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Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists *in vivo*

*,[1,2,3,4,5]Morley D. Hollenberg, [2,4]Mahmoud Saifeddine, [2,4]Sabrina Sandhu, [1,2,4]Steeve Houle & [1,4]Nathalie Vergnolle

[1]Mucosal Inflammation Research Groups, Canadian Institutes of Health Research, Proteases and Inflammation Network (PAIN), Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1; [2]Endocrine-Diabetes Research Group, Canadian Institutes of Health Research, Proteases and Inflammation Network (PAIN), Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1; [3]Smooth Muscle Research Groups, Canadian Institutes of Health Research, Proteases and Inflammation Network (PAIN), Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1; [4]Department of Pharmacology & Therapeutics, Canadian Institutes of Health Research, Proteases and Inflammation Network (PAIN), Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1 and [5]Department of Medicine, Canadian Institutes of Health Research, Proteases and Inflammation Network (PAIN), Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1

1 We evaluated the ability of a number of peptides based on the tethered ligand sequences of human, rat and murine proteinase-activated receptor-4 (PAR_4), to serve as receptor-activating probes or antagonists for bioassays carried out *in vitro* and for *in vivo* models of inflammation.

2 In a rat PAR_4-dependent platelet aggregation assay, the relative potencies of the active sequences (AYPGKF-NH_2 > GYPGKF-NH_2 > GYPGFK-NH_2 > GFPGKP-NH_2) were consistent with an activation of PAR_4.

3 In the aggregation assay, the reverse or partial reverse-sequence peptides (VQGPYG-NH_2, YAPGKF-NH_2 and FKGPYA-NH_2) were inactive, while *trans*-cinnamoyl (Tc)-YPGKF-NH_2, Tc-APGKF-NH_2 and *N*-palmitoyl-SGRRYGHALR-NH_2 (pepducin P4pal-10) were antagonists.

4 However, in an endothelium-dependent NO-mediated rat aorta (RA) relaxation assay and in a gastric longitudinal muscle (LM) contraction assay, these antagonist peptides were agonists as were most other peptides, with distinct orders of potencies that differed for both the RA and LM assays and from the platelet assay.

5 We conclude that PAR_4-derived tethered ligand peptide agonists can act at receptors other than PAR_4 and that a judicious choice of ligands is required to probe for PAR_4 function in bioassay systems and in particular for *in vivo* models.

6 By selecting from these peptides the ones most reliably reflecting PAR_4 activation (AYPGKF-NH_2 as a standard agonist; YAPGKF-NH_2 as a PAR_4-inactive standard), we were able to establish an inflammatory role for the PAR_4-activating peptides acting *via* a non-neurogenic mechanism in a rat paw oedema model.

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Abbreviations: Ach, acetylcholine (amino acids are abbreviated by their one-letter codes: A, alanine; Y, tyrosine, etc. The amino-acid abbreviations for peptide sequences are shown in Table 1.); LM, gastric longitudinal muscle preparation; L-NAME, N^{ω}-nitro-L-arginine-methyl ester; P4pal-10, pepducin, *N*-palmitoyl-SGRRYGHALR-NH_2; PAR, proteinase-activated receptor, with subtypes denoted by subscripts; PAR-AP, PAR-activating peptide; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; RA, rat aorta ring preparation; Rev-P4pal-10, reverse pepducin *N*-palmitoyl-RLAHGYRRGS-NH_2; Tc, *trans*-cinnamoyl

Introduction

Many of the biological actions of the proteinases, thrombin and trypsin, are now known to be due to the proteolytic activation of a novel family of G-protein-coupled receptors, termed proteinase-activated receptors (PARs) (Rasmussen *et al*., 1991; Vu *et al*., 1991; Nystedt *et al*., 1994; Brass & Molino, 1997; Dery *et al*., 1998; Hollenberg, 1999; Coughlin, 2000; MacFarlane *et al*., 2001; Vergnolle *et al*., 2001; Hollenberg & Compton, 2002, Ossovskaya & Bunnett, 2004). The unique mechanism whereby proteinases activate PARs involves the proteolytic unmasking of a nascent N-terminal receptor sequence that acts as a tethered receptor-activating ligand. To date, four receptors belonging to this family have been cloned (PARs 1–4), each of which has a unique proteolytically revealed tethered ligand sequence generated

*Author for correspondence at: Department of Pharmacology & Therapeutics and Department of Medicine, University of Calgary, Faculty of Medicine, Calgary, AB, Canada T2N 1N4;
E-mail: mhollenb@ucalgary.ca
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by thrombin or trypsin (Rasmussen *et al*., 1991; Vu *et al*., 1991; Nystedt *et al*., 1994; Kahn *et al*., 1998; Xu *et al*., 1998). A key discovery that has facilitated the study of the physiology and pharmacology of the PARs was the observation that synthetic peptides with sequences based on the revealed tethered ligand moiety can activate the receptors (so-called PAR-activating peptides (PAR-APs)) and thus mimic the PAR-mediated action of proteinases in a variety of tissues, ranging from platelets to vascular and gastric smooth muscle (Vu *et al*., 1991; Muramatsu *et al*., 1992; Scarborough *et al*., 1992; Vassallo *et al*., 1992; Hollenberg *et al*., 1997; Dery *et al*., 1998; Hollenberg & Compton, 2002). These PAR-APs can prove to be of considerable utility to determine the potential consequences of activating PARs in bioassay systems *in vitro* or in inflammatory or other models *in vivo*.

