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Contractile actions of proteinase-activated receptor-derived polypeptides in guinea-pig gastric and lung parenchymal strips: evidence for distinct receptor systems

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1 We have measured the contractile activities and relative potencies (EC_{50}s) of six thrombin PAR_1 receptor-derived receptor-activating peptides (PAR-APs): AparafluroFRChaCit-y-NH_2 (Cit-NH_2); SFLLRNP(P7); SFLLRNP-NH_2 (P7-NH_2); SFLLR (P5); SFLLR-NH_2 (P5-NH_2); TFLLR-NH_2 (TF-NH_2) and a PAR_2 receptor activating peptide [SLIGRL-NH_2 (SL-NH_2)] (a) in a guinea-pig lung peripheral parenchymal strip preparation and (b) in a gastric longitudinal smooth muscle preparation.

2 The relative potencies of the PAR-APs in the lung preparation (Cit-NH_2 ≅ TF-NH_2 ≅ P5-NH_2 > P7 ≅ P5 ≅ P7-NH_2; SL-NH_2 not active) differed appreciably from their relative potencies in the gastric preparation: Cit-NH_2 ≅ TF-NH_2 ≅ P7-NH_2 ≅ P5-NH_2 > P7 > P5 ≅ SL-NH_2.

3 The contractile actions of the PAR_1-selective peptide, TF-NH_2 in the gastric preparation were entirely dependent on extracellular calcium and were blocked by tyrosine kinase inhibitors (genistein, tyrphostin 47/AG213, PP1) and by the cyclooxygenase inhibitor, indomethacin, whereas in the lung preparation, the PAR_1-mediated contractile response was only partially dependent on extracellular calcium and was refractory to the actions of either tyrosine kinase inhibitors or indomethacin.

4 Partial sequencing of the PAR cDNAs detected by RT–PCR both in whole lung and in the peripheral parenchymal strip bioassay tissue demonstrated the presence of both PAR_1 and PAR_2 mRNA; the expression of PAR_2 was detected by immunohistochemistry.

5 The data point to the presence of distinct receptor systems for the PAR_1-APs in guinea-pig lung parenchymal and gastric smooth muscle and indicate that PAR_2 does not regulate contractile activity in peripheral parenchymal guinea-pig lung tissue
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Abbreviations: Amino acids are abbreviated by their one-letter codes; Cha, cyclohexyl-alanine; Cit, citrulline; Cit-NH_2, AparafluroFRChaCit-y-NH_2; GAPDH, glyceraldehyde phosphate dehydrogenase; HEK, human embryonic kidney cell line; LM, gastric longitudinal smooth muscle preparation; PAR_1, proteinase-activated receptor-1; PAR_2, proteinase-activated receptor-2; PAR-AP, PAR-activating peptide; PAR_1F, forward PCR primer for PAR_1; PAR_1R, reverse PCR primer for PAR_1; PAR_2F1 or PAR_2F2, forward PCR primers for PAR_2; PAR_2R, reverse PCR primer for PAR_2; P5, SFLLR; P5-NH_2, SFLLR-NH_2; P7, SFLLRN; P7-NH_2, SFLLRNP-NH_2; PP1, Src-selective tyrosine kinase inhibitor, 4-amino-5(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; RT–PCR, reverse-transcriptase-polymerase chain reaction; SL-NH_2, SL1GRL-NH_2; TF-NH_2, TFLLR-NH_2

Introduction

In addition to activating proteolytic enzyme cascades (e.g. coagulation caused by thrombin), proteinases such as thrombin, trypsin, tryptase and cathepsin G are now known to regulate target tissues *via* the proteolytic activation of cell surface G-protein-coupled receptors. At this point in time, four members of this unique proteinase-activated receptor (PAR) family have been cloned (PARs 1–4: Vu *et al*., 1991; Rasmussen *et al*., 1991; Nystedt *et al*., 1994; Ishihara *et al*., 1997; Xu *et al*., 1998; Kahn *et al*., 1998; Reviews: Coughlin, 2000; Dery *et al*., 1998; Hollenberg, 1999). A novel feature of the PARs is their mechanism of activation that involves the proteolytic unmasking of a 'tethered' N-terminal receptor activating sequence (Vu *et al*., 1991). Except for PAR_3, short synthetic peptides modeled on the revealed tethered ligand sequences are able to activate the PARs (so-called PAR-activating peptides or PAR-APs). In our own work, we have developed a number of PAR-selective receptor-activating peptide agonists and we have evaluated the originally described thrombin PAR_1 receptor-activating peptides (TRAPs) for their selectivity for PAR_1 compared with PAR_2 (Kawabata *et al*., 1999). With the PAR-APs it has proved possible to assess the effects of selective PAR activation in a variety of intact tissues ranging from neurons

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(Corvera *et al*., 1999) to the vasculature and gastrointestinal tract (Muramatsu *et al*., 1992; Saifeddine *et al*., 1996; Tay-Uyboco *et al*., 1995). Relatively recently, attention has begun to focus on the potential roles that the PARs may play in pulmonary pathophysiology (Cocks *et al*., 1999; Cocks & Moffatt, 2000; Akers, *et al*., 2000; Lan *et al*, 2000).

In the lung, several studies have pointed to a potential role for PAR_2 in asthma (Cocks *et al*., 1999; Akers *et al*., 2000; Lan *et al*., 2000). In one study (Lan *et al*., 2000), both contractile and relaxant responses have been observed for PAR-APs derived from PARs 1, 2 and 4 in murine tracheal smooth muscle preparations that were precontracted with 1 μM carbachol. The predominant response of the tracheal preparations was an indomethacin-blocked transient relaxation, in keeping with the hypothesis that epithelial-derived prostanoids may play a bronchoprotective role in asthma (Cocks *et al*., 1999; Cocks & Moffatt, 2000). Although tracheal smooth muscle segments are frequently used to assess broncho-active agents, previous work has shown that the tracheal preparation need not reflect the responsiveness of alveolar contractile elements, which are of primary importance in regulating pulmonary function (Drazen & Schneider, 1977). Since we were interested in studying the action of PAR_1- and PAR_2-APs on the alveolar smooth muscle elements and terminal bronchioles, we used a parenchymal strip preparation instead of more conventional tracheal or bronchial tissue strips for our study. We selected the guinea-pig as a source of tissue: (1) because this species is frequently used as a model for airway diseases such as asthma and (2) because we have previously assessed in depth the responsiveness of guinea-pig and rat gastric longitudinal smooth muscle (LM) preparations towards PAR_1 and PAR_2 activating peptides (Zheng *et al*, 1998; Al-Ani *et al*., 1995; Saifeddine *et al*., 1996). In a preliminary study, we had observed that the nonselective PAR-AP, SFLLR-NH_2 (P5-NH_2), caused a robust contractile response in both guinea-pig and rat lung parenchymal strips (Mandhane *et al*., 1995). It was the main aim of the study we report here: (a) to use a more extended library of PAR_1APs, including PAR_1-selective agonists, and a selective PAR_2AP to evaluate the PAR_1 and PAR_2 receptor systems present in the lung parenchymal smooth muscle preparation and (b) to compare the results obtained using the lung tissue with structure-activity relationships and signal transduction pathways observed for the same series of peptides in the gastric LM preparation; with which we were already familiar (Muramatsu *et al*., 1988; Hollenberg *et al*, 1993; Al-Ani *et al*., 1995; Yang *et al*., 1992; Zheng *et al*. 1998).

