effect of AH6809 on PGE_2 and SLIGRL-NH_2-induced relaxation

To examine the role of relaxant prostanoid receptors in the mouse trachea, tracheal preparations were incubated with AH6809 (3 μM) or vehicle for 25 min. The preparations were then precontracted to approximately 60% C_{max} with carbachol, after which a cumulative PGE_2 concentration-response curve was generated (3 nM to 1 μM). A similar protocol was used to examine the maximal relaxant response of a bolus dose of PGE_2 (30 nM) or SLIGRL-NH_2 (30 μM).

PGE_2 quantification

The collected bath fluid was thawed, and 50 μl aliquots were assayed for PGE_2 content by enzyme immunoassay (Cayman Chemical Co, Cat. no. 514010), according to the manufacturer's instructions. The assay system has a high specificity for PGE_2, and possesses minimal affinity (<0.01%) for other well-characterized prostanoids such as PGD_2 and $PGF_{2\alpha}$. Samples taken from preparations stimulated with PAR peptides or enzymes were compared to an appropriate time control, and expressed as pg PGE_2 mg^{-1} trachea (Table 1).

Materials

Amide-capped hexapeptides were synthesized by Dr Richard Lipscombe (Protein Facility, University of Western Australia, Perth, Australia). Thrombin was obtained from CSL Limited (Melbourne, Australia), and valeryl salicylate, nimesulide and PGE_2 enzyme immunoassay kits were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Indomethacin, trypsin and carbamycholine chloride (carbachol) were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.), while AH6809 was purchased from BioMol Research Laboratories (Plymouth Meeting, PA, U.S.A.).

Stock solutions of PAR agonists (10 mM), thrombin (1 kU ml^{-1}), trypsin (10 kU ml^{-1}) and carbachol (10 mM) were made in double-distilled water and stored at −20°C. PGE_2 (10 mM) was made in 100% ethanol and stored at −20°C; valeryl salicylate (100 mM) and nimesulide (10 mM) were made in 100% ethanol and stored at room temperature. AH6809 (6 mM) was made in dimethyl sulphoxide and stored at −20°C, while indomethacin was prepared in 5 mM Na_2CO_3 and used immediately. Dilution of drugs were prepared on the day of the experiment in double distilled water or Krebs bicarbonate solution.

Table 1 Effects of trypsin and thrombin on smooth muscle relaxation and PGE_2 release in pre-contracted mouse trachea in the presence or absence of indomethacin

Treatment	*%R*	*%R+ indo**	*[PGE_2]*	*[PGE_2] + indo**
Trypsin (100 U ml^{-1})	57.2±7.2	0±0	51 (12–225)	0.6 (0.3–1.1)
Thrombin (30 U ml^{-1})	38.2±7.0	0±0	57 (21–125)	0.7 (0.1–8.7)

Percentage relaxation (%R) is expressed as mean±s.e.mean (n=3–5). PGE_2 release (pg PGE_2 mg^{-1} trachea) was expressed as the geometric mean with associated 95% confidence limits. Relaxations and PGE_2 production by trypsin and thrombin were abolished by indomethacin (+indo) (*P<0.05, one-way ANOVA).

Analyses

Percentage relaxation (%R) was expressed as the arithmetic mean±s.e.mean. PGE_2 release and EC_{50} values (the concentration that induced 50% of the maximal response) were expressed as the geometric mean with associated 95% confidence limits. Differences between mean responses were assessed using one-way ANOVA and, when appropriate, paired Student's t-tests and Bonferroni's correction for multiple comparisons were used, with P<0.05 being considered statistically significant (SigmaStat, Jandel Corporation, San Rafael, CA, U.S.A.). Drug concentrations described in the text refer to the final molar concentrations in the organ bath.