In previous work, we and others have developed receptor-selective PAR-APs for PAR_1 and PAR_2 (Natarajan *et al*., 1995; Hollenberg *et al*., 1997; Kawabata *et al*., 1999; Maryanoff *et al*., 2001). That said, we have found that some of the PAR-APs can cause effects *via* receptors other than the PARs (Vergnolle *et al*., 1998; McGuire *et al*., 2002). We previously observed that human and murine PAR_4-derived PAR-APs, GYPGQV-NH_2 and GYPGKF-NH_2, which aggregate human and rodent platelets *via* PAR_4 (Kahn *et al*., 1998; Xu *et al*., 1998), are not able to activate either PAR_1 or PAR_2, but are able to cause either an endothelium-dependent nitric oxide-mediated relaxant response in an endothelium-intact rat aorta (RA) preparation or a contractile response in a rat gastric longitudinal muscle (LM) preparation (Hollenberg *et al*., 1999). To characterize further the vascular and gastric receptors responsible for the relaxant and contractile actions of the PAR_4-APs using a pharmacological approach and to assess the utility of such peptides for studies of PAR_4 functions *in vivo*, we synthesized a number of peptide analogues based on the human, murine and rat PAR_4 tethered ligand sequences in addition to the two PAR_4-APs we had previously evaluated (GYPGQV-NH_2 and GYPGKF-NH_2): (1) a more potent PAR_4-AP, based on the murine sequence, AYPGKF-NH_2 (Faruqi *et al*., 2000); (2) the rat PAR_4 tethered ligand sequence, GFPGKP-NH_2 (Hoogerwerf *et al*., 2002); (3) the reverse-sequence human PAR_4-AP, VQGPYG-NH_2; (4) partial and complete reverse-sequence murine-derived PAR_4-APs: YAPGKF-NH_2, and FKGPYA-NH_2; and (5) a partial reverse-sequence murine PAR_4-AP, GYPGFK, that was used as a PAR_4-AP-inactive standard in a previous study (Lan *et al*., 2000). Further, in keeping with the development of an N-acylated peptide antagonist of PAR_1 (Bernatowicz *et al*., 1996), we prepared *trans*-cinnamoyl (Tc)-YPGKF-NH_2 and Tc-APGKF-NH_2. Finally, we prepared the N-palmitoylated PAR_4-derived pepducin peptide *N*-palmitoyl-SGRRY-GHALR-NH_2 (P4pal-10) (Covic *et al*., 2002) and its reverse-sequence peptide *N*-palmitoyl-RLAHGYRRGS-NH_2 (rev-P4pal-10). In our preliminary work with Tc-YPGKF-NH_2 (Hollenberg & Saifeddine, 2001) and in a previous work with P4pal-10 (Covic *et al*., 2002), it has been established that both peptides can antagonize thrombin-triggered, PAR_4-mediated aggregation of rodent platelets, which lack PAR_1. The activities of all peptides were evaluated not only in the rat gastric LM contractile and vascular RA relaxation assays, but also in the rat platelet aggregation assay, in which the aggregation is due to activation of PAR_4 and not PAR_1 (Kahn *et al*., 1998; Xu *et al*., 1998; Rueff *et al*., 2000; Hoogerwerf *et al*., 2002). The working hypothesis of our study was that the structure–activity relationships (SARs) for all synthesized peptides would serve to distinguish specific PAR_4-mediated responses from responses to the peptides that are mediated by receptors distinct from PAR_4. Based on the structure–activity profile information obtained from the platelet, RA and LM assays, we selected the most appropriate standard PAR_4 agonist and a standard PAR_4-inactive peptide to evaluate the potential inflammatory role of PAR_4 and its potential neurogenic mechanism in a rat paw oedema inflammation model, as we had studied previously for PARs 1 and 2 (Vergnolle *et al*., 1999a, b; de Garavilla *et al*., 2001; Steinhoff *et al*., 2000).

Methods

Bioassay procedures

Platelet aggregation assay Aggregation assays were performed exactly as previously described (Wallace & Woodman, 1995; Hollenberg & Saifeddine, 2001) with washed platelets obtained from male albino Sprague–Dawley rats (approx. 250–300 g), treated in accordance with the Canadian Council on Animal Care. In preliminary experiments, it was determined that the activity of the PAR_4-APs was no different in the absence or presence of amastatin (Coller *et al*., 1992). This aminopeptidase inhibitor was therefore not used in the platelet aggregation assay. Animals were anaesthetized with ether and anticoagulated blood was obtained from the inferior vena cava by withdrawing blood into citrate-containing 10 ml syringes (0.5 ml of 3.4% w v^{-1} trisodium citrate per 10 ml blood). This sample was split into two 5 ml portions, which were supplemented further with 1 ml of the 3.4% w v^{-1} trisodium citrate solution. A platelet-rich plasma suspension was then obtained as previously described (Wallace & Woodman, 1995), by centrifugation (900 rpm; $150 \times g_{max}$) at room temperature for 15 min in a Clay Adams Dynac II benchtop centrifuge ($r_{max} = 170$ mm). The platelet-rich plasma supernatant was withdrawn for further use either as a platelet-rich plasma suspension or for the preparation of washed platelets. After withdrawal of the platelet-rich suspension (about 2 ml), further centrifugation of the anticoagulated blood sample at 2100 rpm ($840 \times g_{max}$) for 20 min yielded a platelet-poor plasma (PPP) supernatant, used to resuspend the platelets at a concentration of about $2–3 \times 10^8$ ml^{-1}. To prepare washed platelets, the platelet suspension in PPP was supplemented with 0.8 μM prostaglandin I_2 (PGI_2: 300 ng ml^{-1}), harvested by centrifugation (1800 rpm; $620 \times g_{max}$ for 10 min at room temperature) and resuspended in PGI_2 (0.8 μM)-supplemented calcium-free Tyrode's buffer (pH 7.4) of composition (mM): NaCl (136), KCl (3), $NaHCO_3$ (12), NaH_2PO_4 (0.4), $MgCl_2$ (1) and glucose (6). Platelets were again collected by centrifugation (1400 rpm; $370 \times g_{max}$ for 10 min at room temperature) and resuspended in calcium-free Tyrode's buffer, which was then made up to 1 mM $CaCl_2$ and supplemented with indomethacin (10 μg ml^{-1}; 28 μM) (Wallace & Woodman, 1995). The resulting washed platelet suspension was allowed to stand at room temperature for 1 h to allow for degradation of residual PGI_2 before use as 0.4 ml aliquots ($2–3 \times 10^8$ platelets per ml) in calcium-replete (1 mM) Tyrode's buffer (pH 7.4). Light transmission was monitored with a dual-channel aggregometer (Payton Scientific, Buffalo, NY, U.S.A.). Agonists were added directly to the

0.4 ml suspension at 37°C and aggregation was quantified either as a percentage increase in light transmission (Δ transmission, % max), relative to that of the initial platelet suspension, or as a percentage (% control aggregation) of the maximal aggregation (i.e. maximal increase in light transmission) caused by 25 μM AYPGKF-NH_2, 50 μM GYPGKF-NH_2, 20 μM ADP or 0.25 U ml^{-1} (2.5 nM) human plasma thrombin (catalogue no. 605195, Lot B37722, 3186 NIH U mg^{-1}; Calbiochem, La Jolla, CA, U.S.A.). When present, the Tc analogue Tc-YPGKF-NH_2 was added to the platelet suspension 5 min prior to the subsequent addition of peptide agonists and 10 min prior to the subsequent addition of thrombin or ADP. For calculating the molar concentration of thrombin, 1 U ml^{-1} was taken as equivalent to 10 nM.

