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Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue

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1 The biological activities of the proteinase-activated receptor number 2 (PAR-2)-derived peptides, SLIGRL (PP6) SLIGRL-NH_2 (PP6-NH_2) and SLIGR-NH_2 (PP5-NH_2) were measured in mouse and rat gastric longitudinal muscle (LM) tissue and in a rat aortic ring preparation and the actions of the PAR-2-derived peptides were compared with trypsin and with the actions of the thrombin receptor activating peptide, SFLLR-NH_2 (TP5-NH_2).

2 From a neonatal rat intestinal cDNA library, and from intestinal and kidney-derived cDNA, the coding region of the rat PAR-2 receptor was cloned and sequenced, thereby establishing its close sequence identity with the previously described mouse PAR-2 receptor; and this information, along with a reverse-transcriptase (RT) polymerase chain reaction (PCR) analysis of cDNA derived from gastric and aortic tissue was used to establish the concurrent presence of PAR-2 and thrombin receptor mRNA in both tissues.

3 In the mouse and rat gastric preparations, the PAR-2-derived polypeptides, PP6, PP6-HN_2 and PP5-NH_2 caused contractile responses that mimicked the contractile actions of low concentrations of trypsin (5 u/ml^{-1}; 10 nM) and that were equivalent to contractions caused by TP5-NH_2.

4 The cumulative exposure of the rat LM tissue to PP6-NH_2 led to a desensitization of the contractile response to this polypeptide, but not to TP5-NH_2 and *vice versa*, so as to indicate a lack of cross-desensitization between the receptors responsive to the PAR-2 and thrombin receptor-derived peptides.

5 In the rat gastric preparation, the potencies of the PAR-2-activating peptides were lower than the potency of TP5-NH_2 (potency order: TP5-NH_2 > > PP6-$NH_2 \geqslant$ PP6 > PP5-NH_2); PP6 was a partial agonist in this preparation.

6 The contractile actions of PP6 and PP6-NH_2 in the rat gastric preparation required the presence of extracellular calcium, were inhibited by nifedipine and were blocked by the cyclo-oxygenase inhibitor, indomethacin and by the tyrosine kinase inhibitor, genistein, but not by the kinase C inhibitor, GF109203X. The contractile responses were not blocked by atropine, chlorpheniramine, phenoxybenzamine, propranolol, ritanserin or tetrodotoxin.

7 In a precontracted rat aortic ring preparation, with an intact endothelium, all of the PAR-2-derived peptides caused a prompt relaxation response that was blocked by the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine-methyl ester (L-NAME) but not by D-NAME; in an endothelium-free preparation, which possessed mRNA for both the PAR-2 and thrombin receptors, the PAR-2-activating peptides caused neither a relaxation nor a contraction, in contrast with the contractile action of TP5-NH_2. The relaxation response to PP6-NH_2 was not blocked by atropine, chlorpheniramine, genistein, indomethacin, propranolol or ritanserin.

8 In the rat aortic preparation, the potencies of PP6, PP6-NH_2 and PP5-NH_2 were greater than those of the thrombin receptor activating peptide, TP5-NH_2 (potency order: PP6-$NH_2 \geqslant$ PP6 > PP5-NH_2 > TP5-NH_2).

9 In the rat aortic preparation, the relaxant actions of the PAR-2-derived peptides were mimicked by trypsin, at concentrations (0.5-1 u ml^{-1}; 1-2 nM) lower than those that can activate the thrombin receptor.

10 The bioassay data obtained with the PAR-2 peptides and with trypsin, along with the molecular cloning/RT-PCR analysis, point to the presence of functional PAR-2 receptors that can activate distinct responses in the gastric and vascular smooth muscle preparations. These responses were comparable to those resulting from thrombin receptor activation in the same tissues, so as to suggest that the receptor for the PAR-2-activating peptides may play a physiological role as far reaching as the one proposed for the thrombin receptor.

Keywords: Thrombin receptor; trypsin; protease-activated receptor; gastric smooth muscle; endothelium

Introduction

The protease, thrombin, in addition to acting as a coagulation factor due to the proteolysis of fibrinogen and the aggregation of platelets is now known to regulate target tissues *via* the proteolytic activation of its G-protein-coupled receptor (Vu *et al.*, 1991; Rassmussen *et al.*, 1991; Coughlin *et al.*, 1992). The N-terminal domain of the receptor revealed by proteolysis, and beginning with serine-42 in the human receptor, is believed to act as a 'tethered' or 'anchored' ligand that activates the receptor (Coughlin *et al.*, 1992). Remarkably, peptides based on the revealed N-terminal receptor-activating motif, ranging from five (i.e. SFLLR or TP5) to fourteen amino acids

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(SFLLRNPNDKYEPF, or TP14) (amino acids are designated by their single letter code) have been found to activate the thrombin receptor, so as to mimic the actions of thrombin in a variety of tissues ranging from platelets (Vu *et al.*, 1991; Scarborough *et al.*, 1992; Chao *et al.*, 1992; Hui *et al.*, 1992; Sabo *et al.*, 1992; Vassallo *et al.*, 1992) to vascular and gastric smooth muscle (DeBlois *et al.*, 1992; Muramatsu *et al.*, 1992; Hollenberg *et al.*, 1992; Simonet *et al.*, 1992; Laniyonu & Hollenberg, 1995). In general it can be said that in vascular assay systems, both thrombin and the receptor-activating peptides (so-called TRAPs) can cause either an endothelium-dependent nitric oxide-mediated relaxation (De Mey *et al.*, 1982; Rapoport *et al.*, 1984; White *et al.*, 1984; Walz *et al.*, 1985; Muramatsu *et al.*, 1992; Simonet *et al.*, 1992) or an endothelium-independent contraction (Haver & Namm, 1984; White *et al.*, 1984; Walz *et al.*, 1985; Muramatsu *et al.*, 1992; Simonet *et al.*, 1992; Antonaccio *et al.*, 1993; Ku & Zaleski, 1993; Laniyonu & Hollenberg, 1995). In gastric longitudinal smooth muscle (LM) preparations, thrombin and the TRAPs cause a contractile response that is sensitive to the cyclooxygenase inhibitor, indomethacin and the tyrosine kinase inhibitors, genistein and tyrphostin. Our own structure-activity data have suggested that there are distinct receptor systems for the TRAPs in vascular and gastric smooth muscle preparations (Hollenberg *et al.*, 1993; Laniyonu & Hollenberg, 1995).