Results

Relaxant effects of PAR activators

One hundred μM of PAR-1, -2 and -4 activating peptide induced significantly greater relaxations than the corresponding scrambled peptides (P<0.05, paired t-test; Figure 2). SLIGRL-NH_2 induced the greatest level of relaxation (%R=78.8±2.7, n=9), followed by SFFLRN-NH_2 (%R=22.9±5.6, n=5) and GYPGKF-NH_2 (%R=21.3±3.7, n=4). SFNGGP-NH_2 did not induce significant airway smooth

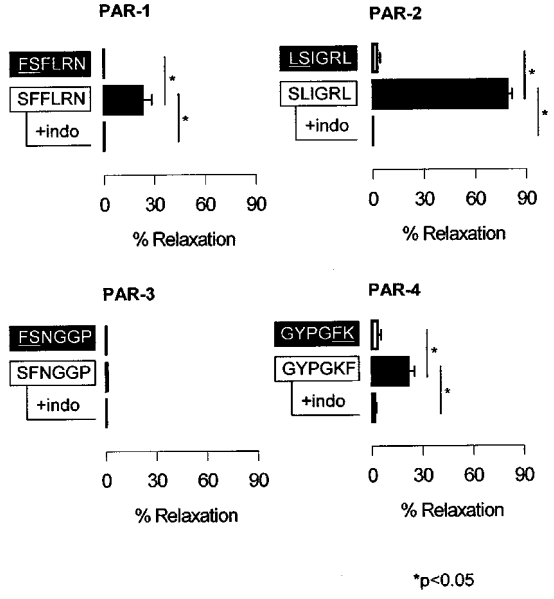

Figure 2 Relaxant effects of 100 μM PAR-1, -2, -3 and -4 peptides on carbachol-precontracted mouse trachea in the absence or presence of 3 μM indomethacin (indo). Data are presented as mean±s.e.mean (n=4–5).

muscle relaxation (%R = 0.8 ± 0.8, $n = 5$; Figure 2). The two enzymatic PAR activators, trypsin and thrombin, induced significant relaxations (%R = 57.2 ± 7.2, $n = 4$ and 38.2 ± 7.0, $n = 5$ respectively; Table 1). Relaxations induced by peptidic and enzymatic activators of PARs were abolished by the cyclo-oxygenase inhibitor, indomethacin (3 μM; Figure 2 and Table 1).

PGE_2 release by PAR activators

Over a 15 min collection period, the basal release of PGE_2 from carbachol-contracted mouse isolated trachea was 3.0 pg mg^{-1} tissue (95% c.l., 2.3 – 4.0 pg mg^{-1} trachea, $n = 3$). One hundred μM of PAR-1, -2 and -4 activating peptides, but not scrambled PAR peptides, stimulated PGE_2 release above basal levels ($P < 0.05$; Figure 3). SLIGRL-NH_2 produced the greatest increase in PGE_2 release (18.4 fold above basal, 95% c.l., 7.9 – 47.8 fold increase, $n = 9$), followed by GYPGKF-NH_2 (5.7 fold above basal, 95% c.l., 2.7 – 16.7 fold increase, $n = 4$) and SFFLRN-NH_2 (2.3 fold above basal, 95% c.l., 1.1 – 9.8 fold increase, $n = 5$). SFNGGP-NH_2 did not significantly modulate PGE_2 release relative to basal secretions (1.2 fold above basal, 95% c.l., 0.9 – 5.8 fold increase, $n = 5$; Figure 3). The two enzymatic activators of PAR, trypsin and thrombin, produced similar increases in PGE_2 release from the mouse trachea (15.9 fold above basal, 95% c.l., 3.9 – 74.1 fold increase, $n = 4$, and 17.9 fold above basal, 95% c.l., 6.9 – 51.6 fold increase, $n = 5$, respectively; Table 1). Indomethacin (3 μM) inhibited PGE_2 release induced by peptidic and enzymatic activators of PARs (Figure 3 and Table 1).