Aorta relaxation assay The endothelium-intact rat aortic ring (RA) assay used to monitor the responses to the PAR_4-derived peptides was the same as that previously described for measuring the actions of PAR_2-APs (Hollenberg *et al*., 1997; 1999). In brief, male Sprague–Dawley rats (250–300 g), cared for in accordance with the Guidelines of the Canadian Council on Animal Care, were killed by cervical dislocation. Clot-free portions of aorta were dissected free of adherent connective tissue and endothelium-intact rings (approx. 5 mm length × 2 mm outer diameter) were cut for use in the bioassay. Aortic rings were equilibrated at 1 g resting tension for 1 h at 37°C in a gassed (5% CO_2, 95% O_2) modified Kreb–Henseleit buffer, pH 7.4, of the following composition (mM); NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgCl_2$ (1.2), $NaHCO_3$ (25), KH_2PO_4 (1.2) and glucose (10). The relaxant actions of the PAR_4-derived peptides (20–1000 μM) were measured in endothelial-intact aortic rings that were precontracted with 1 μM phenylephrine. A relaxant response to 10 μM acetylcholine (Ach) (60–95% of phenylephrine contraction) was taken as a positive index for an intact endothelium. To assess the contribution of the endothelium to the relaxation response, endothelium-free preparations were used, in which the endothelium was destroyed by rolling the aortic rings against a thin wire. The absence of endothelium was verified by observing an absence of a relaxant response to 10 μM Ach. Peptides were added directly to the organ bath (4 ml cuvette) and the development of tension and subsequent relaxation exhibited by the rings was monitored using either Grass or Statham force-displacement transducers. When present, N^{ω}-nitro-L-arginine-methyl ester (L-NAME; 0.1 mM) was added to the organ bath 20 min prior to the addition of other reagents. The relaxant action of agonist peptides was expressed as a percentage (% Ach) of the relaxation caused in the same preparation by 10 μM Ach. The EC_{50} values for each agonist were estimated directly from the concentration–effect curves shown in Figure 6, as the concentration causing a relaxant effect that was 50% of the relaxation caused by 10 μM Ach.

Gastric longitudinal muscle contraction assay The gastric LM contractile assay was done essentially as previously described for assessing the contractile activities of PAR_2-APs (Hollenberg *et al*., 1993; 1999 ; Al-Ani *et al*., 1995). Rats were killed as for the RA assay and the stomach was rapidly removed for use in the bioassay. The LM strips were cut from the body of the stomach in a region free from overlying secretory mucosa. The remnant of the mucosal layer was removed and the tissue was equilibrated in the same gassed buffer described for the RA assay above. The responsiveness of the LM strip was tested by the addition of 50 mM KCl to the organ bath (4 ml, as above) and the response to peptide agonists was similarly monitored using either Statham or Grass force-displacement transducers. Amastatin (10 μM; Sigma, St Louis, MO, U.S.A.) was added to the organ bath to minimize peptide degradation (Coller *et al*., 1992), a precaution that was not necessary for the RA and platelet assays (Hollenberg *et al*., 1993 and data not shown). Contractile responses were monitored directly in terms of the increase in tension (% KCl) relative to the contractile response caused in the same preparation by 50 mM KCl. Concentration–effect measurements for both the RA and LM assays were done by monitoring noncumulatively, the responses to increasing concentrations of peptides, followed by washing the tissue three times and allowing a 20 min re-equilibration period before the next addition of agonist. The relative potencies of each agonist were expressed (EC_{50}) as the concentration causing a contractile response 50% of that generated by 50 mM KCl, as estimated directly from the concentration–effect curves shown in Figure 8. To assess the effects of signal pathway inhibitors (indomethacin, 1 μM; PP1, 1 μM; genestein, 20 μM), multiple tissues obtained from three or more different animals were pretreated or not for 20 min with each inhibitor, followed by the addition of either AYPGKF-NH_2 (100 μM) or Tc-YPGKF-NH_2 (1 μM) as contractile agonists. Contractions monitored in the presence of each inhibitor were expressed as a percentage of the contraction observed in the absence of inhibitor in tissues derived from the same animal on a given day. A per cent inhibition was calculated accordingly. Values of % inhibition represent the means (±s.e.m.) for measurements carried out in six or more tissues coming from different animals.

Paw oedema inflammation model Paw oedema was evaluated as previously described (Vergnolle *et al*., 1999a, b) using male Wistar rats (200–250 g). The paw diameter of each animal was measured using a caliper, before (time 0) and after intraplantar injections (under light halothane anaesthesia) of 0.1 ml of control or peptide-containing (200 μg per paw) 0.9% saline. The change in paw thickness (mm) was monitored hourly, over a 6 h time period, and was expressed as the difference (△) between the thickness at each time point and that measured immediately before (time 0) the injection of peptide. Prior to measurements of paw oedema, some animals were treated with vehicle alone or with compound 48/80 to deplete mast cells (0.6 mg kg^{-1} administered morning and evening intraperitoneally for 4 days prior to an experiment; Di Rosa *et al*., 1971; Vergnolle *et al*., 1999a, b) or with capsaicin to ablate sensory C-fibres (McCafferty *et al*., 1997; Steinhoff *et al*., 2000; de Garavilla *et al*., 2001) (three subcutaneous doses under halothane anaesthesia of 25, 50 and 50 mg kg^{-1}, at 0, 8 and 32 h; total dose, 125 mg kg^{-1} in a solution of 10% ethanol, 10% Tween, 80% isotonic buffered saline). Intraplanatar injections were administered 10 days after the last capsaicin treatment. The effectiveness of capsaicin treatment was evaluated by the ocular administration of 0.1 mg ml^{-1} capsaicin, and subsequently monitoring an absence of wiping movements. Control animals were treated with capsaicin or compound 48/80 vehicle alone.

Peptides and other reagents The PAR_4-derived peptides and their complete or partial reverse control sequences are designated in the figures and table as GYPGQV-NH_2 (◆), VQGPYG-NH_2 (◇), GYPGKF-NH_2 (○), GYPGFK-NH_2 (●), GFPGKP-NH_2 (▼), AYPGKF-NH_2 (■), YAPGKF-NH_2 (△), FKGPYA-NH_2 (□), Tc-YPGKF-NH_2 (SYMBOL 88 \f "MacSgml2" \s 16), Tc-APGKF-NH_2 (◁), the PAR_1-AP (TFLLR-NH_2: ×), as well as the palmitoylated PAR_4 antagonist P4pal-10 (*N*-palmitoyl-SGRRYGHALR-NH_2: ▶) (Covic *et al*., 2002) and its reverse peptide, rev-P4pal-10 (*N*-palmitoyl-RLAHGYRRGS-NH_2: ◀). All peptides were prepared by standard solid-phase synthesis procedures either by the Core Peptide Synthesis Laboratory at the Department of Biochemistry, Queen's University, Kingston, Ontario, or by Dr Denis McMaster, Peptide Synthesis Facility, University of Calgary, Faculty of Medicine, Calgary, Alberta. Peptides were greater than 95% pure by HPLC chromatographic analysis and mass spectral criteria. The concentration and composition of stock peptide solutions, dissolved in 25 mM HEPES buffer (pH 7.4), were verified by quantitative amino-acid analysis. Ach, indomethacin, phenylephrine, L-NAME, human thrombin (3000 U mg^{-1}, catalogue no. T6759) and amastatin were from Sigma (St Louis, MO, U.S.A.).

Statistical analysis

Values recorded in the text and in the figures, except as otherwise indicated, represent the average ± s.e.m. for the number of independent measurements indicated in brackets. For the structure–activity figures, the bars at each data point represent the s.e.m. In general, values were obtained from four or more tissue assays coming from three or more different animals. Changes in paw volume in the oedema assay were analysed with a one-way analysis of variance followed by Dunnett's test. For the statistical analysis, $P<0.05$ was considered significant.