Recently, a reduced stringency hybridization search of a mouse genomic library with a substance K receptor-derived oligonucleotide probe led to the identification of a novel putative G-protein-coupled receptor related to, but distinct in sequence from the thrombin receptor (Nystedt *et al.*, 1994; 1995). This putative receptor contained in its sequence a target for proteolysis by trypsin (...SKGR/SLIGRLETQ...), so as to reveal a potential anchored receptor-activating sequence, SLIGRLDTN... The new receptor, when expressed in *Xenopus* oocytes was activated not only by low concentrations of trypsin (0.3–3 nM) but also by the receptor-derived peptide, SLIGRL (PP6) with an EC_{50} of about 5 μM (Nystedt *et al.*, 1994). The new receptor, termed *P*rotease-*A*ctivated-*R*eceptor No. *2*, or PAR-2, was resistant to activation by thrombin at concentrations as high as 100 nM (10 u ml^{-1}). Conversely, trypsin, at low concentrations (10–50 nM or about 5–25 u ml^{-1}), was unable to activate the thrombin receptor (Vu *et al.*, 1991). Furthermore, the PAR-2-receptor-activating peptide, PP6 which lacks a phenylalanine pharmacophore at position 2, essential for thrombin receptor activation (Hollenberg *et al.*, 1993; Natarajan *et al.*, 1995), was unable to stimulate the thrombin receptor present in CHO cells (Nystedt *et al.*,1995). The presence of appreciable mRNA for the PAR-2 receptor detected in mouse stomach (Nystedt *et al.*, 1994), suggested that PAR-2-activating peptides (PAR-2-APs) might lead to a contractile response, as do the TRAPs (Yang *et al.*, 1992) in this tissue. Further, we wondered if the PAR-2-APs might regulate vascular contractility, as is the case for the TRAPs. To evaluate these possibilities, the PAR-2-APs, SLIGRL (PP6), SLIGRL-NH_2 (PP6-NH_2) and SLIGR-NH_2 (PP5-NH_2) were synthesized and evaluated for their actions in a mouse and rat gastric longitudinal muscle preparation and in a rat aortic ring preparation, using the approach developed previously for the TRAPs, SFLLR (TP5) and SFLLR-NH_2 (TP5-NH_2) (Hollenberg *et al.*, 1992; 1993). The actions of PP6, PP6-NH_2 and PP5-NH_2 were compared with the effects of low concentrations of trypsin (0.5–5 u ml^{-1} or 1–10 nM) on these two tissues. To confirm the presence of the PAR-2 receptor in the gastric and aortic tissues used for the bioassays, the rat receptor was first cloned and sequenced from a neonatal rat intestinal cDNA library and from adult rat intestinal and kidney-derived cDNA. Then, based on this sequence information, a reverse-transcriptase/polymerase chain reaction (RT-PCR) approach was used to evaluate the concurrent presence of the PAR-2 and thrombin receptor mRNA in both gastric and aortic tissues.

Methods

Bioassay procedures

Male Sprague-Dawley animals (200–250 g) and Swiss Webster mice (50–70 g) cared for in accordance with the guidelines of the Canadian Council on Animal Care, were killed by cervical dislocation and the assay tissues were rapidly removed for use in the bioassay procedures. The aortic ring and gastric longitudinal muscle assays were performed essentially as previously described (Hollenberg *et al.*, 1993). In brief, rat aortic rings (2 mm × 3 mm), either intact or rubbed free of endothelium, were equilibrated for 1–2 h at 37°C in a gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer (3 ml) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_3$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2. glucose 10. Rings were precontracted with 1 μM phenylephrine (PE), and the presence or absence of a functional endothelium was ascertained by monitoring the relaxation response to 1 μM acetylcholine. Tissues were exposed to PE at 35 min time intervals, and the relaxation responses to trypsin, the TRAPs and PAR-2-APs were monitored during a 5 min interval, begun at the plateau of the PE-induced contraction. The gastric longitudinal muscle strips were cut from the body of the mouse and rat stomach in a region that was free from overlying secretory mucosa. The tissue was dissected further from adhering mucosal tissue and was equilibrated in the buffer described above. The integrity of the LM preparation was monitored by measuring the contractile response to 50 mM KCl. Agonists were added to the organ bath at 35 min intervals. For the gastric assay, amastatin (10 μM) was added to the organ bath to minimize peptide degradation; this precaution was not necessary for the aorta assay (Hollenberg *et al.*, 1993). When present, the enzyme inhibitors, genistein (GS) indomethacin (Indo) and GF109203X were added to the organ bath 20 min prior to the addition of contractile agonists. For all preparations, tension (baseline, 1 g) was monitored isometrically with either Statham or Grass force-displacement transducers. In the gastric LM assay, the contractile responses to TP5-NH_2 and the PAR-2-APs were expressed as a percentage (% KCl) of the increase in tension caused by 50 mM KCl. In the aorta relaxation assay, the responses to TP5-NH_2 and the PAR-2-APs were expressed as a percentage (% ACh) of the relaxation caused by 1 μM acetylcholine.

Peptides and other reagents

The PAR-2 and thrombin receptor activating peptides were prepared by standard solid phase synthesis procedures either by the Core Peptide Synthesis Laboratory at the Department of Biochemistry, Queens University, Kingston, ON, Canada or by BioChem Therapeutic, Laval, PQ, Canada, with the assistance of Dr John DiMaio. Peptides were >95% pure by chromatographic and mass spectral criteria. The concentration and composition of stock peptide solutions, dissolved in 50 mM phosphate buffer pH 7.4, were verified by quantitative amino acid analysis. PCR primers were obtained from the core DNA services facility at The University Of Calgary (Calgary, AB, Canada). Acetylcholine, amastatin, chlorpheniramine, indomethacin, nifedipine, phenylephrine, propranolol, N^{ω}–nitro-L-arginine methyl ester (L-NAME) and its D-amino acid analogue (D-NAME), porcine trypsin (14,900 u mg^{-1}, Cat. No.T7418) and tetrodotoxin were from Sigma (St. Louis, MO, U.S.A.). A maximum specific activity of 20,000 u mg^{-1} for the porcine trypsin preparation (Sigma Cat. No. T7418) was used to calculate the approximate molar concentrations of trypsin in the bioassay organ bath. Genistein was from ICN Biochemicals (Costa Mesa, CA, U.S.A.). Phenoxybenzamine and ritanserin were from Research Biochemicals International (Natick, MA, U.S.A.).

Cloning and PCR detection of PAR-2 receptor

RNA was isolated from freshly dissected adult rat gastric and aortic tissue prepared exactly as for a bioassay as well as from