Relationship between SLIGRL-NH_2, COX-isoforms, PGE_2 release and smooth muscle relaxation

Over the concentration range of 3 to 100 μM, SLIGRL-NH_2 concentration-dependently induced smooth muscle relaxation (EC_{50} = 15.8 μM, 95% c.l., 9.2 – 27.2 μM, $n = 6$) and PGE_2 release (EC_{50} = 20.3 μM, 95% c.l., 4.4 – 93.5 μM, $n = 4$; Figure 4a). The mean EC_{50} values of SLIGRL-NH_2-induced smooth muscle relaxation and SLIGRL-NH_2-induced PGE_2 release were not statistically different (one-way ANOVA of log EC_{50}). One hundred μM SLIGRL-NH_2-induced PGE_2 release and smooth muscle relaxation were markedly attenuated by the selective COX-2 inhibitor nimesulide (1 μM), and by the non-selective COX inhibitor indomethacin (3 μM). In contrast, the selective COX-1 inhibitor valeryl salicylate (1 mM) had no significant effect on SLIGRL-NH_2-induced relaxations or PGE_2 release ($P < 0.05$; Figure 4b,c).

Effect of AH6809 on PGE_2 and SLIGRL-NH_2-induced relaxations

Concentration-dependent relaxations induced by the cumulative addition of exogenous PGE_2 (EC_{50} = 60.3 nM, 95% c.l., 34.9 – 104.0 nM, $n = 10$) were inhibited by the DP/EP_1/EP_2 antagonist, AH6809 (3 μM; Figure 5a). AH6809 also significantly attenuated the relaxant responses to single-concentration additions of PGE_2 (30 nM) or SLIGRL-NH_2 (30 μM; Figure 5b,c).

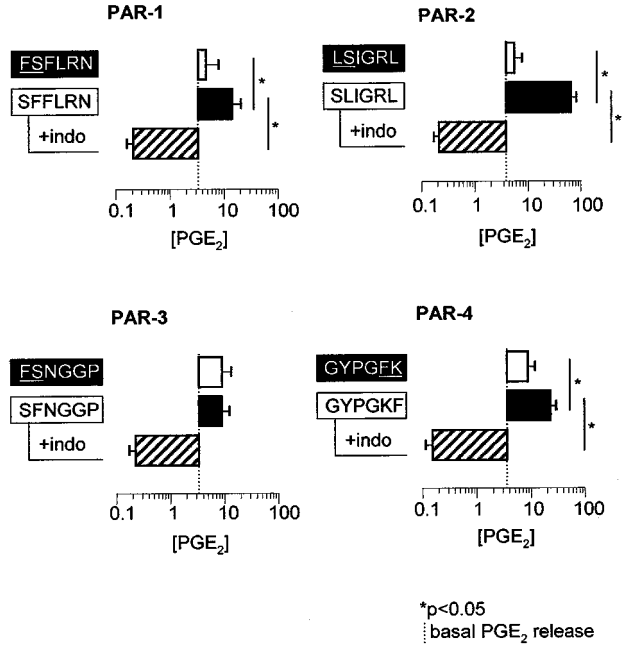

Figure 3 Effects of 100 μM PAR-1, -2, -3 and -4 peptides on PGE_2 release from carbachol-precontracted mouse trachea in the absence or presence of 3 μM indomethacin (indo). Data are presented as mean ± s.e.mean ($n = 4 – 9$).

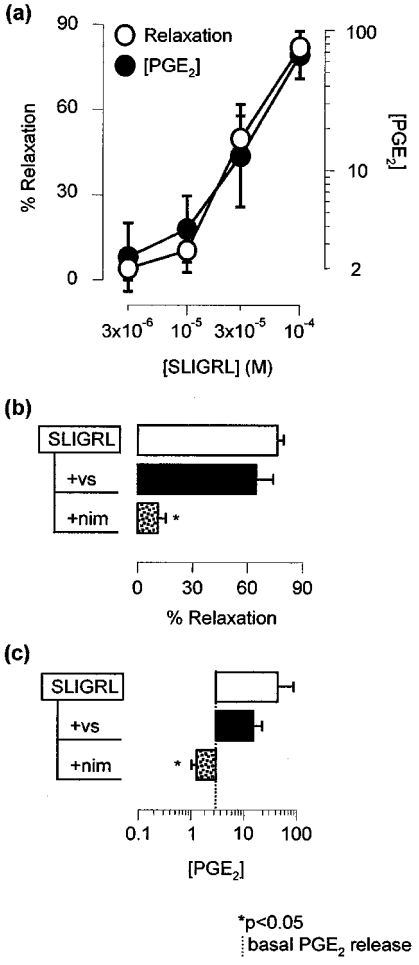

Figure 4 (a) Concentration-response curve of SLIGRL-NH_2-induced smooth muscle relaxation and PGE_2 release from precontracted mouse isolated trachea ($n = 4 – 6$). (b,c) Effects of 1 mM valeryl salicylate (vs), 1 μM nimesulide (nim), or 3 μM indomethacin (indo) on SLIGRL-NH_2-induced smooth muscle relaxation and PGE_2 release ($n = 5 – 7$).