Results

Activity of PAR_4-derived peptides in the platelet aggregation assay

The rat platelet aggregation assay, known to depend on PAR_4 activation, was used to validate the activities of the various PAR_4-derived peptides. In keeping with other studies of human and murine platelet aggregation, the PAR_4-derived peptides GYPGKF-NH_2 (Kahn *et al*., 1998), AYPGKF-NH_2 (Faruqi *et al*., 2000), GYPGQV-NH_2 (Xu *et al*., 1998) and GFPGKP-NH_2 (Hoogerwerf *et al*., 2002) were active (Figures 1–3, Table 1 and data not shown). To our surprise, the partial reverse-sequence murine PAR_4-derived peptide GYPGFK-NH_2, which was formerly described to be inactive as a PAR_4 agonist in a murine tracheal preparation (Lan *et al*., 2000), also caused rat platelet aggregation (Figures 1 and 2 and Table 1), albeit with lower potency than the nonreverse peptide GYPGKF-NH_2. In contrast, both of the N-acylated peptides, Tc-YPGKF-NH_2 and Tc-APGKF-NH_2, not only failed to cause platelet aggregation but were, as heralded by our preliminary data (Hollenberg & Saifeddine, 2001), antagonists in the assay triggered by either thrombin or AYPGKF-NH_2 (Figure 4, Table 1 and data not shown). Like the Tc derivatives, the palmitoylated pepducin P4pal-10 peptide *N*-palmitoyl-SGRRYGHALR-NH_2, but not its PAR_4-inactive reverse-sequence peptide rev-P4pal-10, blocked thrombin- or AYPGKF-NH_2-induced platelet aggregation, as expected (Covic *et al*., 2002) (Figure 3, bottom tracing and data not shown for rev-P4-pal-10). The reverse-sequence human PAR_4-derived peptide VQGPYG-NH_2 also failed to cause platelet aggregation (Figure 2 and Table 1). Similarly, the partial reverse murine-derived PAR_4-AP YAPGKF-NH_2 as well as the complete reverse sequence FKGPYA-NH_2 failed to cause platelet aggregation (Figure 2). As expected, the PAR_1-AP TFLLR-NH_2 was not active in the assay (Kinlough-Rathbone *et al*., 1993), confirming the dependence of aggregation on PAR_4 activation by thrombin. The concentration–effect curves

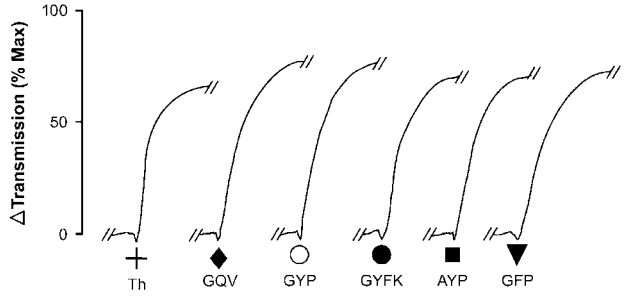

Figure 1 Platelet aggregation triggered by PAR_4-derived peptides. Representative tracings are shown for aggregation (increase in light transmission, △: % max) caused by the addition of the indicated PAR_4-derived peptides or thrombin (+, 0.25 U ml^{-1}; 2.5 nM) to the stirred platelet suspension, as outlined in Methods: GYPGQV-NH_2 (GQV), 200 μM; GYPGKF-NH_2 (GYP), 200 μM; GYPGFK-NH_2 (GYFK), 200 μM; AYPGKF-NH_2 (AYP), 50 μM; GFPGKP-NH_2 (GFP), 400 μM. The data are representative of three or more independently conducted experiments with different isolated platelet preparations.

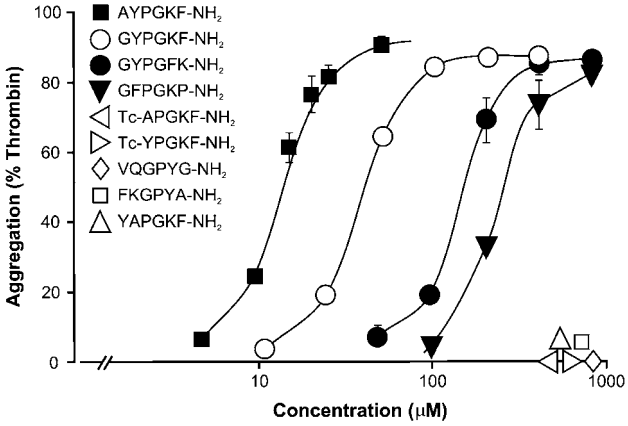

Figure 2 Concentration–effect curves for platelet aggregation caused by increasing concentrations of PAR_4-derived peptides. The effects of increasing concentrations of the PAR_4-derived peptides, for which the symbols are shown in the inset, were determined as a percentage (% thrombin) of the aggregation caused in the same preparation by thrombin (0.25 U ml^{-1}; 2.5 nM). Values represent the mean ± s.e.m. (bars) for three or more independent measurements of aggregation carried out with different platelet preparations. No bars appear for values for which the s.e.m. was smaller than the size of the symbols. Compounds without aggregating activity (open symbols, lower right) were tested at concentrations ⩾500 μM.