whole kidney and from small intestine, using the TRI-reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). Aortic tissue was processed both before and after removing the endothelium. The RNA was reversed 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; 3 μl of this solution was used with sets of overlapping primers designed on the basis of the published mouse PAR-2 receptor sequence (Nystedt *et al.*, 1994; 1995) for polymerase chain reaction (PCR) amplification employing 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 was allowed to proceed for 35 cycles, beginning with a 1 min denaturation period at 94°C, followed by a 1 min reannealing time at 55° and a primer extension period of 1 min at 72°C. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The forward PCR primers used for the PAR-2 receptor were: (PF1): 5′ AGA AGT CTT ATT GGC AGA TT3′; (PF2): 5′ TTG CCC AGT AAT GGT ATG GC3′; (PF3): 5′ CAC CAC CTG TCA CGA TGT GCT3′; (PF4), a 5′ degenerate N-terminal primer: 5′ ATG (C/A)GN (A/T)(G/C)N (C/T)TN (A/T)(G/C)N (C/T)TN GCN TGG3′ and (PF4A), a non-degenerate N-terminal primer containing a Hind III restriction site and a sequence to improve translation initiation: 5′ T CAA GCT TCC ACC ATG CGA AGT CTC AGC CTG GC3′. The reverse PAR-2 primers were: (PR1): 5′ AGC ACA TCA/G TGA CAG GTA/G GTG3′; (PR2): 5′ CCC GGG CTC AGT AGG AGG TTT TAA CAC3′; (PR3): 5′ ACG CTG AGG CAG GTC ATG AA3′ and (PR4): 5′ GAC CGC GGA/G AGA AAG ACC/G GTG GTC AG3′. The forward primers for the PAR-2 receptor were targeted to the putative activating peptide sequence, SLIGRL (PF1), to an oligonucleotide sequence spaning residues L92 to A98 of the mouse receptor (PF2), to a domain in common between the mouse PAR-2 and thrombin receptors (amino acid residues 222 to 232 of the mouse receptor) (PF3) and to the N-terminal sequence of the receptor, beginning with methionine (PF4 and PF4A). The reverse PAR-2 receptor primer (PR1) was targeted to the same domain (residues 222–232) in common between the thrombin and PAR-2 receptors. Reverse primer (PR2), containing a Sma I restriction site, was targeted to the PAR-2 C-terminal sequence (TSVKTSY). Finally, reverse primers PR3 and PR4 were targeted to nucleotide sequences 5′- to PR1, so as to be nested between PF1 and PR1. Primer pairs (PF1)/(PR1) and (PF3)/(PR2) were used for screening the aorta and gastric tissues; primer pairs (PF2)/(PR1), (PF1)/(PR1), (PF3)/(PR2) and (PF4)/(PR4) were used to confirm the identity of the expected PAR-2 PCR products, and to confirm the identity of the cDNA clone isolated from a neonatal rat intestinal cDNA library λZAP II (Stratagene, La Jolla, CA, U.S.A.). Primer PF4A was used along with the set of reverse primers for the PCR cloning of the 30 amino acid residue N-terminal sequence of the receptor, that was absent from the intestinal library clone. The RT-PCR products used for sequencing were obtained from kidney and small intestine RNA preparations. The signals yielded from the gastric and aorta tissue RNA extracts by the PAR-2 PCR primer pairs were normalized to the PCR signal generated concurrently by an actin primer pair (Watson *et al.*, 1992) that spans an actin intron: forward primer: 5′ CGT GGG CCG CCC TAG GCA CCA3′; reverse primer: 5′ TTG GCC TTA GGG TTC AGG GGG3′. The detection of a 243 bp PCR product using this primer pair can confirm the absence of intron sequences in the RT product obtained from tissue RNA. For comparison with data obtained with the PAR-2 receptor-targeted primers, we also prepared a thrombin receptor primer pair spanning a portion of the N-terminal 1/3 of the receptor as outlined below.

The rat intestinal cDNA library was screened with a PCR fragment cloned from mouse intestinal cDNA, using primer pairs (PF3) and (PR2). The identity of the ~ 3 kb cDNA cloned from the intestinal library was confirmed using a nested PCR approach, employing the primer pairs described above to yield PCR products of the expected size. Sequencing of cDNA, subcloned into the p Bluescript SK^- phagemid, was done using the dideoxynucleotide sequencing method (Sanger *et al.*, 1977), employing a T7DNA polymerase sequencing kit (Pharmacia). Sequencing was done in both the 5′- and 3′-directions.

PCR primers for the thrombin receptor

The primers used to detect thrombin receptor mRNA using the RT-PCR approach were designed according to the published rat receptor sequence (Zhong *et al.*, 1992) and were targeted to the N-terminal domain of the receptor, beginning with the thrombin cleavage site and spanning a portion of the first transmembrane domain: forward primer (TF1), 5′ CCC GCT CAT TTT TTC TCA GGA A3′ and reverse primer (TR1), 5′ CAA TCG GTG CCG GAG AAG T3′.

Results

Responses of the mouse and rat gastric LM preparation

The effects of the PAR-2-APs, PP6-NH_2 and PP6 were first evaluated in a murine gastric longitudinal muscle preparation. Like the thrombin receptor-activating peptide, TP5-NH_2, the PAR-2-derived peptides, PP6-NH_2 and PP6, caused reproducible contractile responses in the mouse LM preparation (Figure 1a); this contractile response mimicked the effect of a low concentration of trypsin (1 u ml^{-1} or about 2 nM; Figure 1). The responses of the rat LM preparation to PP6 and PP6-NH_2 were comparable to those of the mouse LM (Figure 1b). The rat LM preparation was used for continued studies.

Lack of cross-desensitization between PP6-NH_2 and TP5-NH_2

Although exposure of the rat gastric LM preparation to either PP6-NH_2 or TP5-NH_2 at intervals of 30 min or greater followed by washing caused a reproducible contractile response, cumulative exposure at shorter time intervals, without washing the tissue free from peptide resulted in a diminished response (Figure 2 and data not shown), indicating a short-term desensitization of the tissue. To determine whether or not short-term desensitization to a TRAP (TP5-NH_2) would also desensitize the tissue to a PAR-2-AP (PP6-NH_2) and *vice versa*, the following experiment was done. A standard response of a tissue to the two peptides (PP6-NH_2 and TP5-NH_2) was obtained first (see Figure 2) and the preparation was then exposed cumulatively to a repetitive addition of one or other peptide (e.g., PP6-NH_2, Figure 2a), each time allowing tension to return towards baseline. Upon observing a diminished response to the second addition of the first peptide, the second peptide (e.g. TP5-NH_2, Figure 2a) was added to the organ bath. As shown in Figure 2a, the preparation desensitized to PP6-NH_2 remained fully responsive toTP5-NH_2 and *vice versa* (Figure 2b), indicating a lack of cross-desensitization. If anything, activation of the thrombin receptor system by TP5-NH_2 potentiated the subsequent response to PP6-NH_2 (Figure 2b) and *vice versa* (Figure 2a).

Role of extracellular calcium

The contractile response of the rat LM preparation to the PAR-2-AP, PP6-NH_2 was markedly attenuated in the absence of extracellular calcium; upon replenishing extracellular calcium in the continued presence of PP6-NH_2, a robust contractile response developed (Figure 3a). In accord with this result, pretreatment of the LM preparation with the calcium channel

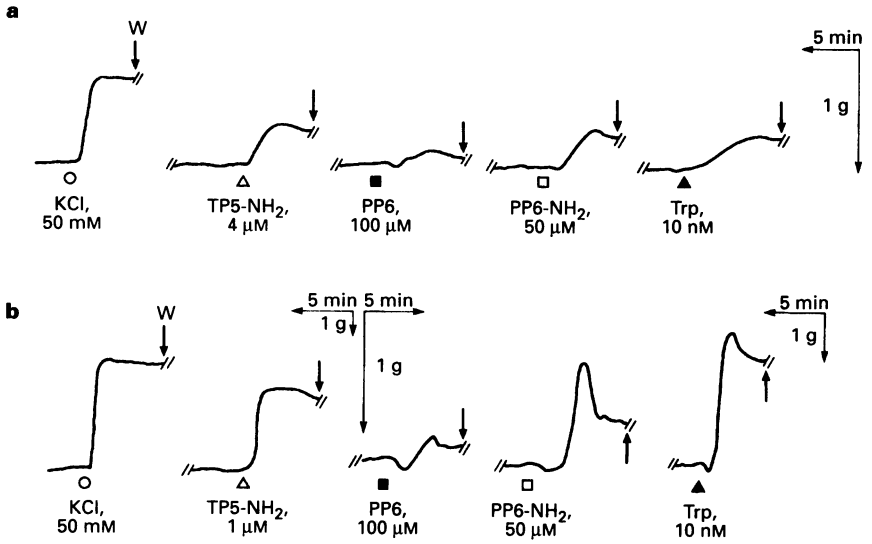

Figure 1 Contractile activity of PAR-2 and thrombin receptor activating peptides in mouse and rat gastric LM tissue: comparison with the action of trypsin. The contractile effects of the TRAP, TP5-NH_2 (△, 4 μM) the PAR-2-APs, PP6 (■, 100 μM) and PP6-NH_2(□, 50 μM) as well as trypsin (▲, 10 nM or 5 u ml^{-1}) were monitored sequentially in individual mouse (a) and rat (b) gastric longitudinal muscle strips as outlined in Methods. The contractile responses were compared with the effects of 50 mM KCl (○). The tracings are representative of four independently conducted assays showing comparable responses. The scales for time and tension are shown for each tracing. Note the changes of sensitivity for different portions of an individual tracing. W (arrow) = tissue wash.