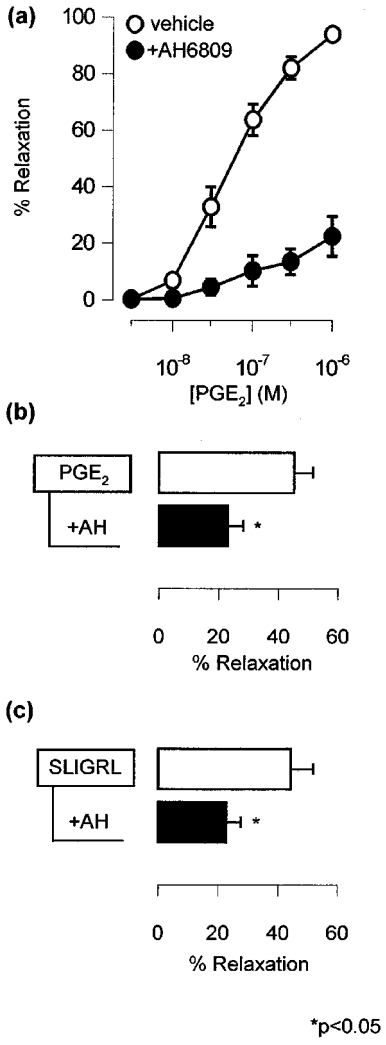

Figure 5 (a) Effect of 3 μM AH6809 on the cumulative concentration-response relationship generated by exogenous PGE_2 in the precontracted mouse isolated trachea ($n=5-10$). (b,c) Effect of 3 μM AH6809 (AH) on the single concentration addition of PGE_2 (30 nM) or SLIGRL-NH_2 (30 μM) in the precontracted mouse isolated trachea ($n=6-13$).

Discussion

In this study, synthetic peptidic activators of PAR-1, -2 and -4, as well as trypsin and thrombin, induced indomethacin-sensitive release of PGE_2 and airway smooth muscle relaxation of murine isolated trachea. The PAR-2 activator, SLIGRL-NH_2, induced concentration-dependent PGE_2 release and smooth muscle relaxation, and exogenously applied PGE_2 induced marked relaxation responses in this isolated airway preparation. Furthermore, SLIGRL-NH_2-induced relaxations were inhibited by the COX-2 selective inhibitor nimesulide and a mixed $DP/EP_1/EP_2$ receptor antagonist, AH6809. Taken together, these findings provide strong supportive evidence for COX-2-derived PGE_2 playing an important mediator role in PAR-induced relaxations in mouse isolated trachea.

Several recent studies have demonstrated that stimulation of airway PARs modulates airway smooth muscle tone (Cocks *et al*., 1999; Lan *et al*., 2000; Ricciardolo *et al*., 2000). For example, PAR-2 activators have been reported to induce relaxation in isolated smooth muscle preparations from mouse bronchi and trachea (Cocks *et al*., 1999; Lan *et al*., 2000), guinea-pig trachea and main bronchi (Ricciardolo *et al*., 2000) and rat trachea, main bronchi and interpulmonary bronchi (Chow *et al*., 2000). In contrast, PAR-2 activation induced contractile responses in guinea-pig intrapulmonary bronchi and epithelium-denuded main bronchi (Ricciardolo *et al*., 2000). In the current study, PAR-2 activation was associated solely with relaxation responses, although PAR-1- and PAR-4-mediated relaxations were typically preceded by a transient contractile response (Lan *et al*., 2000). PAR-1-mediated contractile responses have also been reported in epithelium-denuded mouse bronchi (Cocks *et al*., 1999) and rat trachea, and main and intrapulmonary bronchi (Chow *et al*., 2000). Furthermore, alpha-thrombin, which is an enzymatic activator of PAR-1 (-3 and -4), induced contraction of human isolated bronchial rings in a receptor-specific and dose-dependent manner (Hauck *et al*., 1999). Thus, stimulation of PARs in the airways may result in profound changes in bronchomotor tone, but the magnitude and nature of the response depends on which particular PAR is stimulated, and is subject to significant regional and species differences, many of which have yet to be fully elucidated in human airways.