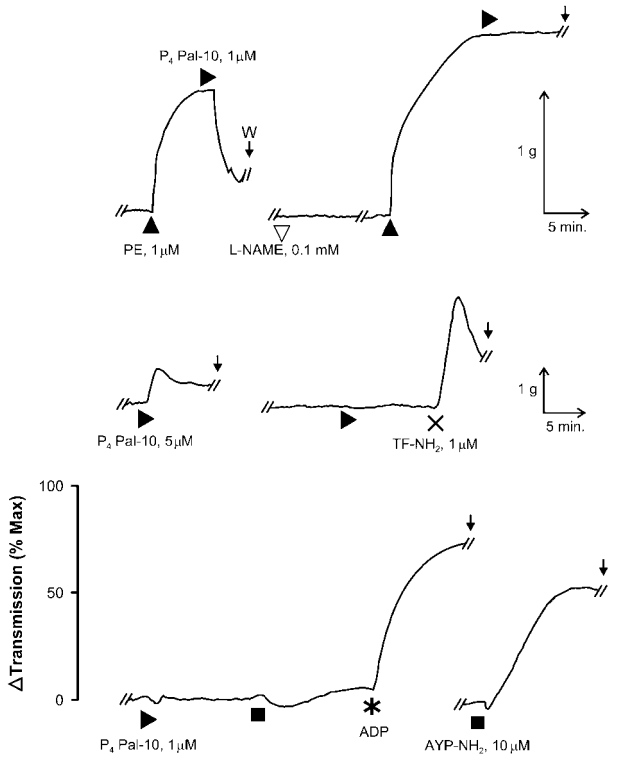

Figure 3 Action of pepducin P4pal-10 in three bioassays: RA relaxation (upper); gastric LM contraction (middle) and platelet aggregation (lower). Upper: A phenylephrine (1 μM) preconstricted endothelium-intact RA ring was treated with P4pal-10 (1 μM) either before (left) or after (right) the addition of 0.1 mM L-NAME. The scales for tension (g) and time (min) are shown by the inset. The tracings are representatives of three independently conducted experiments with different RA rings. Middle: A gastric LM preparation was exposed twice to a desensitizing concentration of P4pal-10 (5 μM), followed by the addition of a contractile concentration of the PAR_1-AP TFLLR-NH_2 (TF-NH_2, 1 μM). The scales for time (min) and tension (g) are shown by the inset. The tracings are representative of three independently conducted experiments with different tissue preparations. Lower: Aggregation ($\triangle$, increased light transmission: % max) was monitored for platelet suspensions treated with AYPGKF-NH_2 (AYP-NH_2, 10 μM) with (left tracing) or without (right tracing) the prior addition of pepducin P4pal-10 (1 μM) to the stirred suspension. A suspension in which aggregation caused by AYPGKF-NH_2 (10 μM) was blocked by pepducin P4Pal-10 (1 μM: left-hand tracing) did nonetheless aggregate in response to the subsequent addition of ADP (10 μM).

for the various peptides in the platelet aggregation assay are shown in Figure 2, wherein the relative peptide potencies were AYPGKF-NH_2 > GYPGKF-NH_2 > GYPGFK-NH_2 > GFPGKP-NH_2. The activities of the various peptides in the platelet aggregation assay are summarized in Table 1. In view of the inhibitory action of Tc-YPGKF-NH_2, its K_i for inhibiting aggregation caused by AYPGKF-NH_2 was determined (Figure 4). The K_i of Tc-YPGKF-NH_2 shifted to the right upon increasing the concentration of the agonist (10 or 25 μM AYPGKF-NH_2; Figure 4), pointing to a pseudocompetitive mechanism of inhibition. The inhibitory activity of Tc-APGKF-NH_2 was not studied further, since in preliminary work, its potency appeared lower than that of Tc-YPGKF-NH_2. The inhibitory activity of P4pal-10, previously reported in platelet aggregation assays to be inhibitory in the 1–10 μM range (Covic *et al*., 2002), was not studied further in the rat platelet assay.

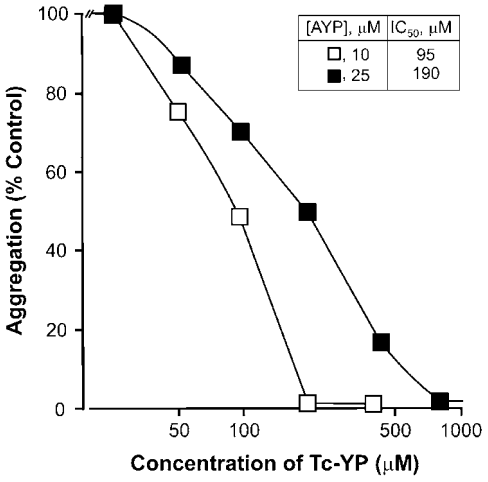

Figure 4 Concentration–inhibition curve for the inhibition of AYPGKF-NH_2-induced platelet aggregation by Tc-YPGKF-NH_2. Platelet aggregation (% control) was monitored in suspensions that were treated with increasing concentrations of Tc-YPGKF-NH_2 (Tc-YP) followed by the addition of either 10 or 25 μM AYPGKF-NH_2 (AYP). Values represent the averages of duplicate determinations for each concentration of Tc-YPGKF-NH_2.

Table 1 Relative potencies of PAR_4-derived peptides in the platelet aggregation, aorta relaxation (RA) and gastric (LM) contraction assays

Sequence	*Abbreviation*	*Symbol*	*Assay potency (EC_{50}) (μM)*		
			Platelet	*RA*	*LM*
AYPGKF-NH_2	AYP	■	13	11	110
FKGPYA-NH_2	FKG	□	NA	>300	22
YAPGKF-NH_2	YAP	△	NA	>500	>500
GYPGKF-NH_2	GYP	○	40	>800	>500
GYPGFK-NH_2	GYFK	●	160	>500	650
Tc-YPGKF-NH_2	Tc-YP	▷	Antagonist	64	1
GFPGKP-NH_2	GFP	▼	260	>800	ND
Tc-APGKF-NH_2	Tc-AP	◁	Antagonist	380	23
VQGPYG-NH_2	VQG	◇	NA	600	NA

EC_{50} values were obtained from the data shown in Figures 2, 6 and 8. NA: not active; ND: not done.

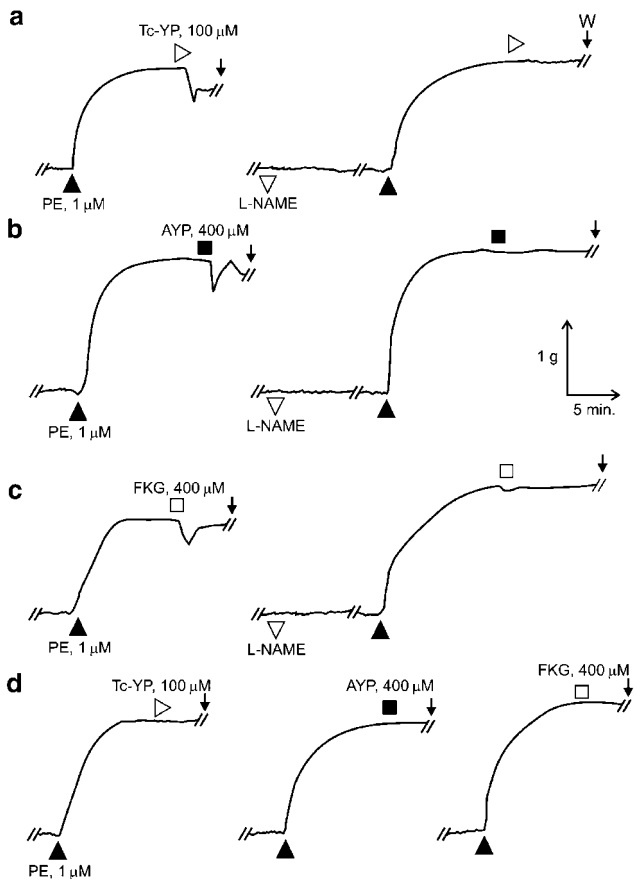

Figure 5 Relaxant actions of PAR_4-derived peptides in the RA assay. The relaxant actions of Tc-YPGKF-NH_2 (Tc-YP), AYPGKF-NH_2 (AYP) and FKGPYA-NH_2 (FKG, □) were measured in RA preparations either with (a–c) or without (d) a functional endothelium (response to 10 μM Ach present (a–c) or absent (d)). Tissues, treated or not with L-NAME (0.1 mM, a–c), were preconstricted with phenylephrine (PE: 1 μM) and PAR_4-derived peptides were added to the organ bath at 10 min, by which time the tension (upward deflection) had reached a plateau. The scales for time (min) and tension (g) are shown by the inset. Tracings are representative of three or more independently conducted experiments with tissues derived from different animals.