Methods

Bioassay preparations and assay procedures

All procedures were conducted according to the recommendations of the Canadian Council on Animal Care, concerning animal experimentation, as approved by an institutional committee for animal care. Hartley strain male guinea-pigs (250–400 g) were sacrificed by cervical dislocation and the thoracic cavity was opened. The animal was then immediately anticoagulated by the intracardiac administration (right ventricle) of heparin [5 ml of 1 mg ml^{-1} heparin (174 Units/ml, ICN Biomedicals Inc., Aurora OH, U.S.A.) in physiological saline] followed by perfusion of the lung (as evidenced by blanching) *via* the right ventricle with Krebs-Henseleit buffer pH 7.4, of composition (mM): NaCl, 115; KC1, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 2.5; KH_2PO_4, 1.2 and glucose, 10. Lungs were then removed *en block* and parenchymal strips were cut (about 2×10 mm) from the peripheral edge of each blanched pulmonary lobe preparation. This tissue contained mainly parenchymal elements, with few scattered terminal bronchioles and very few vascular structures (see Results). Strips were suspended in a 4 ml plastic organ bath maintained at 37°C and gassed with 95% O_2/5% CO_2. Tissue was subjected to a tension of 0.5 mN (determined to be optimal for response monitoring) and contractile force was recorded isometrically, using Grass or Statham force-displacement transducers. Identical lung strips were obtained for fixation and histological staining as well as for the preparation of RNA. The gastric longitudinal muscle strips were prepared as outlined elsewhere (Muramatsu *et al*., 1988; Yang *et al*., 1992; Hollenberg *et al*., 1993) and were used for bioassay. Isometric contractile responses to the receptor-derived peptides were monitored in the LM prepration as described previously (Muramatsu *et al*., 1988; Yang *et al*., 1992) under a tension of 1 mN. Agonists were added to the organ bath at 25–30-min intervals, and tension was allowed to develop over a 5–10 min time period, followed by washing the tissue and re-equilibration in fresh buffer. To monitor the response of tissues in the absence of extracellular calcium, the preparations were washed once with calcium-free Krebs-Henseleit buffer containing 0.2 mM EGTA (5 min) and were equilibrated in calcium-free buffer containing 0.2 mM EGTA for a further 5 min prior to exposure of the tissues to agonists. Contractile responses were expressed as a percentage (% KCl) of the response of the tissue to 50 mM KCl (in complete calcium-containing buffer). Values reported for the concentration-effect curves or for the histograms represent the means ($\pm$s.e.mean, bars in figures) for measurements done with 4–10 individual tissue preparations coming from three or more different animals. Receptor-activating peptides based on the human PAR_1 sequence: SFLLR-NH_2 (P5-NH_2), SFLLR (P5), SFLLRNP-NH_2 (P7-NH_2), SFLLRNP (P7), TFLLR-NH_2 (TF-NH_2) and Apara-fluroFRChaCit-y-NH_2 (Cit-NH_2) and on the rat PAR_2 sequence: SL1GRL-NH_2 (SL-NH_2), were prepared by standard solid phase synthesis procedures by the core peptide synthesis laboratory at the University of Calgary Faculty of Medicine, Calgary AB, Canada. Peptides were >95% pure by HPLC and mass spectral criteria. The concentrations and amino acid compositions of stock peptide solutions (1 to 3 mM), dissolved in 25 mM HEPES buffer, pH 7.4 were verified by quantitative amino acid analysis. The above described receptor activating peptides have been shown to activate PARs in a number of species including the hamster, rat, guinea-pig and humans and have to date been found to be the most potent for doing so. It was based on previously published work by us (Hollenberg *et al*., 1992; 1993; 1997) and by others (Scarborough *et al*., 1992; Vassallo *et al*., 1992) that this spectrum of receptor-activating peptides was selected for purposes of comparing the gastric and pulmonary smooth muscle responses. Amastatin, nifedipine, indomethacin and human thrombin (3000 NIH units/mg; cat no. T-6759) and heparin were from sigma (St. Louis MO, U.S.A.). Genistein was from ICN biochemical (Costa Mesa CA, U.S.A.). Tyrphostin 47/AG 213 (Levitzki & Gazit, 1995) and the

Src-tyrosine kinase-selective inhibitor, PP1 (Hanke *et al*., 1996) were from Calbiochem (La Jolla CA, U.S.A.).

Reverse transcriptase polymerase chain reaction (RT–PCR) detection of PAR_1 and PAR_2 and partial sequencing of guinea-pig PAR_1 and PAR_2

To assess the presence of PAR_1 and PAR_2 mRNA in the lung tissue, total RNA was prepared either from a dissected lung segment or from a parenchymal strip, prepared as for a bioassay, using the TRI^R reagent (Molecular Research Center, Cincinnati OH, U.S.A.). RNA samples were then digested for 15 min at room temperature with DNase I (10 units in 10 μl: Pharmacia FPLCpure™, Amershan Pharmacia Biotech, Baie D'Urfé QC, Canada) to eliminate any possible contaminating DNA. DNase I was then inactivated by heating at 68°C for 15 min in the presence of 2.5 μM EDTA. The RNA was reverse-transcribed (RT) with a first-strand cDNA synthesis kit using pd(N)6 primer (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to manufacturers recommendations at 37°C for 60 min; 2 μl of this solution was used with primer pairs derived from Rat PAR_1 and PAR_2. For PAR_1, the primer pairs were: forward primers, (a) PAR_1F: 5′-**AAAAGCTT**CCCGCTCATTTTT-TCTCAGGAA-3′; (b) PAR_1F1 (lacking the *Hind*III site present in PAR_1F: bold letters): 5′-CCCGCTCATTTTTTCT-CAGGAA-3′ and reverse primer, PAR_1R: (containing an *Eco*RI site shown in bold) 5′-**GGGAATT-C**AATCGGTGCCGGAGAAGT-3′ (expected length of PCR product for PAR_1F/PAR_1R, 409 nucleotides). For PAR_2, the primer pairs were: forward primer, PAR_2F1: 5′-CACCACCTGTCACGATGTGCT-3′ and reverse primer, PAR_2R: 5′-CCCGGGCTCAGTAGGAGGTTTTAACAC-3′ (expected length of PCR product, 527 nucleotides). A second PAR_2 forward primer pair, about 330 nucleotides down-stream of PAR_2F1, based on the determined sequence of guinea-pig PAR_2 [PAR_2F2: 5′-CAACAGCTGCATT-GACCCCTT-3′ (expected length of PCR product, when used with PAR_2R, 196 nucleotides)] was also used to confirm that the PCR product yielded by the PAR_2F1/PAR_2R primer pair did indeed represent guinea-pig PAR_2. The signal yielded by the PAR_2 primer pairs was normalized to the PCR signal generated from the same RT product using a primer pair for glyceraldehyde phosphate dehydrogenase (GAPDH), with the sequences: forward primer, GAP-F: 5′-CGGAGTCAACG GATTTGGTCGTAT-3′ and reverse primer, GAP-R: 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (expected length of PCR product, 309 nucleotides). Polymerase-chain reaction (PCR) amplification was achieved using 2.5 units of Taq DNA polymerase (Promega, Madison WI, U.S.A.) in a 10 mM Tris.HCl buffer, pH 9.0 (0.05 ml final vol), containing $MgCl_2$ (1.5 mM), KCl (50 mM), 0.1% v/v Triton X-100 and 0.2 mM each of deoxynucleotide triphosphates. Amplification for 35 cycles began with a 1 min denaturation period at 94°C, followed by a 1 min reannealing time at 55°C and a primer extension period of 1 min at 72°C. PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR products obtained with primer pairs PAR_1F/PAR_1R and PAR_2F1/PAR_2R were 'gene-cleaned' (Magic™ PCR Preps DNA purification system, Promega, Madison WI, U.S.A.) and ligated (Ready To Go™ T4 ligase, Amersham Pharmacia Biotech Inc. Baie D'Urfé QC, Canada) into the PGEM-T vector (Promega, Madison WI, U.S.A.). This ligation mixture (2 μl) was used to transform *E. coli* strain DH5α to produce permanent clones for both manual and automated sequencing by the dideoxynucleotide chain termination method (Sanger *et al*., 1977), employing a T7DNA sequencing kit (Pharmacia, Dorval QC, Canada) or *via* the DNA Services Facility at the University of Calgary Faculty of Medicine.