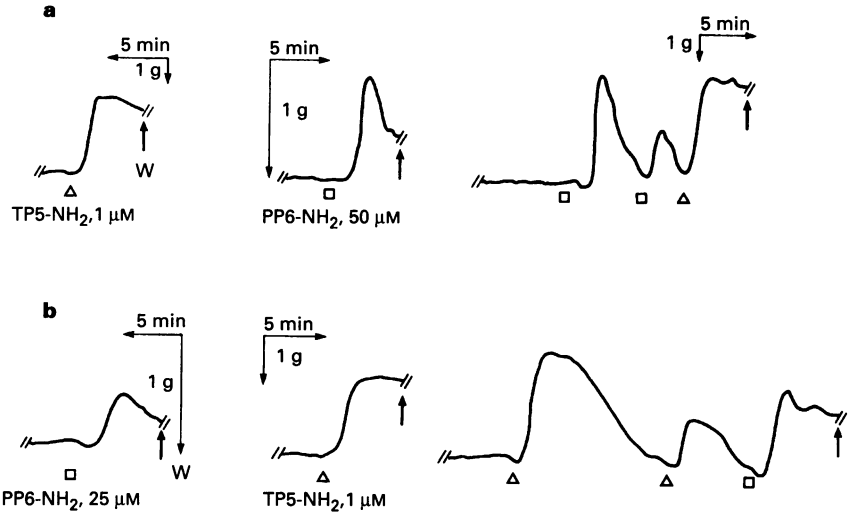

Figure 2 Lack of cross-desensitization between the TRAP, TP5-NH_2 and the PAR-2-AP, PP6-NH_2, in the rat gastric contractile assay. In each tissue strip, a standard contraction was first measured in response to PP5-NH_2 and PP6-NH_2 using an intermittent dosing protocol followed by a tissue wash (W, arrow). The tissue was then exposed cumulatively first to two repeated additions to the organ bath of either PP6-NH_2 (□) (50 μM): a) or TP5-NH_2 (△) (1 μM: b). Then, upon observing a diminished response to the repeated addition of the first agonist, the second agonist (either TP5-NH_2, a; or PP6-NH_2, b) was added concurrently to the organ bath. The magnitude of the final tissue response to either TP5-NH_2 or PP6-NH_2 was compared with the previously measured 'standard' response to the same agonist monitored by the intermittent exposure protocol described in Methods. Note the changes in the scale for tension (vertical arrows) necessitated to monitor accurately the gastric responses to TP5-NJ_2, compared with PP6-NH_2. The individual tissue strip tracings are representative of three independently conducted experiments.

blocker, nifedipine (1 μM), blocked the PP6-NH_2-induced contractile response (Figure 3b). The contractile response of the tissue to 50 mM KCl was also blocked by 1 μM nifedipine.

Effects of inhibitors of cyclo-oxygenase, tyrosine kinase and other agents in the gastric LM assay

In the rat gastric LM assay, both the cyclo-oxygenase inhibitor, indomethacin (Indo) and the tyrosine kinase inhibitor, genistein (GS) completely blocked the contractile actions of PP6-NH_2 (Figure 3c and d) and trypsin (not shown), without affecting the contractile responses causes by KCl (Figure 3c and d) and carbachol (not shown). The potent kinase C inhibitor, GF109203X (1 μM), had no effect on PP6-NH_2-induced contractions. Further, the contractile effects of PP6-NH_2 in the LM preparation were not blocked by 1 μM of the following agents: atropine, chlorpheniramine, phenoxybenzamine, propranolol, ritanserin and tetrodotoxin (data not shown).

Concentration-effect curves in the LM preparation

Concentration-effect curves were obtained for the contractile actions of PP6, PP6-NH_2 and PP5-NH_2 in the rat gastric LM