Indomethacin, a non-selective inhibitor of cyclo-oxygenase, completely prevented PAR-1, -2 and -4-mediated relaxation responses in mouse trachea (current study; Lan *et al*., 2000). PAR-2-mediated relaxations were also at least partially inhibited by indomethacin in isolated airways preparations of mouse and rat bronchi (Cocks *et al*., 1999; Chow *et al*., 2000), and guinea-pig trachea (Ricciardolo *et al*., 2000), suggesting that these PAR-induced responses were mediated *via* the actions of cyclo-oxygenase product(s). Moreover, results from the current study provide strong evidence that the cyclo-oxygenase product responsible for PAR-1, -2 and -4-mediated relaxations in mouse isolated tracheal preparations was PGE_2. Firstly, each of the PAR stimulants that induced a significant relaxant response (SFFLRN-NH_2, SLIGRL-NH_2, GYPGKF-NH_2, trypsin and thrombin) also induced a significant increase in PGE_2 release. In contrast, the synthetic peptidic activator of PAR-3, SFNGGP-NH_2, induced neither relaxation nor PGE_2 release. Secondly, there was a strong positive relationship between the amount of PGE_2 released by each of the synthetic peptidic PAR stimulants and the magnitude of the relaxant response. The relative order of effect for both relaxation and PGE_2 release was SLIGRL-NH_2 > GYPGKF-NH_2 $\approx$ SFFLRN-NH_2 > SFNGGP-NH_2. Thirdly, the PAR-2 stimulant SLIGRL-NH_2 induced concentration-dependent increases in both PGE_2 release and relaxation. Moreover, the concentration-effect relationships were superimposable, indicating that SLIGRL-NH_2-induced increases in PGE_2 release and smooth muscle relaxation occurred over the same concentration range. Finally, the exogenous application of PGE_2 induced potent, concentration-dependent relaxations in carbachol-contracted mouse isolated tracheal preparations, confirming previous studies that PGE_2 is an effective spasmolytic agent in this preparation (Li *et al*., 1998). Thus, these findings collectively suggest that the prostanoid PGE_2 mediates PAR-induced relaxations in the mouse airway.

An initial step in the synthesis of PGE_2 is the metabolism of arachidonic acid by the two cyclo-oxygenase isoforms, COX-1 and COX-2. Despite earlier indications that COX-2 only plays a role in pathological conditions, there is accumulating evidence of both COX-1 and COX-2 being required for normal cellular function. Constitutive COX-2 expression has been detected in non-inflamed bronchial tissue including the airway epithelium (Watkins *et al*., 1999), as well as smooth muscle cells of the pulmonary vasculature (Ermert *et al*., 1998). In the present study, we showed that SLIGRL-NH_2-induced PGE_2 release and smooth muscle relaxation in tracheal preparations obtained from non-diseased mice were largely attenuated by the selective COX-2 inhibitor nimesulide (Pang & Knox, 1997; Range *et al*., 2000), but not by selective COX-1 inhibitor valeryl salicylate (Bhattacharyya *et al*., 1995; Johnson *et al*., 1995). These findings suggest that PAR-2-mediated PGE_2 generation and subsequent smooth muscle relaxation was dependent on COX-2 rather than COX-1. However, the COX isoform(s) that regulate PAR-1 and PAR-4-induced PGE_2 release and smooth muscle relaxation remain unknown and is currently being investigated.