Immunohistochemistry

Perfused dissected lung lobules and peripheral parenchymal strips (2 × 10 mm), excised as for a bioassay, were fixed for about 24 h at room temperature in 10% isotonic buffered formalin solution, pH 7.4, followed by paraffin embedding. Tissue sections (4 μm) were cut, mounted on silane-coated slides, dried overnight, deparaffinized and treated with 3% H_2O_2 for 10 min at room temperature to destroy endogenous tissue peroxidase. The PAR_2 epitope against which the B5 antibody was developed (Al-Ani *et al*., 1999), was unmasked by treatment of the slides with dilute pepsin (Digest-All 3, Zymed, San Francisco CA, U.S.A.) for 10 min at 37°C followed by washing with isotonic buffered saline, pH 7.4. After pre-blocking slides with avidin/biotin treatment, tissue was preabsorbed for 10 min with 10% (vol^{-1} vol^{-1} in buffered saline) non-immune goat serum (Zymed, San Francisco CA, U.S.A.), washed and then exposed to a 1/1000 dilution of B5 anti-PAR_2 antiserum overnight at 4°C, without or with preadsorption of the antiserum for 2 h with the immunizing peptide (final concentration 20 μg ml^{-1}). Immunoreactivity was visualized with the use of biotinylated goat anti-rabbit IgG followed with streptavadin-conjugated peroxidase (Sigma, St. Louis MO, U.S.A.) and colour generation with diamino-benzidine (25 μg ml^{-1}, 10 min, room temperature). Tissue was counter-stained with haematoxylin (Fisher, Fair Lawn NJ, U.S.A.), and the image was recorded by digital photomicrography.

Results

Responses of the pulmonary strip preparation to PAR_1-activating peptides: potentiation by amastatin, desensitization of thrombin action and comparison of activities and concentration-effect curves with the gastric preparation

Both thrombin and the thrombin receptor-derived peptides P5-NH_2 and TF-NH_2 caused a contractile response of the pulmonary strip preparation (Figure 1 and data not shown for P5-NH_2). Previous work has shown that at a concentration of 3–4 μM, P5-NH_2 selectively activates PAR_1 compared with the PAR_2 and that TF-NH_2 is highly selective for PAR_1 compared with PAR_2 (Kawabata *et al*., 1999). Because of the known presence of peptidase activity in lung tissue and because of the recognized susceptibility of PAR-APs to amino-peptidase (Coller *et al*., 1992) we monitored the response to TF-NH_2 and P5-NH_2 in the absence and presence of the amino-peptidase inhibitor, amastatin (Figure 1). The protease inhibitor significantly (at least 2 fold) potentiated the response to P5-NH_2 and TF-NH_2 (Figure 1A and data not shown). Concentrations of amastatin higher than 10 μM caused no further potentiation of the response; this concentration of

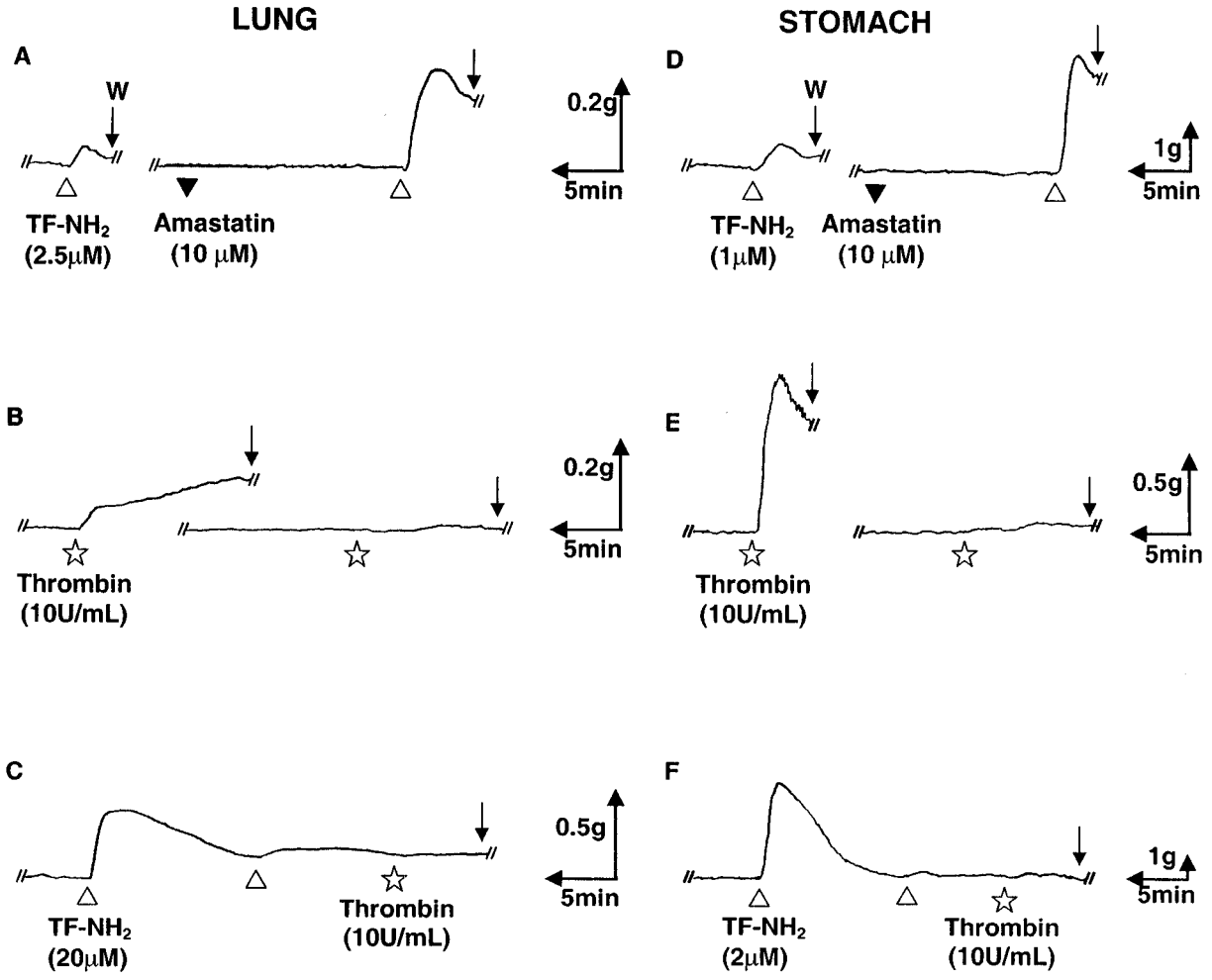

Figure 1 Contractile responses of the guinea-pig lung and stomach LM preparations to thrombin and the selective PAR_1AP, $TF\text{-}NH_2$: potentiation by amastatin and desensitization of the thrombin response by $TF\text{-}NH_2$. The contractile responses to $TF\text{-}NH_2$(△) and thrombin (☆) were monitored in the lung parenchymal strip (left-hand tracings, A–C) and stomach LM preparation (right-hand tracings, D–F), as outlined in Methods. In each preparation, the contractile response was potentiated by the addition of amastatin (10 μM, ▼: tracings A and D). Desensitization of the tissues by the repeated addition of $TF\text{-}NH_2$ to the organ bath also desensitized the tissues to thrombin (Tracings C and F). The scale for time (min) and tension (g, mN) is also shown for each tracing. W (Arrow) = tissue wash.

amastatin was therefore added to the organ bath for all subsequent experiments with the receptor-activating peptides. For purposes of comparison, this concentration of amastatin was also added to the organ bath for the gastric contractile assays in which this amino-peptidase inhibitor also potentiated the contractile action of $TF\text{-}NH_2$ (Figure 1D and see below). Other protease inhibitors (e.g. phosphoramidon, leupeptin, captopril) either with or without amastatin did not potentiate the contractile actions more than the potentiation observed with amastatin alone (not shown).