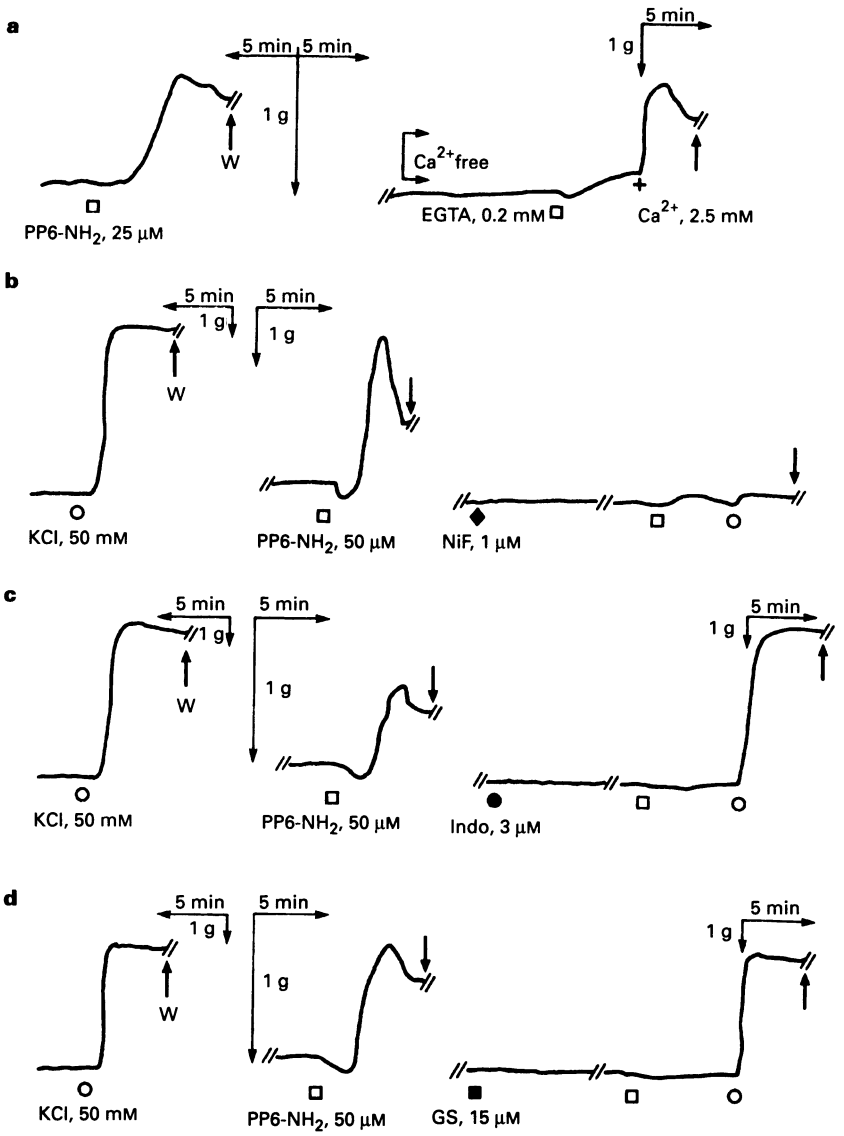

Figure 3 Role of extracellular calcium and effects on indomethacin and genistein in the rat gastric LM contractile assay. Tissues were exposed to PP6-NH_2 (□) both before and after removal of extracellular calcium (a: wash and switch to Ca^{2+}-free buffer containing 0.2 mM EGTA) or treatment for 15 min with nifedipine (NiF: ◆ 1 μM, b), indomethacin (Indo, ● 3 μM, c) or genistein (GS:■ 15 μM, d). The effectiveness of nifedipine was monitored by exposing the tissue concurrently to 50 mM KCl (○, b). The lack of effect of either indomethacin or genistein on overall tissue contractility was also assessed by monitoring a response to 50 mM KCl in the continued presence of these inhibitors (c and d). The contractile effect of replenishing the buffer with calcium (+, a) is also shown. The control responses to KCL (○) are shown on the left for (b), (c) and (d). The scales for time and tension are shown for each tracing, with indications of changes in sensitivity during an experiment with an individual tissue strip. The tracings are representative of three or more independently conducted experiments with different tissue strip preparations.

preparation for comparison with the actions of TP5-NH_2 (Figure 4a). The relative order of potencies of the peptides in this preparation was: TP5-NH_2 > > PP6-NH_2 ≥ PP6 > PP5-NH_2. The intrinsic activity of PP6 appeared to be lower than that of PP6-NH_2. The potency of PP5-NH_2 was too low in this preparation to evaluate its intrinsic activity with confidence.

Responses of the rat aortic ring preparation

In the endothelium-intact rat aortic ring preparation, trypsin (0.5 u ml^{-1}; about 1 nM) and PP6-NH_6 (1 μM) caused a comparable relaxation in a tissue that had been precontracted with 1 μM phenylephrine (Figure 5). An equivalent response was observed for higher concentrations of the other two PAR-2-APs. The relaxation responses to the three PAR-2APs were more prolonged than the transient relaxation caused by TP5-NH_2. The relaxation response of the tissue to PP6-NH_2 was not desensitized after multiple exposures and was reproducible in an individual tissue preparation over a time span of 6 to 8 h (not shown). Although the preparation was not desensitized to PP6-NH_2, desensitization of the relaxation response to the cumulative addition of trypsin (1 u ml^{-1}; 2 nM) was observed after 4 to 6 exposures; the preparation that had been desensitized by cumulative exposures to trypsin no longer responded to PP6-NH_2 but did respond to TP5-NH_2 (not shown). In a preparation that was desensitized to thrombin, by repeated exposure to this enzyme, trypsin (0.5 u ml^{-1}) or PP6-NH_2 still caused a prompt relaxation (Figure 5b).

Role of the endothelium in the response of the rat aortic preparation

In the endothelium-intact aortic preparation, the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine-methyl ester (L-NAME) (but not the inactive analogue, D-NAME) abolished

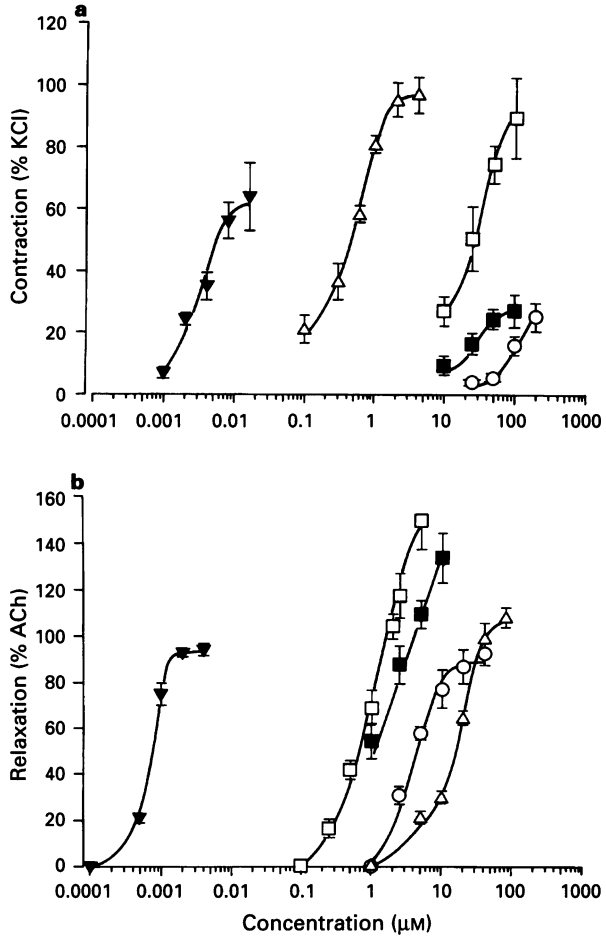

Figure 4 Concentration-effect curves for PAR-2-APs and TP5-NH_2 in the gastric LM contraction assay (a) and the aorta relaxation assay (b). Individual gastric LM tissue strips were exposed intermittently (followed by a tissue wash) to increasing concentrations of PP6-NH_2 (□), PP6 (■), PP5-NH_2 (○) or TP5-NH_2 (△) and a contractile response was measured (% KCl) relative to the contractile response caused by 50 mM KCl. The contractile responses to different concentrations of trypsin (▼) relative to 50 mM KCl were measured in individual tissue strips that were exposed intermittently no more than twice to the enzyme. The relaxation responses (b) to different concentrations of the same agonists were measured in aortic ring preparations that had been precontracted with 1 μM phenylephrine. The relaxation response was expressed as a percentage (% ACh) of the relaxation caused by 1 μM acetylcholine in the same endothelium-intact preparation. For both (a) and (b), the responses at each concentration represent the average (±s.e.mean) of measurements obtained from a minimum of 8 and maximum of 55 independently conducted measurements on separate tissue preparations taken from a minimum of two or more different animals.

the relaxant actions of either PP6-NH_2 (Figure 5c) or trypsin (not shown). The relaxant action of acetylcholine was, as expected, also blocked by L-NAME (Figure 5c). Further, in an endothelium-free aortic preparation, PP6-NH_2 caused neither a relaxation of a precontracted tissue, nor an increase in baseline tension (Figure 5d). The TRAP, TP5-NH_2, did nonetheless cause a contraction in an endothelium-free preparation that was insensitive to PP6-NH_2 (Figure 5d). Neither indomethacin nor genistein had any effect on the relaxant action of PP6-NH_2 in an endothelium-intact preparation (not shown). The relaxant response was also unaffected by 1 μM of the following agents: atropine, chlorpheniramine, phenoxybenzamine, propranolol and ritanserin (data not shown).