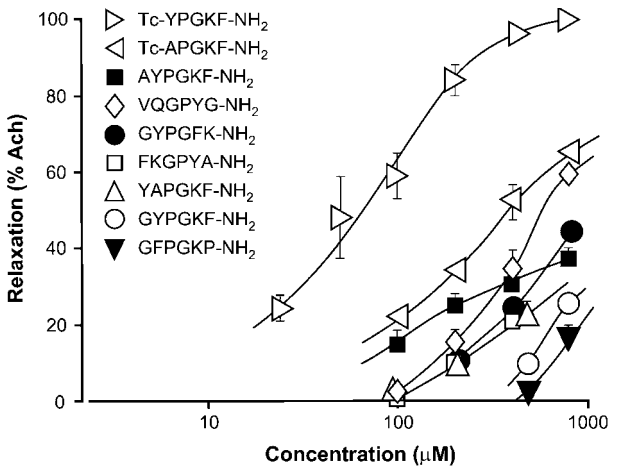

Figure 6 Concentration–effect curves for the relaxant actions of PAR_4-derived peptides using the RA assay illustrated in Figure 5. The relaxant actions of increasing concentrations of the peptide sequences shown in the inset were measured and expressed as a percentage of the relaxation caused in the same preparation by 10 μM Ach (% Ach). Values represent the average ± s.e.m. (bars) for measurements carried out at each peptide concentration using four or more tissue preparations from two or more different animals.

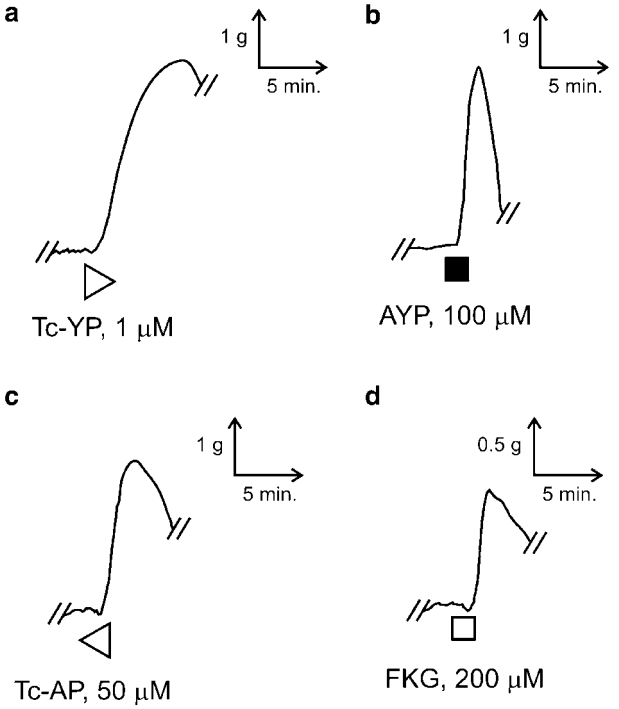

Figure 7 Contractile actions of PAR_4-derived peptides in the gastric LM assay. The contractile actions of (a) Tc-YPGKF-NH_2 (Tc-YP, 1 μM), (b) AYPGKF-NH_2 (AYP, 100 μM), (c) Tc-APGKF-NH_2 (Tc-AP, 50 μM) and (d) FKGPYA-NH_2 (FKG, 200 μM) were measured as outlined in Methods. The scales for tension (upward deflection, g) and time (min) are shown by the inset. The tracings are representative of three or more independently conducted experiments with different tissue preparations.

Responses of vascular and gastric tissues to PAR_4-APs

In keeping with our previously reported data using the PAR_4-APs GYPGQV-NH_2 and GYPGKF-NH_2 (Hollenberg *et al.*, 1999), both the RA preparation (relaxation; Figures 5 and 6 and Table 1) and the gastric LM preparation (contraction; Figures 7 and 8 and Table 1) responded to the following peptides: AYPGKF-NH_2, GYPGFK-NH_2 and GFPGKP-NH_2. Significantly, the two compounds that were antagonists in the platelet assay, Tc-YPGKF-NH_2 and Tc-APGKF-NH_2, were the most potent agonists in the RA and LM assays (Figures 6 and 8 and Table 1). Both of the pepducin peptides, P4pal-10 and revP4pal-10, also caused responses in the RA and LM assays (Figure 3 and data not shown for revP4pal-10). The representative responses to Tc-YPGKF-NH_2 and AYPGKF-NH_2 shown in Figures 5 and 7 were qualitatively the same as the responses to the other peptides (data not shown). Although the reverse PAR_4-AP VQGPYG-NH_2 failed to cause a contractile response in the LM preparation (Figure 8), it did cause a prominent relaxation response in the RA preparation (Figure 6). The RA but *not* the LM preparation also responded (relaxation) to the reverse human PAR_4-AP sequence VQGPYG-NH_2 (Figures 6 and 8 and data not shown), and the LM but not the RA preparation

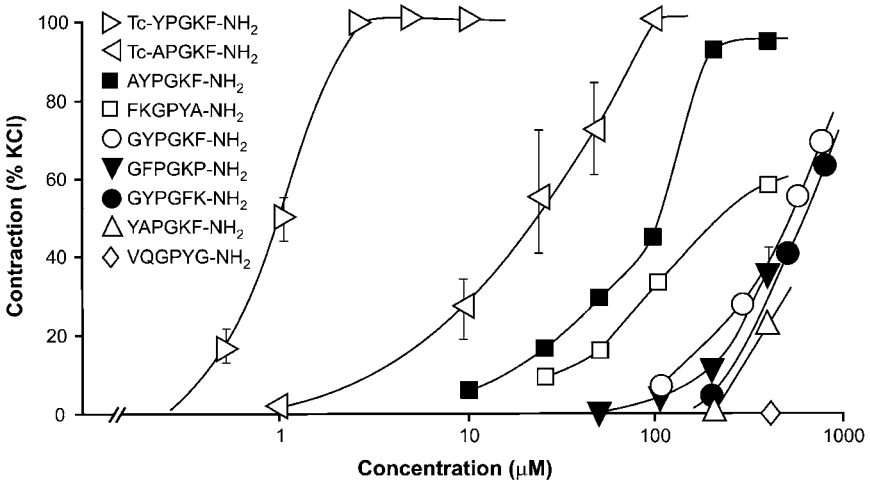

Figure 8 Concentration–effect curves for the contractile actions of PAR_4-derived peptides in the gastric contractile LM assay. The contractile responses to increasing concentrations of the indicted PAR_4-derived peptides were measured as a percentage of the contractile response of each preparation to 50 mM KCl (% KCl). Values represent the means ± s.e.m. (bars) for three or more measurements carried out at each peptide concentration with tissues obtained from two or more animals. Bars are absent for values wherein the s.e.m. was smaller than the symbols.