The response to thrombin showed considerable tachyphylaxis in both tissue preparations, even after a 1 h re-equilibration period (Figure 1 and data not shown). Desensitization of the contractile response of the lung preparation (but not the gastric preparation) was also observed after repeated exposures of the tissue to relatively high concentrations of $TF\text{-}NH_2$ (10–20 μM) (data not shown). In the stomach LM preparation, as observed by us previously, a 25 min–1 h re-equilibration period allowed for a re-sensitization to the PAR-APs, but not to thrombin (Figure 1 and data not shown). A lung preparation desensitized by the cumulative addition of the selective PAR_1AP, $TF\text{-}NH_2$ to the organ bath no longer responded to thrombin (Figure 1C). A similar result was observed with the LM preparation (Figure 1F). Nonetheless, tissues which were desensitized to thrombin subsequently did respond to $TF\text{-}NH_2$ or $P5\text{-}NH_2$ (not shown and see Hammes & Coughlin, 1999). Because of the partial desensitization observed in the lung preparation at relatively high concentrations of $P5\text{-}NH_2$ and $TF\text{-}NH_2$, even after a 40 min to 1 h re-equilibration period, concentration-effect curves for the lung preparation were obtained by monitoring only the first response to each receptor-activating peptide, subsequent to standardizing the tissue's response to 50 mM KCl. For the gastric LM preparation a re-equilibration period of 25 min to 1 h allowed for complete resensitization and therefore permitted the measurement of responses to multiple concentrations of the agonists in a single tissue preparation. The contractile responses to increasing concentrations of the PAR-APs in both the lung and stomach tissues were

expressed as a percentage (% KCl) of the contractile response caused in each preparation by 50 mM KCl (Figure 2).

The concentration-effect curves for the six PAR_1-APs in the lung preparation (Figure 2B) revealed the following order of peptide potencies: cit-$NH_2 \cong$ TF-$NH_2 \cong$ P5-$NH_2 >$ P7 $\cong$ P5 $\cong$ P7-NH_2. This order of peptide potencies was clearly distinct form the order measured concurrently in the stomach longitudinal muscle strip assay (Figure 2A): Cit-$NH_2 \cong$ TF-$NH_2 \geqslant$ P7-$NH_2 \geqslant$ P5-$NH_2 >$ P7 $>$ P5. Apart from the distinct order of potencies for the PAR_1APs in the two preparations, there were differences in the maximum responsiveness of the lung preparation to the different PAR-APs: P5 and P7-NH_2 appeared to be partial agonists, compared with the other PAR_1APs. There were also differences in the maximum responsiveness of the gastric LM preparation, with P7, P5 and P5-NH_2 behaving as partial agonists (Figure 2).

Actions of PAR2 APs in the pulmonary and gastric smooth muscle preparations

In the gastric LM preparation, the PAR2-selective agonist, SL-NH_2 caused a contractile response, as we have observed previously (Al-Ani *et al*., 1995 and Figure 3C). The potency of SL-NH_2 in the stomach preparation was equivalent to that of P5 (Figure 2A). In contrast, in the lung preparation, when care was taken to prepare parenchymal strips 2 mm or less in width from the very periphery of the lobe, a contractile response to the PAR_2AP, SL-NH_2, was not observed in any of more than 75 preparations at concentrations as high as 100 μM (Figure 3A). Such tissues contracted in response to much lower concentrations (e.g. 2.5 μM) of the PAR_1AP, TF-NH_2 (Figure 3A). In a lung strip that was pre-constricted with carbachol, the PAR_2AP, SL-NH_2, failed to cause either a contraction or relaxation, whereas the selective PAR_1AP, TF-NH_2, caused a further increase in tension (Figure 3B). On occasion (not shown), a contractile response was caused by SL-NH_2 ($\geqslant$100 μM) in a lung strip preparation that was

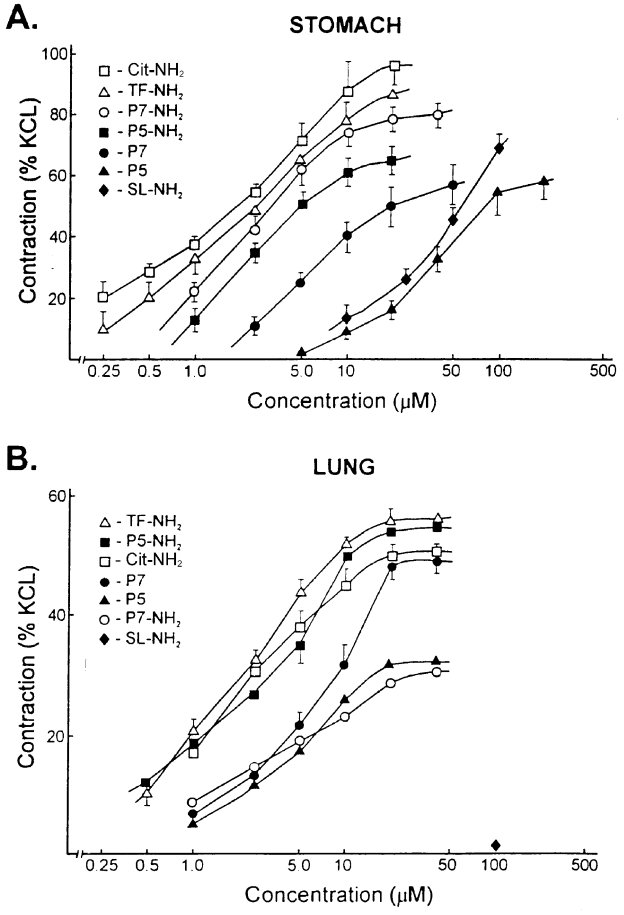

Figure 2 Concentration-effect curves for PAR-APs in the stomach (A, upper) and lung (B, lower) preparations. Contractile responses to increasing concentrations of the PAR-APs were measured as outlined in Methods, relative to the contraction caused in each preparation by 50 mM KCl (% KCl). Each data point represents the mean $\pm$ s.e.mean (bars) for observations obtained from six or more independent tissue preparations coming from three or more different animals.

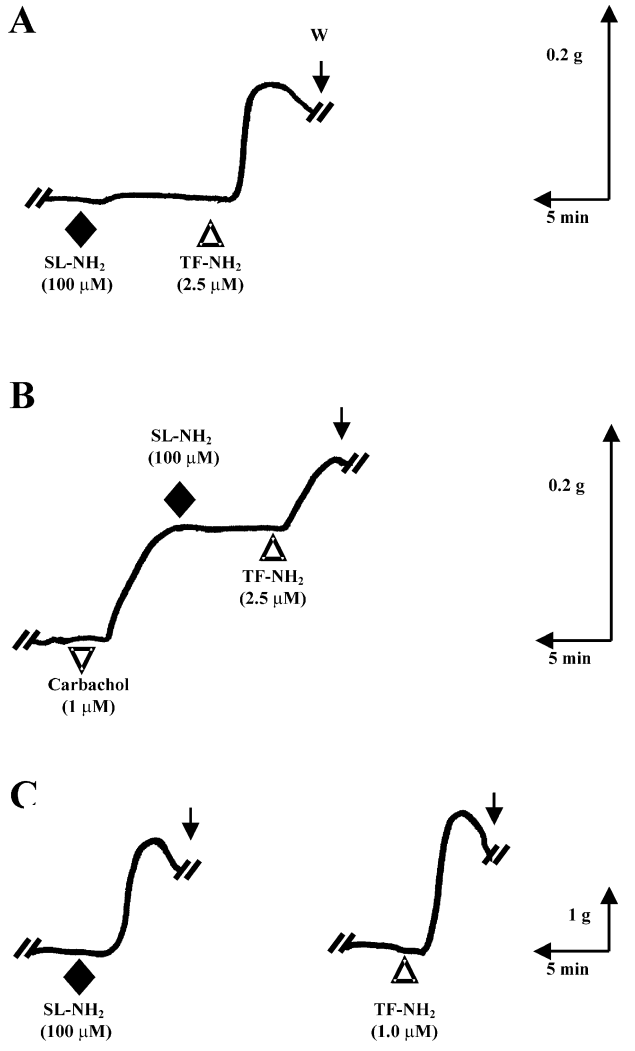

Figure 3 Action of the PAR_2-selective agonist, SL-NH_2 in lung and stomach tissue: comparison with the action of TF-NH_2. The lung parenchymal (A,B) and gastric LM tissues (C) were exposed to SL-NH_2 (◆) or TF-NH_2 (△), either singly (C, LM tissue) or in succession (lung tissue, A,B). The effects of the two PAR-APs were also tested in a lung preparation that had been pre-constricted with carbachol (▽, tracing B). The tracings are representative of three or more independent experiments done with tissues coming from three or more separate animals. The scales for time (min) and tension (g, mN) are shown along with the tracings. W (arrow) = tissue wash.

wider than 3 mm, possibly containing higher order bronchial elements (see histology, below).