PGE_2 is the predominant prostaglandin in the upper respiratory tract (Karim *et al*., 1967; Knight *et al*., 1995a) and is the most potent relaxant prostaglandin in airway preparations (Coleman & Kennedy, 1980, Knight *et al*., 1995b). In the current study, PGE_2 induced potent concentration-dependent relaxation in murine isolated tracheal preparations. These findings are consistent with a recent report by Li *et al*. (1998) using murine trachea, and with a raft of other studies using animal (Kennedy *et al*., 1982; Gardiner, 1986; Lydford & McKechnie, 1994) and human (Norel *et al*., 1999) isolated airway smooth muscle preparations.

Somewhat less clear is the subtype(s) of prostanoid receptor that mediate these relaxant effects, since PGE_2 can activate four different subtypes of EP receptor (EP_1, EP_2, EP_3 and EP_4) and the distribution of these receptors in the murine airway is unknown. Nevertheless, EP_1 and EP_3 receptor subtypes are unlikely to be involved in PGE_2-induced airway smooth muscle relaxation since they are typically linked to transduction mechanisms that induce smooth muscle contraction *via* the increase of phosphoinositide tunover, elevation of intracellular free Ca^{2+} and inhibition of adenylate cyclase (Coleman *et al*., 1994).

In contrast, stimulation of EP_2 and EP_4 receptors is associated with increases in adenylate cyclase activity and intracellular cyclic AMP levels, a well-established pathway for mediating airway smooth muscle relaxation. Although the EP_4 receptor has been implicated in PGE_2-induced relaxations in rat isolated trachea (Lydford & McKechnie, 1994), it is the EP_2 receptor that has been proposed to be the predominant relaxant EP receptor in human bronchus (Norel *et al*., 1999) and several animal airway preparations (Kennedy *et al*., 1982; Gardiner, 1986). Our findings that AH6809, an antagonist at EP_2 but not EP_4 receptors, markedly inhibited PGE_2-induced relaxations suggests that EP_2 rather than EP_4 receptors were also involved in relaxation of murine trachea. Although further studies are required to clearly classify the relaxant EP receptor in murine trachea, it was interesting to note that AH6809 inhibited relaxations induced by PGE_2 and SLIGRL-NH_2 to a similar extent. These latter findings provide further support for the postulate that the relaxant actions of the PAR-2 activating peptide are mediated by PGE_2.

In addition to bronchodilatory effects, PGE_2 also possesses anti-inflammatory properties. For example, PGE_2 inhibits leukotriene production (Christman *et al*., 1993) and IgE synthesis (Pene *et al*., 1988), and blocks both the early and late responses to allergen challenge in asthmatics (Pavord *et al*., 1993). Furthermore, the recruitment, activation and survival of various inflammatory cells into the lung is inhibited by PGE_2 and PGE_2-mimetics (Smith *et al*., 1996; Chouaib *et al*., 1987; Alam *et al*., 1993). PGE_2 may also exert favourable effects on airway remodelling since PGE_2 (Florio *et al*., 1994) and COX-2 induction (Belvisi *et al*., 1998) both exert powerful anti-proliferative effects on airway smooth muscle cells and fibroblasts (McAnulty *et al*., 1997). In light of the evidence that endogenous PGE_2 may possess bronchoprotective and anti-inflammatory roles in the airway (for review, see Pavord & Tattersfield, 1995), release of PGE_2 by PAR activation may possess potential therapeutic benefit in respiratory diseases such as allergic rhinitis and asthma.

In conclusion, we have shown that peptidic and enzymatic activators of PAR-1, -2 and -4 induced concomitant release of PGE_2 and smooth muscle relaxation in murine trachea. Strong, positive relationships were observed between the amount of PGE_2 released and the extent of smooth muscle relaxation produced. Moreover, inhibition of PAR-2-induced PGE_2 release by the COX-2 inhibitors indomethacin and nimesulide was associated with marked attenuation of smooth muscle relaxation. Finally, exogenous PGE_2 induced marked concentration-dependent relaxations in mouse isolated tracheal smooth muscle, and both PGE_2- and SLIGRL-NH_2-mediated relaxations were inhibited by AH6809. These findings strongly suggest that COX-2-derived PGE_2 is an important mediator of PAR-2-induced relaxations in mouse isolated trachea.

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