responded, although minimally, to the partial reverse PAR_4-AP sequence YAPGKF-NH_2 (Figures 6 and 8). In the RA preparation, the relaxation caused by all activators was blocked by the nitric oxide synthase inhibitor L-NAME (Figure 5a–c and data not shown), and was absent from preparations that were denuded of endothelium (Figure 5d and data not shown; Hollenberg *et al*., 1999). In the LM preparation, the contractile response caused by the peptides exhibited marked tachyphylaxis, often being absent after the first exposure of the tissue to the PAR-APs. Moreover, on occasion (e.g. in two of 10 preparations), the LM tissue failed to respond at all to the first exposure to the PAR_4-APs. In contrast, a nondesensitizing contractile response to the PAR_1-AP TFLLR-NH_2 was observed in all LM preparations studied (data not shown; Hollenberg *et al*., 1997), whether or not they responded to the PAR_4-APs. At sufficiently high concentrations (e.g. 200–400 μM), all peptides that did cause a contractile response in the LM assay also caused relaxation response in the RA assay. Table 1 summarizes the actions of the various peptides in the RA and LM preparations.

Relative potencies of the PAR_4-derived peptides in the RA and LM assays

Concentration–response curves for the above-described peptides in the two bioassays (Figures 6 and 8) revealed the following order of relative potencies (Table 1): (1) in the RA assay (relaxation; Figure 6): Tc-YPGKF-NH_2 $\gg$ Tc-APGKF-NH_2 $\geqslant$ AYPGKF-NH_2 $\geqslant$ VQGPYG-NH_2 $\simeq$ GYPGFK-NH_2 $\simeq$ FKGPYA-NH_2 $\simeq$ YAPGKF-NH_2 $\geqslant$ GYPGKF-NH_2 $\geqslant$ GFPGKP-NH_2; (2) in the LM assay (contraction; Figure 8): Tc-YPGKF-NH_2 $\gg$ Tc-APGKF-NH_2 > AYPGKF-NH_2 > FKGPYA-NH_2 > GYPGKF-NH_2 $\simeq$ GFPGKP-NH_2 $\simeq$ GYPGFK-NH_2 $\simeq$ YAPGKF-NH_2. In the RA assay, AYPGKF-NH_2 did not appear to be a full agonist. In the LM contraction assay, YAPGKF-NH_2 was only minimally active and VQGPYG-NH_2 was without activity at concentrations at or below 500 μM.

Signalling pathways triggered by Tc-YPGKF-NH_2 and AYPGKF-NH_2 in the LM preparation

We have routinely found that the PAR-induced relaxation in the RA preparation is due to an endothelium-dependent, nitric oxide-mediated mechanism (as for the PAR_4-derived peptides described above). However, work with a guinea pig LM preparation has shown that a tyrosine kinase-mediated, cyclooxygenase-dependent process involving Src is involved in PAR-AP-induced contractions (Zheng *et al*., 1998). We therefore tested the sensitivity of the contractile response caused by Tc-YPGKF-NH_2 and AYPGKF-NH_2 to inhibitors of tyrosine kinase (Src-targeted PP1 (1 μM) and genestein (20 μM)) and cyclooxygenase (indomethacin (1 μM)). These concentrations of the enzyme inhibitors were selected based on our previous experience in optimizing their inhibitory actions and avoiding nonspecific actions (Zheng *et al*., 1998). The contractile response to both peptide agonists was partially blocked by prior treatment of the tissue with 1 μM indomethacin: 42 ± 14% (mean ± s.e.m. for $n = 8$) inhibition of contraction caused by AYPGKF-NH_2; 43 ± 13% (mean ± s.e.m. for $n = 9$) inhibition of contraction caused by Tc-YPGKF-NH_2. These results were in contrast with the complete block of PAR_1-mediated contractions by 1 μM indomethacin in a guinea pig gastric LM preparation (Zheng *et al*., 1998). Further, the Src-targeted tyrosine kinase inhibitor PP1 (1 μM) (Hanke *et al*., 1996) had no effect on contractions caused by the PAR_4-derived peptides in six independent LM tissue assays, as opposed to the complete inhibition by PP1 of contractions activated in a guinea pig and gastric LM preparation by the selective PAR_1 agonist TFLLR-NH_2 (Zheng *et al*., 1998). In contrast, the general tyrosine kinase inhibitor genestein (20 μM) completely blocked the contractile responses to both PAR_4-derived peptides in six independently conducted experiments. Thus, the peptides AYPGKF-NH_2 and Tc-YPGKF-NH_2 appeared to induce the activation of similar contractile signalling pathways involving tyrosine kinases different from Src and partially involving cyclooxygenase.

Crossdesensitization studies with the PAR$_4$-derived peptides in the LM assay

In previous work with the LM assay preparation, it has been possible to use a crossdesensitization approach to evaluate receptor specificity (Saifeddine *et al*., 1996; Hollenberg *et al*., 1999). Thus, prior treatment of the LM preparation with the PAR$_1$-AP SFLLR-NH$_2$ markedly reduces the tissue's response to a further exposure to the PAR$_1$-AP, but does not affect the tissue's response to activation with a selective PAR$_2$-AP, like SLIGRL-NH$_2$ (Saifeddine *et al*., 1996). In a similar way, we found that desensitization of the LM preparation to Tc-YPGKF-NH$_2$ by repeated exposures did not prevent contraction caused by the subsequent addition of AYPGKF-NH$_2$ (and *vice versa*) or by the subsequent addition of the PAR$_1$-AP TFLLR-NH$_2$ to the organ bath (and *vice versa*; Figure 9 and data not shown). Thus, in the LM preparation, Tc-YPGKF-NH$_2$ appeared to act *via* receptors distinct from those activated by AYPGKF-NH$_2$ and TFLLR-NH$_2$.