Effects of tyrosine kinase inhibitors, indomethacin and removal of extracellular calcium in lung and gastric preparations

In our previous work with the PAR-activating peptides, we had observed that the PAR_1-mediated contractile response of gastric smooth muscle preparations required the presence of extracellular calcium and was inhibited by the tyrosine kinase inhibitor, genistein and the cyclo-oxgenase inhibitor indomethacin (Zheng *et al*., 1998). We were therefore interested to determine if the contractile responses for the lung parenchymal strip to PAR_1APs required extracellular calcium and were sensitive to the same enzyme inhibitors. As illustrated in Figure 4A and B, the contractile response of the lung preparation to the PAR_1AP, TF-NH_2, was refractory to the action of indomethacin (neither inhibited, nor potentiated at 3 μM) and was only minimally affected by genistein (15 μM). Similarly, neither the Src-targeted tyrosine kinase inhibitor, PP1 (1 μM: Hanke *et al*., 1996), nor the non-specific tyrosine kinase inhibitor, tyrphostin 47/AG213 (20 μM: Levitzki & Gazit, 1995) affected the PAR_1AP-stimulated contractions in this tissue (not shown). In contrast, under the same conditions, the contractile responses of the gastric preparation to the PAR_1APs were abolished by indomethacin and by each of the three tyrosine kinase inhibitors tested (Figure 4D and E and data not shown). In the absence of extracellular calcium, there was an appreciable response of the lung preparation to TF-NH_2 (on average, 30–40% of control: Figures 4C and 5), whereas the response of the gastric longitudinal muscle strip was abolished in the absence of extracellular calcium (Figures 4F and 5). An increase in tension in both preparations exposed to a PAR_1 agonist was observed upon replenishing extracellular calcium (Figure 4C and F). In keeping with these results, the calcium channel blocker, nifedipine, caused a more marked inhibition of the PAR_1AP-mediated response in the gastric preparation (>65% inhibition: Figure 5) than it did in the pulmonary strip preparation (<30% inhibition: Figure 5).

Detection of PAR_1 and PAR_2 by RT–PCR, partial sequences of guinea-pig PAR_1 and PAR_2

To determine if both PAR_1 and PAR_2 were present in the lung tissue, RNA was obtained both from freshly dissected whole lung lobules and from parenchymal strip preparations as obtained for the contractile bioassay. RT–PCR using primer pairs targeted to either PAR_1 or PAR_2 yielded oligonucleotide products having the sizes anticipated from the published sequences of the mouse, human and rat receptors. The relative signal intensities of the PAR_1 and PAR_2 RT–PCR products obtained from the whole lung

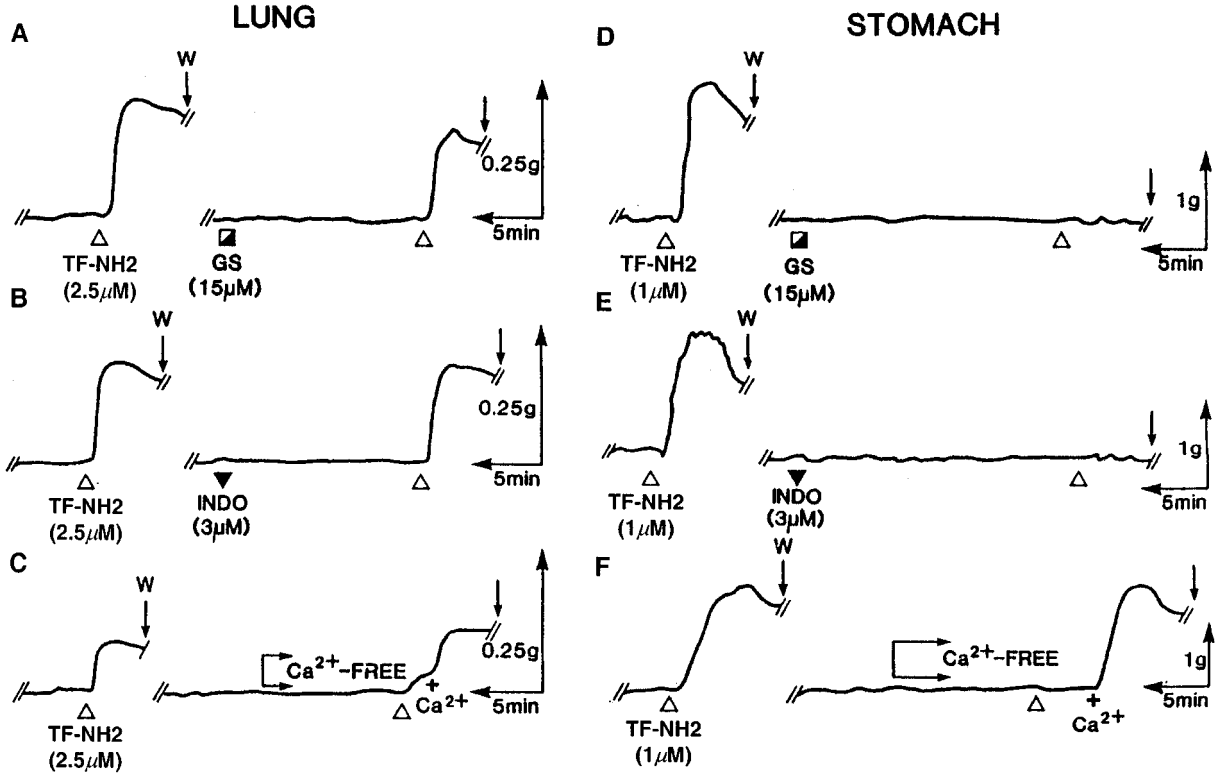

Figure 4 Effects of indomethacin, genestein and removal of extracellular calcium on TF-NH_2-mediated contractions in the lung (left) and stomach (right) preparations. Contractile responses to TF-NH_2 were monitored in the lung (A–C) and stomach (D–F) both before and after exposure of the tissues for 20 min to genistein (GS ◪, 15 μM), indomethacin (▼, 3 μM) or after the removal of extracellular calcium (Ca^{2+}-free). The tension was also monitored upon replenishing the organ bath with 2.5 mM calcium ($+Ca^{2+}$). Tissues were washed and re-equilibrated for 1 h after the first exposure to TF-NH_2. The tracings are representative of three or more independent experiments done with tissues coming from different animals. The scales for time (min) and tension (g, mN) are also shown. W (arrow) = tissue wash.

tissue, compared with the RT–PCR signal for glyceraldehyde phosphate dehydrogenase (GAPDH), are shown in Figure 6. The relative intensity of the PCR signals for PAR_1 and PAR_2 obtained for the parenchymal strip (not shown) were comparable to those shown in Figure 6 for whole lung tissue. Sequencing of the RT–PCR products obtained from the lung tissue, using primer pairs PAR_1F/PAR_1R for PAR_1 and PAR_2F1/PAR_2R for PAR_2 confirmed (Figure 7) that they did indeed represent partial sequences of guinea-pig PAR_1 and PAR_2. The identity of the PAR_2 PCR product was supported further by an independent RT–PCR procedure, using a second forward PCR primer (PAR_2F2) based on the