Concentration-effect curves in the rat aortic preparation

The concentration-effect curves for relaxant actions of PP6, PP6-NH_2 and PP5-NH_2 in the rat aortic preparation were compared with the one for TP5-NH_2 (Figure 4b). In this preparation, the intrinsic activity of PP6 appeared to be equivalent to that of PP6-NH_2. Both of these peptides caused a maximal relaxation of the preparation to baseline tension at concentrations of 5 to 10 μM (Figure 4b). The relative order of potencies of the peptides in the aortic preparation was: PP6-$NH_2 \geqslant$ PP6 > PP5-HN_2 > TP5-NH_2.

Receptor sequence obtained from intestinal cDNA

The clone isolated by the screening procedure from the λ ZAP II neonatal rat intestinal library revealed an insert of about 3 kb that was unequivocally identified as the receptor sequence, using diagnostic nested PCR analyses with the several primer pairs described in Methods. Sequencing of the insert from the 5′ end over a region of about 1400 nucleotides demonstrated that this clone contained almost the entire coding region of the rat PAR-2 receptor, except for the short N-terminal amino acid sequence corresponding to exon I of the mouse PAR-2 receptor. The remainder of the N-terminal sequence of the rat receptor sequence was determined with both kidney and intestine RNA, using an RT-PCR approach, employing the 5′-primer, PF4A, along with reverse primers PR1 and PR3. The PCR products contained a nucleotide sequence corresponding to the N-terminal domain of the receptor along with downstream nucleotide sequences overlapping precisely with the sequence previously obtained from the λ ZAP II library clone. The composite nucleotide sequence spanning about 1400 nucleotides, along with the translated amino acid sequence for the coding region of the receptor is shown in Figure 6.

Thrombin and PAR-2 receptor message in gastric and aortic tissue

The sets of primer pairs for the PAR-2 and thrombin receptors and for actin yielded comparable PCR products from the mouse and rat tissue cDNA (Figure 7). Relative to the actin signal (A, Figure 7), the signals for the PAR-2 (P, Figure 7) and thrombin receptors (T, Figure 7) appeared to be equivalent in the gastric tissue samples (Figure 7, lanes MS and RS). In the aortic tissue, removal of the endothelium caused a substantial diminution of the signal for the thrombin receptor (lane RA^-, Figure 7) without a comparable reduction in the PCR signal for the PAR-2 receptor (compare lanes RA^+ and RA^-, Figure 7). The PCR signal for the thrombin receptor from the endothelium-denuded aorta preparation was readily visualized under ultraviolet irradiation, but failed to reproduce well photographically (Figure 7, lane RA^-). The size of the PAR-2 receptor PCR product yielded by primer pairs (PF3)/(PR2) was the same for the rat and mouse tissue samples (compare lanes $RA^+/^-$, MS and RS, Figure 7); and the size of this PCR product was the same as the one obtained with the identical primer pair from the λ ZAP II library-derived receptor clone (not shown). In control PCR experiments, no bands were observed in the absence of cDNA. In a brief survey of rat tissues a variable abundance of mRNA was detected for both receptors by RT/PCR; but no tissue yet examined lacked either mRNA (data not shown).

Discussion

The main finding of our study was that the gastric LM and aortic smooth muscle preparations, that we had studied previously for their response to thrombin and the TRAPs, also responded to the receptor-activating peptides derived from the PAR-2 receptor (Nystedt *et al.*, 1994; 1995). The same assay tissues also contained PAR-2 mRNA. Since the RT/PCR data revealed comparable amounts of the PAR-2 and thrombin receptor mRNAs in the two assay tissues, it was important to determine that the activities of the PAR-2-APs and trypsin were not due to the activation of the thrombin receptor. Sev-

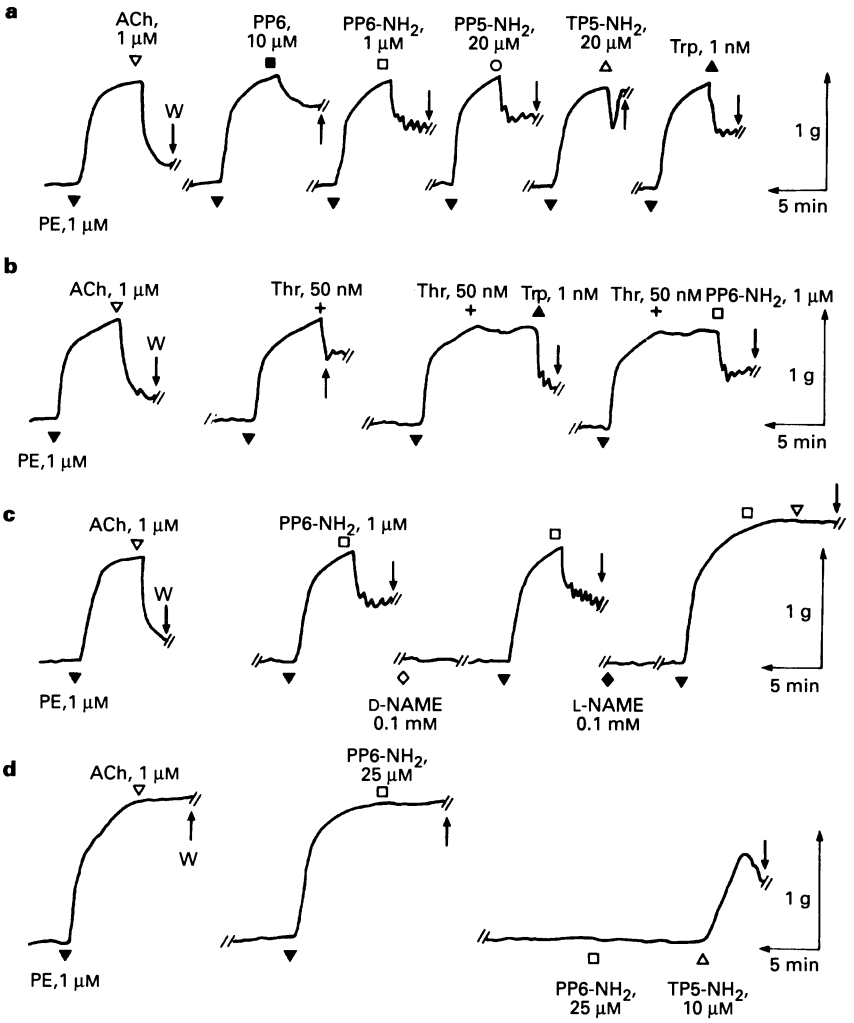

Figure 5 Representative responses of the aortic ring preparation to trypsin, thrombin, PAR-2-APs and TP5-NH_2: role of the endothelium, lack of cross-desensitization of thrombin and trypsin and inhibitory effect of L-NAME. Aortic ring preparations with (a-c) or without (d) an intact endothelium were precontracted with phenylephrine (PE, ▼, 1 μM) and the presence or absence of an intact endothelium was monitored by measuring the relaxation response caused by 1 μM acetylcholine (ACh, ▽). Similarly (a), the relaxant response caused by PP6 (■, 10 μM), PP6-NH_2 (□, 1 μM) PP5-NH_2 (○, 20 μM), TP5-NH_2 (△,20 μM) and trypsin (Trp) (▲, 1 nM or 0.5 u ml^{-1}) were observed sequentially in a single tissue strip. The ability of a thrombin (Thr) (+, 50 μM)-desensitized preparation to respond to trypsin (▲, 1 nM or 0.5 u ml^{-1}) or PP6-NH_2 (□, 1 μM) was ascertained (b) and the ability of L-NAME (◆, 0.1 mM) (but not D-NAME: ◇, 0.1 mM) to block the relaxant action of PP6-NH_2 and ACh was determined (c). Further (d), the lack of either a relaxant or contractile action of PP6-NH_2 (□, 25 μM) in an endothelium-free preparation (no response to 1 μM ACh, ▽) that otherwise exhibited a contractile response to TP5-NH_2 (△, 10 μM) was demonstrated. Each tracing, representing the response of an individual aortic ring preparation, is representative of three or more independently conducted experiments.