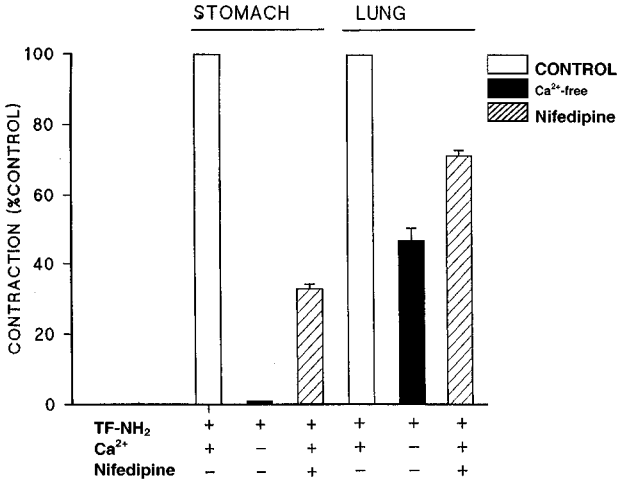

Figure 5 Effects of nifedipine and removal of extracellular calcium on the TF-NH_2-mediated contractile response in stomach and lung tissue. The inhibitory effects of either removing extracellular calcium, or of adding nifedipine (1 μM) to the organ bath on the contractile actions of TF-NH_2 were monitored as outlined in Figure 4. Responses in the absence of calcium (filled histograms) or in the presence of nifedipine (1 μM, hatched histograms) were expressed as a percentage (% control) of the contractile response observed in the presence of 2.5 mM calcium and in the absence of nifedipine (open histograms). The histograms represent the averages (±s.e.mean, bars) for observations done with six or more tissue preparations coming from different animals.

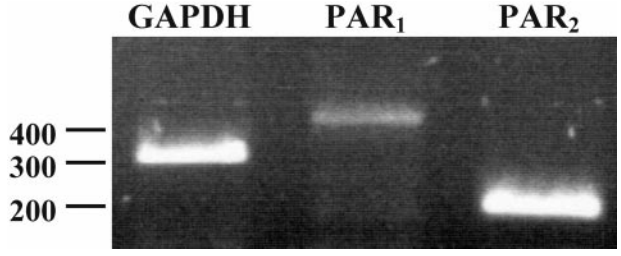

Figure 6 Reverse-transcriptase-polymerase-chain reaction detection of PAR_1 and PAR_2 in lung tissue. RNA was isolated from whole lung tissue and reverse-transcribed as outlined in Methods. RT–PCR was performed as described in Methods, using primer pairs targeted to PAR_1 (PAR_1F1/PAR_1R), to PAR_2 (PAR_2F2/PAR_2R) and to GAPDH (GAP-F/GAP-R). The positions of the oligonucleotide size markers (in nucleotides, nt) are shown to the left of the separating gel. The identity of each PCR product of the expected size is indicated at the top of each lane for GAPDH (expected size, 306 nt), PAR_1 (expected size, 401 nt) and PAR_2 (expected size, 196 nt).

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AMINO ACID SEQUENCES:

Proteinase Activated Receptor 1 (PAR1)

GpPAR1 SFFLRNPSEDTFEQFPLGDEGEKNESIPLEGRAVVLNKSRFPPMPPPPFISEDASGYLTSP
RPAR1  SFFLRNPSEDTFEQFPLGDEEEKNESIPLEGRAVVLNKSRFPPMPPPPFISEDASGYLTSP

GpPAR1 WLALFIPSVYTFVFIVSLPLNILAIAVFVFRMKVKKP
RPAR1  WLTLFIPSVYTFVFIVSLPLNILAIAVFVFRMKVKKP

GpPAR1 AVVYMLHLAMADVLFVSVLPFKISYYFSGTD
RPAR1  AVVYMLHLAMADVLFVSVLPFKISYYFSGTD

Proteinase Activated Receptor 2 (PAR2)

GpPAR2 TTCHDVLPEEVLVGDMFSYFLS
RPAR2  TTCHDVLPEEVLVGDMFSYFLS

GpPAR2 LAIGVFLFPALLTASSYVLMIRALRSSTMDEHSVRKRRAVK
RPAR2  LAIGVFLFPALLTASAYVLMIKTLRSSAMDEHSEKKRRRAIR

GpPAR2 LIITVLATYLICFTPSNLLLVVHYFLIKSRQQSHVYAL
RPAR2  LIITVLSMYFICFAPSNVLLVVHYFLIKSQRQSHVYAL

GpPAR2 YITALCLSTLNSCIDPFIYYFV
RPAR2  YLVALCLSTLNSCIDPFVYYFV

GpPAR2 SQDFRHHAKNTILCRSVRTVKRIQVSHTSTKSSRKSSSYSSSSTSVKTSY
RPAR2  SKDFRDQARNALLCRSVRTVKRMQISLTSNKFSRKSSSYSSSSTSVKTSY

NUCLEOTIDE SEQUENCES:

Gplungpar1
1   AAAAGCTTCC CGCTCATTTT TTCTCAGGAA
    TCCCAGTGAA GATACATTTG
51  AACAGTTCCC CCACGGGGAT GAGGGGGAGA
    AAAATGAAAG CATACCGCTC
101 GAGGGCAGGG CAGTCTACTT AAATAAAAGC
    CGTTTTCCTC CCATGCCGCC
151 TCCTCCCTTC ATCTCCGAGG ACGCCTCCGG
    ATATCTGACC AGCCCCTGGC
201 TGGCGCTCTT CATACCCTCC GTGTACACGT
    TTGTGTTCAT AGTCAGCCTT
251 CCCCTGAACA TCCTGGCCAT CGCTGTGTTT
    GTCTTTCGGA TGAAGGTCAA
301 GAAGCCGGCC GTGGTGTACA TGCTGCACCT
    GGCCATGGCC GATGTCCTCT
351 TCGTGTCCGT GCTCCCCTTC AAGATCAGCT
    ACTACTTCTC CGGCACCGAT
401 TGAATTCCC