eral lines of evidence indicate that the results we describe here for the PAR-2-APs and trypsin were not due to activating the thrombin receptor. First, it has been clearly demonstrated by peptide structure-activity studies that TRAP peptides lacking an aromatic residue at position 2 (as in the PAR-2-APs) are unable to activate the thrombin receptor in platelets (Scarborough *et al.*, 1992; Vassallo *et al.*, 1992; Natarajan *et al.*, 1995), in smooth muscle assay systems (Hollenberg *et al.*, 1993), or in the CHO-DG44 cells used for PAR-2 receptor transfection experiments (Nystedt *et al.*, 1995). Secondly, in the endothelium-free aorta preparation that responded to the TRAP, TP5-NH_2, PP6-NH_2 failed to cause a contraction at concentrations well above those required to cause a relaxation response (Figure 5d). The endothelium-free aorta preparation has been shown previously to respond to thrombin as well (Laniyonu & Hollenberg, 1995). Third, in the gastric LM assay, partial desensitization of the contractile response to TP5-NH_2 did not desensitize the preparation to PP6-NH_2 and *vice versa* (Figure 2). Similarly, an aortic preparation that was desensitized by repeated exposure to thrombin still responded to a low concentration of trypsin and to PP6-NH_2 (Figure 5b). Finally, in both the gastric LM and aorta assay tissues, trypsin was able to cause a response (contraction or relaxation) at concentrations ($\leqslant$10 nM) well below those required to activate the thrombin receptor ($\geqslant$50 nM: Vu *et al.*, 1991). We were thus led to conclude that the contractile (LM) and relaxant (aorta) actions of trypsin and the PAR-2-APs were not due to the activation of the thrombin receptor but were due to the selective activation of a separate receptor system, most likely represented by PAR-2 itself.

Another key question to ask is: do the functional responses of the gastric and aortic tissues to the PAR-2-APs result from the direct activation of the PAR-2 receptor shown biochemically to be present in the tissues; or, might the functional responses be due to the activation of another receptor apart from or along with PAR-2? Given the ability of the PAR-2-APs to activate the PAR-2 receptor selectively in a cell transfection assay (Nystedt *et al.*, 1994; 1995), there is no reason to doubt that the PAR-2 receptor, shown to be present in the vascular and gastric tissue, would be activated by PAR-2-APs. Further, it has been shown that CHO-DG44 cells, which possess a variety of pharmacological receptors including the thrombin

receptor, but which evidently do not possess PAR-2 receptors, do not respond to PAR-2-APs unless transfected with PAR-2 cDNA (Nystedt *et al.*, 1995). Thus, the most straightforward interpretation of our data is that the functional responses observed in the gastric and vascular tissue were due to the activation of the PAR-2 *per se*. This conclusion is supported further by the inability of a variety of receptor antagonists to block the functional tissue responses caused by the PAR-2-

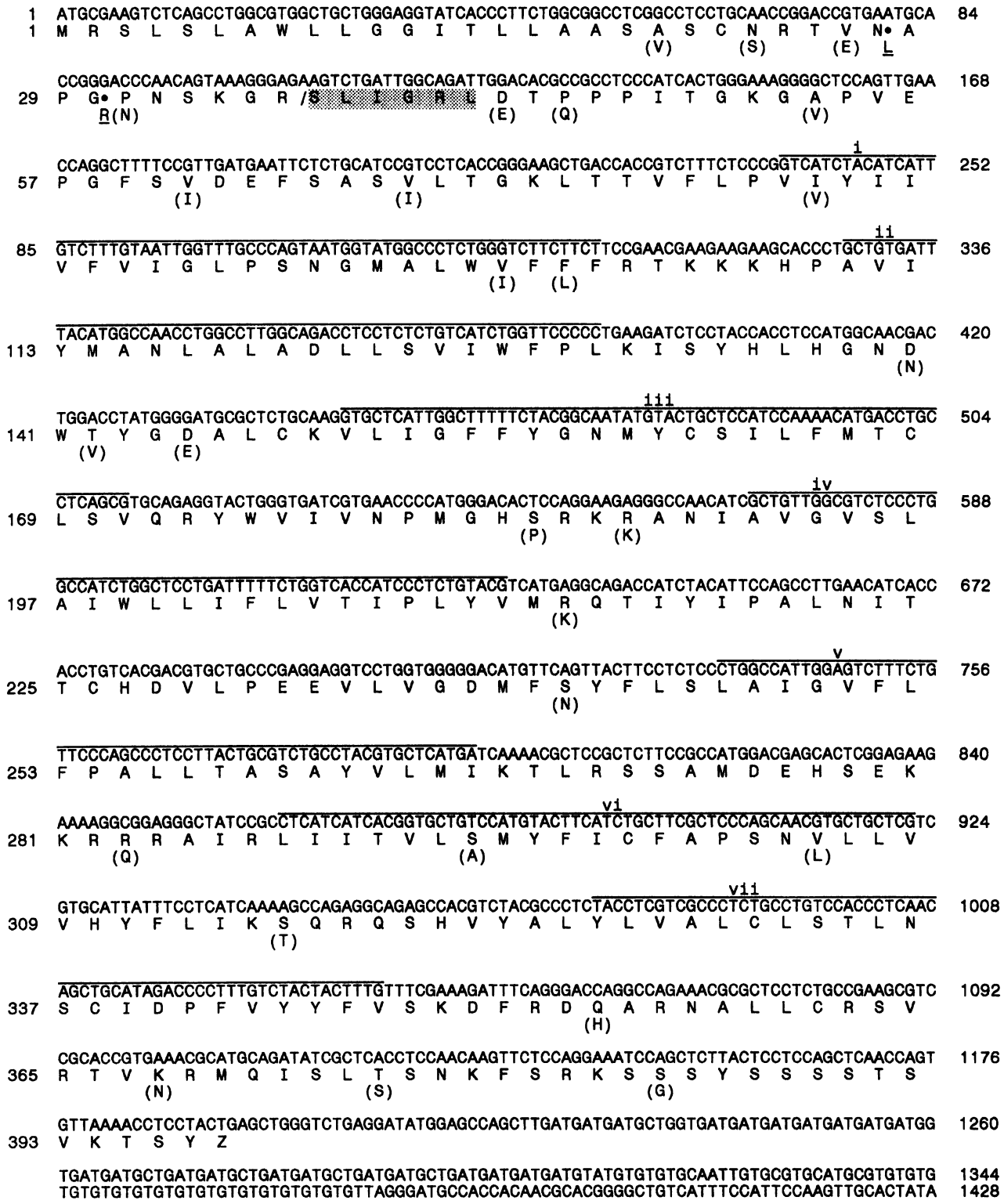

Figure 6 Oligonucleotide cDNA sequence and translated amino acid sequence of the rat PAR-2 receptor: comparison with mouse PAR-2 sequence. Amino acids are represented by their single letter codes. Differences between the translated amino acid sequences of the rat and mouse PAR-2 receptors are indicated by the mouse amino acid residues in parentheses. All other residues were identical in the two receptors, except for the arginine and leucine residues (underlined) present in the mouse receptor, but absent (dot in sequence) in the rat receptor. The putative tethered activating peptide sequence (shaded) and the site of trypsin cleavage (/) are also shown, as are the seven putative transmembrane domain sequences (lines denoted by Roman numerals). The numbering for nucleotides is on the right; number for amino acid residues is on the left.