gplungpar2
1   CACCACCTGT CACGATGTGC TGCCCGAGGA
    GGTGCTGGTG GGGGACATGT
51  TCAGCTACTT CCTCTCCTTG GCCATCGGAG
    TCTTTCTGTT CCCAGCCCTC
101 CTCACGGCGT CGTCCTATGT GCTCATGATC
    CGAGCGCTCC GGTCCTCGAC
151 CATGGATGAG CACTCAGTGA GGAAGAGGCG
    CAGGGCCGTC AAGCTGATCA
201 TCACCGTCCT GGCCACGTAC CTCATCTGCT
    TCACACCTAG TAACCTACTC
251 CTCGTGGTGC ATTACTTCCT GATCAAGAGC
    CGTGGCCAGA GCCATGTCTA
301 CGCCCTGTAC ATCACCGCCC TCTGCCTGTC
    CACTCTCAAC AGCTGCATTG
351 ACCCCTTCAT CTACTACTTC GTCTCTCAGG
    ACTTCAGGCA CCATGCCAAG
401 AATACCATCC TCTGTCGCAG CGTCCGCACG
    GTAAAGCGGA TTCAGGTCTC
451 CCACACCTCC ACCAAGTCCT CCCGGAAGTC
    CAGCTCTTAC TCCTCAAGCT
501 CCACCAGTGT TAAAACCTCC TACTGAG
```

Figure 7 Translated amino acid sequences (upper) and oligonucleotide sequences (lower) for guinea-pig PAR PCR products. The oligonucleotide sequences (lower left and right) for the PCR products obtained by RT–PCR from lung RNA samples, using primer pairs PAR_1F/PAR_1R for PAR_1 (gplungpar1: lower left) and primer pairs PAR_2F1/PAR_2R for PAR_2 (gplungpar2: lower right) were determined as outlined in Methods. Nucleotide residues resulting from the added *Hind*III and *Eco*RI restriction sites, that are not part of the PAR_2 receptor sequence, are shown in boldface type. The translated amino acid sequences for guinea-pig PAR_1 and PAR_2 are shown in the upper panel, compared with the equivalent rat PAR sequences (RPar1; RPar2). The asterisks denote differences between the rat and guinea-pig deduced amino acid sequences.

determined guinea-pig oligonucleotide sequence shown in Figure 7. This second forward primer targets a receptor sequence about 330 nucleotides (110 amino acids) downstream from the 5′-primer (PAR_2F1), that begins at the position of the amino acid sequence, TTCHDVL in extracellular loop 2. In comparing the partial predicted amino acid sequences we obtained from the guinea-pig tissue with the predicted amino acid sequences of rat PAR_1 and PAR_2, we observed only two amino acid differences between rat and guinea-pig PAR_1 (out of 137 amino acid residues), despite a number of differences in the oligonucleotide sequences (not shown); there were 29 differences between rat and guinea-pig PAR_2 (out of 176 amino acid residues: asterisks, Figure 7). Although there was a lack of a contractile response of the parenchymal strip for the PAR_2AP, a strong PCR product was detected both for the lung segment used to prepare RNA for PCR-based sequencing (Figure 6) and for the parenchymal strip tissue prepared exactly as for a bioassay (results comparable to those shown in Figure 6). The PCR signal for PAR_2 appeared much stronger than that obtained for PAR_1.

Immunohistochemical detection of PAR_2 using antireceptor antibody B5

The haematoxylin stain of the peripheral strip sections used for the bioassay revealed the presence principally of scattered terminal bronchioles and very few arterial elements, amidst abundant parenchymal alveolar structures that have been described previously (Figure 8E,F, data not shown and see Kapanci *et al*., 1974). Given the absence of arterial and bronchial components observed histologically, it was evident that the contractile responses observed with the peripheral parenchymal strip preparation must have been due to the intrinsic contractility of the airway smooth muscle itself and not due to the contractility of the bronchiolar or vascular tissue components. In contrast, the histology of the whole lung segments revealed the expected presence of the primary and secondary bronchi with a prominent epithelial lining as well as numerous vascular structures (Figure 8A – D and data not shown). In the whole lung tissue containing primary and secondary bronchioles, the B5 antiserum revealed prominent reactivity in the bronchial epithelium (Figure 8A: granular bead-like staining at the cell periphery, open arrowhead; diffuse cytoplasmic staining, solid arrowhead) as well as in cells with the morphology of type II pneumocytes (Figure 8C), that exhibited granular staining at the cell periphery (solid arrowhead, Figure 8C). Pre-adsorption with the immunizing B5 peptide eliminated the staining in these cells (Figure 8B,D, solid and open arrows), indicating specificity. In contrast, in the parenchymal strip tissue, only diffuse cytoplasmic PAR_2 immunoreactivity that was partially blocked by peptide pre-adsorption was observed in the alveolar components. Notwithstanding, in the occasional terminal bronchiole that was present in the strip, granular immunoreactivity (not shown), completely eliminated by peptide pre-adsorption, was detected in the epithelial elements as described above for Figure 8A. Some, but not all cells with the morphology of alveolar type II pneumocytes present in the strip preparation (not shown) also showed light immunoreactivity at the cell periphery; this reactivity was also eliminated by peptide pre-adsorption. In the parenchymal strip tissue, immunoreactivity neutralized by peptide pre-adsorption (Figure 8F, solid arrow) was detected in addition in cells at the very periphery of the strips (Figure 8E, solid arrowhead), most likely representing mesothelial cells of the pleural tissue. Thus, in contrast with the lobular tissue wherein PAR_2-expressing cells were abundant and readily identified, in the parenchymal strip tissue used for the bioassay, it was possible to detect PAR_2-expression by immunohistochemistry with confidence only in the mesothelial cells, type II pneumocytes and in the epithelial cells of the rare terminal bronchial elements.

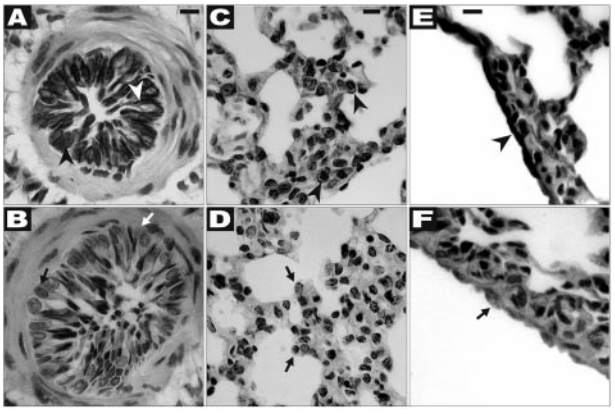

Figure 8 Immunohistochemistry of whole lung and peripheral strip tisssues. Sections of either whole lung (A – D) or of the peripheral strip preparation (E,F) were prepared from paraffin embedded fixed tissue specimens as outlined in Methods. Immunoreactivity was detected using the B5 anti-PAR_2 antiserum as described, either without (A, C and E) or with (B, D and F) peptide pre-adsorption. (A) Shows bronchial epithelium immunoreactivity with a granular pattern at the cell periphery (open arrowhead) as well as in the cytoplasm (solid arrowhead); peptide pre-adsorption eliminated this reactivity (B, open and solid arrows). (C) Shows granular immunoreactivity at the periphery of a cell with morphology consistent with that of a Type II pneumocyte (solid arrowheads); this reactivity was eliminated by peptide pre-adsorption (solid arrows, D). (E) Shows the immunoreactivity of pleural mesothelial cells (solid arrowhead); this reactivity was neutralized by peptide pre-adsorption (F, solid arrow). Magnification was approximatately $\times 400$ for A,B,E and F; and about $\times 200$ for C and D. The solid bar at the top of A,C and E represents 10 μm, which scale also applies to each panel directly underneath (B,D and F).

Discussion

The main findings of our study were: (1) that the contractility of peripheral lung parenchymal strips was stimulated by a number of PAR_1-activating peptides, but was not affected by the selective PAR_2-activating peptide, SLIGRL-NH_2 and (2) that there were differences between the pulmonary and gastric preparations in terms of the contractile signal transduction pathways and in terms of the structure-activity relationships for a number of PAR_1-activating peptides (Figure 2). Our new data considerably extend our preliminary observations of the contractile activities of P5-NH_2 and P7-NH_2 in the lung strips assay (Mandhane *et al*., 1995). Further, our data differed somewhat from the findings of others (Cocks *et al*., 1999; Lan *et al*., 2000; Ricciardolo *et al*., 2000) in terms of a lack of response to the PAR_2-activating peptide, SLIGRL-NH_2.

Peptide potencies and possible role of tissue peptidase activity

As with a platelet assay system (Coller *et al*., 1992), it was necessary to prevent peptide degradation by the addition of the amino-peptidase inhibitor, amastatin, to observe the full effects of the PAR-activating peptides. Other peptidase inhibitors added in addition to amastatin had no further effect than did amastatin alone. This result suggested that aminopeptidase activity *per se* may play a significant role in the peripheral pulmonary strip tissue. In the presence of amastatin, the concentration range over which the PAR_1-activating peptides caused a contractile response in the lung strip preparation was comparable to the concentration range over which the same peptides have been found to regulate contractility in smooth muscle preparations derived from other tissues (vascular or gastric tissue: Muramatsu *et al*., 1992; Hollenberg *et al*., 1993; Glusa & Paintz, 1994). Given the presence of peptidase activity in the lung and gastric tissue, the possibility cannot be entirely discounted that

differential susceptibility of the PAR-activating peptides to the peptidases, albeit unlikely in the presence of amastatin, may possibly have affected the relative potencies of the PAR-activating peptides that were used. This theoretical (but unlikely) differential susceptibility to proteolysis of the peptide agonists with comparable sequences (e.g. P5, P7, P5-NH_2, P7-NH_2) would be difficult to document and was therefore not explored in the present study. Notwithstanding, the order of potencies for the contractile actions of the PAR-activating peptides in the lung preparation (Cit-$NH_2 \cong$ TF-$NH_2 \cong$ P5-NH_2 > P7 $\cong$ P5 $\cong$ P7-NH_2; SL-NH_2 not active) clearly differed from the order of peptide potencies observed in the gastric LM preparation (Cit-$NH_2 \cong$ TF-$NH_2 \geqslant$ P7-$NH_2 \geqslant$ P5-NH_2 > P7 > P5 $\cong$ SL-NH_2), particularly in terms of the activity of P7-NH_2, relative to Cit-NH_2 and TF-NH_2.