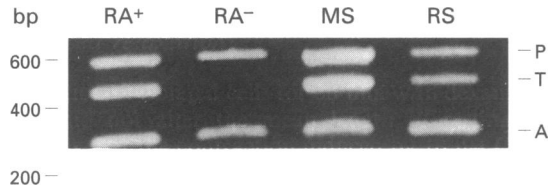

Figure 7 Analysis of tissue content of mRNA for PAR-2 and thrombin receptors in rat aorta (RA) as well as rat (RS) and mouse (MS) stomach LM preparations. Tissues were prepared as for a bioassay and RNA was then isolated for RT-PCR analysis as outlined in Methods. Analyses were done for identical RNA samples evaluated concurrently with primer pairs for actin (A), the thrombin receptor (primer pair TFI/TRI:T) (T) and the PAR-2 receptor (primer pair PF3/PR2:P). The positions of the gel calibration markers are shown (base pairs, bp) on the left. The positions of the gel calibration markers are shown (base pairs, bp) on the left. The positions corresponding to the PAR-2 (P) and thrombin (T) receptors as well as for actin (A) are shown on the right. Analyses were done for aortic samples that were either intact (RA^+) or denuded of endothelium (RA^-).

APs. Nonetheless, as indicated by the experiment with the endothelium-free aortic preparation which does possess PAR-2 mRNA (Figure 5d), but which did not respond to PP6-NH_2, the mere presence of the PAR-2 receptor in a tissue need not confer a response indicated by a change in tissue tension. Thus, without the availability of a selective PAR-2 antagonist, it was not possible to exclude unequivocally the possibility that the functional responses of the aortic and gastric tissues might have been due to the activation of a separate receptor, exclusive of or in addition to PAR-2.

Although the PAR-2 and thrombin receptor-activating peptides stimulated two distinct receptors in the assay systems we have studied, there is nonetheless a striking similarity between the pharmacodynamics of the two receptors. For instance, activation of both receptors causes an endothelium-dependent nitric oxide-mediated relaxation in the aortic assay (this study and Muramatsu *et al.*, 1992). Further, in the gastric contractile assay, the responses to both TRAPs and the PAR-2-APs require extracellular calcium and are blocked by the tyrosine kinase inhibitor, genistein and the cyclo-oxygenase inhibitor, indomethacin. Finally, for both receptor systems, the activating peptides with a free carboxyl are less potent than their carboxyamide counterpart (this work and Hollenberg *et al.*, 1993). Taken together, the data suggest that the two receptor systems can couple efficiently to the same G-proteins (G_i, G_q) so as to activate comparable downstream signal transduction pathways. It will be of considerable interest to identify the seemingly common non-receptor tyrosine kinase pathway that is evidently involved in the contractile action of the TRAPs and PAR-2-APs in the gastric longitudinal smooth muscle preparation.

Although the relative potencies of the three PAR-2-APs in the gastric contractile and aortic relaxation assays were comparable (PP6-$NH_2 \geqslant$ PP6 > PP5-NH_2), there was a considerable difference between the two tissues in terms of their relative sensitivities towards the PAR-2-APs on the one hand and the TRAP, TP5-NH_2 on the other (i.e. PAR-2-APs more potent than TP5-NH_2 in the aorta and less potent than TP5-NH_2 in the gastric tissue: Figure 4). Further, PP6 appeared to be a full agonist in the relaxation assay, but only a partial agonist in the gastric contractile assay. The differences in sensitivity (about 10 fold) of the two tissues towards the PAR-2-APs would not appear to be due to marked differences in PAR-2 receptor content, since the PCR results pointed to equivalent amounts of receptor mRNA in the two tissues (Figure 7). Most likely the differences in tissue sensitivities may be attributed to the distinct signal transduction pathways activated by the PAR-2-APs in the gastric smooth muscle elements, compared with the vascular endothelial cells. In this regard, it was of interest that, despite the apparent presence of much more mRNA for the PAR-2 receptor compared with the thrombin receptor in the endothelium-free aorta preparation (lane RA^-, Figure 7), the PAR-2-AP, PP6-NH_2 failed to cause a contractile response, whereas the TRAP, TP5-NH_2 did so. Evidently, even a comparatively low abundance of thrombin receptor mRNA in the endothelium-free aortic preparation was sufficient to confer tissue sensitivity to both thrombin and the TRAPs. This result suggests that even in an individual tissue, the PAR-2 and thrombin receptors may be able to activate different signalling pathways, even with quite disparate receptor abundance. These potential differences in signal coupling (a) for the PAR-2 receptor situated in different tissues and (b) between the thrombin and PAR-2 receptors in an individual tissue merit further study.

The deduced amino acid sequence of the rat PAR-2 receptor revealed considerable amino acid sequence identity with the deduced sequence of the murine receptor (Figure 6). Most of the differences between the sequences of the two receptors can be attributed to 'conservative' amino acid substitutions which would not, *a priori*, be expected to alter receptor function dramatically. Overall, there were fewer differences in sequence between the rat and mouse PAR-2 receptors than there are in comparing the rat and mouse thrombin receptor sequences (Zhong *et al.*, 1992 and protein sequence database). Significantly, the putative tethered PAR-2 receptor-activating sequence (SLIGRL) is identical in the rat and mouse receptors.

Although the endogenous protease that may activate the PAR-2 receptor has not yet been identified, the sensitivity of the vascular and gastric preparations to trypsin (nanomolar range) was comparable to the sensitivity we previously measured for thrombin in equivalent bioassays (Hollenberg *et al.*, 1992). Nonetheless, the PAR-2 receptor would appear to be much more sensitive to trypsin than thrombin (Figure 5 and Nystedt *et al.*, 1994; 1995); and *vice versa* for the thrombin receptor (Vu *et al.*, 1991). This sensitivity to trypsin may account for the hypotensive action of trypsin when administered intravenously to rats and dogs in amounts equivalent to thrombin (Williams *et al.*, 1991). That trypsin itself would be the physiological activator of the PAR-2 receptor is unlikely, but still possible. Nonetheless, the concurrence of the thrombin and PAR-2 receptors in the two tissues we have studied, and the comparable responses of the two tissues, upon selective activation by the PAR-2-APs would suggest that the PAR-2 receptor system (and its cognate endogenous protease) may play a physiological role as far reaching as the one proposed for the thrombin receptor system.

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