Comparison with previous structure-activity studies with PAR_1-activating peptides

It can be noted that the relative potencies of the PAR_1-activating peptides, modelled on the human tethered ligand, have been found to be comparable for activating PAR_1 in both human and rat tissues (Hollenberg *et al*., 1993; Kawabata *et al*., 1999; Vassallo *et al*., 1992; Scarborough *et al*., 1992). Indeed, the order of potencies observed for the peptides in the guinea-pig gastric LM preparation was in accord with data obtained previously with a rat gastric LM assay (Hollenberg *et al*., 1993) and with a human cultured HEK cell assay (Kawabata *et al*., 1999). In the gastric LM preparation we have established previously, by a receptor cross-desensitization approach, that the response to the PAR_1-APs peptides can be attributed principally to the activation of PAR_1 and not PAR_2. In agreement with these previous observations, the EC_{50}s of all of the PAR_1-APs except for P5 were lower than that of the PAR_2-AP, SL-NH_2 (Figure 2). The distinct relative potencies and different apparent intrinsic activities of the PAR_1-activating peptides in the lung versus the gastric preparation therefore suggest the presence of a functionally distinct receptor subtype, in keeping with classical receptor criteria established some time ago (Ahlquist, 1948). Since the PAR_1-AP, TF-NH_2, was able to desensitize the lung preparation completely to subsequent thrombin stimulation (Figure 1), it was clear that the preparation did not possess functional PAR_4, which is not affected by PAR_1-APs (Xu *et al*., 1998; Kahn *et al*., 1998). Notwithstanding, the partial sequence that we have determined for guinea-pig PAR_1 was essentially the same as that for rat PAR_1 (Figure 7), including the putative tethered ligand activating sequence. Thus, the distinct order of peptide potencies for the PAR_1-APs in the lung and LM tissues and the differences in signal transduction pathways between the lung and gastric preparations may possibly be due either, as suggested above, to the presence of a distinct receptor subtype that was not detected by RT–PCR or to a distinct post-translational modification of the lung PAR_1 receptor (e.g. differential glycosylation) that could in theory alter peptide specificity. The distinct order of PAR_1-AP potencies we have found in comparing the guinea-pig lung and gastric tissues is in keeping with previous work we have done with human placental vascular preparations, wherein the PAR_1-activating peptide structure activity (SAR) profiles differ from the one observed in human platelets (Tay-Uyboco *et al*., 1995). As in the present study, our previous work with the human placental tissue displaying distinct PAR-AP structure activity relationships, did not reveal the presence of a distinct PAR_1 receptor mRNA sequence. It can be noted that the same receptor present in different tissues can in theory yield different agonist relative potencies because of differences in receptor coupling, leading to distinct agonist intrinsic efficacies. Thus, receptor antagonists are required to distinguish clearly between receptor subtypes. Unfortunately, pure receptor antagonists for PAR_1, such as the one recently described (Andrade-Gordon *et al*., 1999) are not yet readily available to explore in more depth the receptors in the two PAR_1AP-responsive tissues we have described in this study. Further work will thus be required to determine the molecular basis for the functionally distinct receptor present in the lung parenchymal tissue.

Comparison with previous work using non-selective PAR_1 activating peptides

Although previous work has documented a constrictor effect in lung tissue with non-selective PAR-activating peptides (e.g. SFLLRN-NH_2: Lum *et al*., 1994; Cocks *et al*., 1999; Lan *et al*., 2000) or with thrombin, none of these previous studies used PAR_1-selective agonists like TF-NH_2 or Cit-NH_2. Nonetheless, our data obtained using the selective PAR_1APs, which were able to desensitize the tissue completely to the contractile action of thrombin (thus, ruling out the presence of functional PAR_4, as mentioned above), were in keeping with previous studies with tracheal, bronchial and perfused lung preparations (Cocks *et al*., 1999; Lan *et al*., 2000; Lum *et al*., 1994). Taken together, all of the studies suggest that the contractile action of thrombin in the lung tissue can be attributed principally to the activation of PAR_1 and not PAR_4 that is activated with the assistance of PAR_3. In the lung strip preparation, inhibition of cyclo-oxygenase with indomethacin did not either potentiate or block the PAR_1-mediated contractile response. In contrast, in the work reported previously using a murine bronchial preparation, indomethacin potentiated a contracile response caused by the PAR_1-activating peptide, SFLLRNP-NH_2 (Cocks *et al*., 1999). Our data suggest that this epithelium-dependent PAR_1-mediated relaxant response involving prostaglandins does not occur in the peripheral guinea-pig lung tissue. The ability of PAR_1 activation to cause a contractile response in the lung strip preparation *in vitro* indicates that PAR_1-mediated responses in lung tissue *in vivo* could result from a direct action of thrombin on lung tissue, in addition to the reported ability of thrombin or PAR_1-activating peptides to cause bronchoconstriction indirectly *via* platelet activation (Cicala *et al*., 1999). In this regard, the intense desensitization towards repeated thrombin activation (Figure 1B) would suggest that *in vivo*, the effect of thrombin itself to alter alveolar function in the lung periphery may be transient.

Comparison with previous work with pulmonary preparations and PAR_2 agonists

Our result with the parenchymal strip preparation can be compared with data obtained by others with rodent tissues (rat, mouse) using either isolated tracheal or bronchial preparations (Cocks, *et al*., 1999; Lan *et al*., 2000) or

guinea-pig preparations employing either perfusion (Lum *et al*., 1994) or intratracheal/intravenous administration (Ricciardolo *et al*., 2000) of PAR-agonists. Significantly, in our work with the guinea-pig pulmonary strip preparation, we were not able to observe either a contractile or a relaxant response to the PAR_2AP, $SL\text{-}NH_2$, in contrast with the PAR_2-mediated epithelium-dependent relaxation of tracheal or bronchial preparations observed by others in rodent tracheal and bronchial preparations (Cocks *et al*., 1999; Lan *et al*., 2000). Possibly these differences are due to species differences (mouse or rat in previous work with $SL\text{-}NH_2$, compared with guinea-pig tissue for our own study). Importantly, the study of Ricciardolo *et al*. (2000), that appeared upon completion of our work, used the identical PAR_2-targeted antiserum to localize the receptor in guinea-pig airway epithelial cells. That study documented a tachykinin-mediated (presumably neurally-triggered) PAR_2-regulated bronchoconstriction caused by agonists administered *in vivo*, as well as both a mixed bronchomotor effect of PAR_2-agonists *in vitro*, resulting in a prostanoid/epithelium-dependent relaxation of isolated trachea and main bronchi and a contraction of intrapulmonary bronchi. Our data with the peripheral parenchymal strip indicate that the prostanoid-dependent PAR_2-mediated relaxant response and the neurally-regulated contractile response observed by Ricciardolo *et al*. (2000) are not present in the peripheral alveolar tissue, wherein contractile elements are thought to be important for matching ventilation to perfusion (Kapanci *et al*., 1974). Thus, overall, there would appear to be distinct regional differences in the various roles that PAR_2 may play in the setting of pulmonary function.

Differences in signal transduction pathways between the pulmonary and gastric preparations

It is of interest that the signal transduction pathway for PAR_1-mediated contractions of the lung strip preparation differed from those leading to contraction of the gastric LM preparation in terms of: (1) blockade of the gastric, but not the lung preparation by indomethacin and the tyrosine kinase inhibitors and (2) a complete dependence of the contractile response of the gastric but not the lung preparation on extracellular calcium. Thus, as pointed out briefly above, there were differences between the lung and gastric smooth muscle preparation not only in terms of the PAR_1-activating peptide structure-activity relationships, but also in terms of the receptor signal transduction pathways leading to contraction. From a practical point of view, the distinct functional receptor systems for the PAR_1 activating peptides present in the guinea-pig lung and gastric tissues, if also present in humans, may allow for the development of tissue-selective PAR receptor agonists and antagonists that could prove of use in a pathophysiological setting.

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