Activity of peptides in the paw oedema inflammation model

Given the relatively high potency of AYPGKF-NH$_2$ in the PAR$_4$-dependent platelet aggregation assay and the low activity of the corresponding partial or complete reverse-sequence peptides YAPGKF-NH$_2$ and FKGPYA-NH$_2$ in the RA and LM assays, these three peptides were selected for evaluation in the rat paw oedema assay. As shown in Figure 10, AYPGKF-NH$_2$ caused a prompt increase in paw thickness that was maximal at about 1 h and persisted for a further 4–6 h. In contrast, the partial reverse peptide YAPGKF-NH$_2$ caused only a minimal response. However, the complete reverse peptide FKGPYA-NH$_2$ caused an increase in paw thickness that was equivalent to that caused by AYPGKF-NH$_2$ over the first hour, but was significantly lower over the remaining 5 h (Figure 10a). In animals chronically treated with compound 48/80 to deplete connective tissue mast cells of their granule content, the changes in paw thickness observed at 1 h in animals treated with either AYPGKF-NH$_2$ or the reverse-sequence peptide FKGPYA-NH$_2$ were not significantly different from control vehicle-treated animals (Figure 10b). However, in the 48/80-treated animals (open symbols, Figure 10b), as well as in vehicle-treated animals (solid symbols,

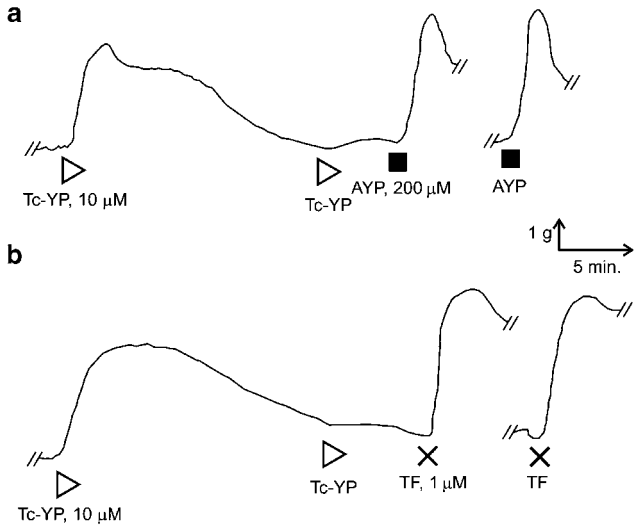

Figure 9 Lack of crossdesensitization of PAR$_4$- and PAR$_1$-derived peptides in the gastric LM contractile assay. Individual gastric LM preparations were treated first with two separate exposures to Tc-YPGKF-NH$_2$ (Tc-YP, 10 μM) to desensitize the contractile response, followed by the addition to the organ bath of either (a) the PAR$_4$-derived peptide AYPGKF-NH$_2$ (AYP, 200 μM) or (b) the PAR$_1$-derived peptide TFLLR-NH$_2$ (TF, 1 μM). The response of parallel LM preparations to either the AYP or TF peptides without prior desensitization with Tc-YPGKF-NH$_2$ is shown on the right in each tracing. The data are representative of three or more independently conducted experiments with different tissue preparations.

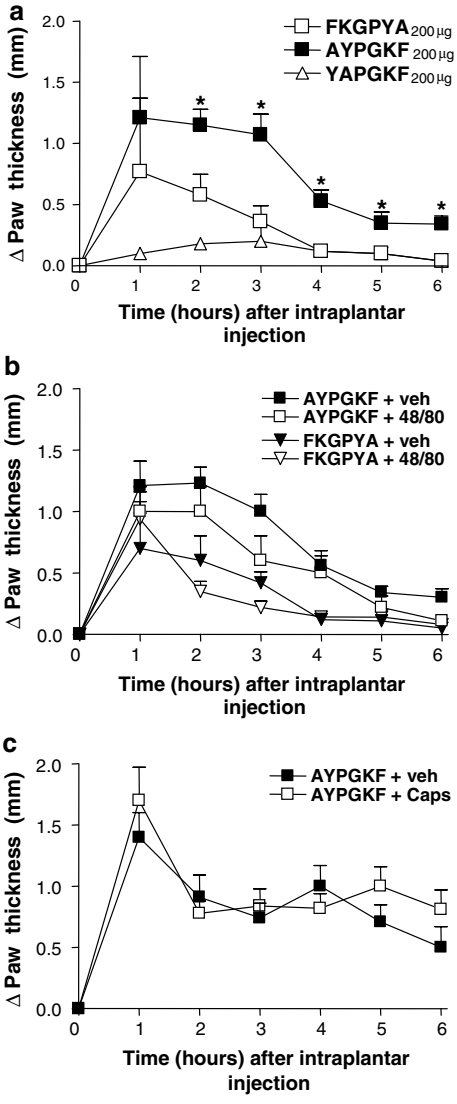

Figure 10 Inflammatory actions of PAR$_4$-derived peptides in the rat paw oedema model. Animals were either untreated (a) or pretreated with either (b) compound 48/80 or (c) capsaicin (Caps), with reference to the vehicle (Veh) used for these agents, as outlined in Methods. The PAR$_4$-AP AYPGKF-NH$_2$ or its reverse-sequence peptides (FKGPYA-NH$_2$ or YAPGKF-NH$_2$) that cannot activate PAR$_4$ were then administered by intraplantar injection and the increase in paw thickness ($\triangle$) was measured over a 6 h time period at hourly intervals. Values represent the mean$\pm$s.e.m. for six or more animals in each group. The asterisk (*) denotes the time points at which the values for AYPGKF-NH$_2$ differed ($P<0.05$) from values obtained for the reverse PAR$_4$-inactive peptides.

Figure 10b), the oedema induced by intraplantar administration of the reverse-sequence peptide diminished more rapidly than in animals treated with the PAR_4-AP AYPGKF-NH_2 (compare open triangles with open squares in Figure 10b). Further, the swelling in the reverse peptide-treated animals approached the baseline values slightly more rapidly in the 48/80-treated animals than in the vehicle-treated controls (compare open with closed triangles in Figure 10b). That said, pretreatment of the animals with compound 48/80 did not reduce the oedema caused by the complete reverse-sequence peptide FKGPYA-NH_2 to the minimal level elicited by the partial reverse-sequence peptide YAPGKF-NH_2. Both of these reverse-sequence peptides have the same overall amino-acid composition. Pretreatment of the animals with capsaicin to ablate sensory nerve C-fibres had no effect on the oedema caused by the PAR_4 agonist AYPGKF-NH_2 (Figure 10c). This result differed markedly from the ability of capsaicin treatment to mitigate paw oedema in a PAR_1- or PAR_2-dependent murine paw oedema assay (de Garavilla *et al*., 2001; Steinhoff *et al*., 2000).

Discussion

One principal finding of our study was that N-acylated peptide derivatives of the murine PAR_4 tethered ligand (Tc-YPGKF-NH_2 and Tc-APGKF-NH_2) were antagonists of both thrombin and the PAR_4-AP AYPGKF-NH_2 in a PAR_4-dependent rat platelet aggregation assay. In contrast, the same peptides were quite active as agonists in the RA and LM assays. The same type of result was obtained with the pepducin P4pal-10, a recognized antagonist for PAR_4-stimulated platelet aggregation (Covic *et al*., 2002), that was an agonist in the RA and LM assays. In platelets, the structure–activity profiles of the PAR_4-derived peptides and of other peptides modelled on the human, murine and rat PAR_4 tethered ligands were entirely in accord with those expected for a PAR_4-mediated process (platelets activated by AYPGKF-NH_2, GYPGKF-NH_2, GYPGQV-NH_2 and GFPGKP-NH_2, but not by the reverse-sequence PAR_4-inactive peptides YAPGKV-NH_2, VQGPYG-NH_2 and FKGPYA-NH_2, or by the PAR_1-related peptides TFLLR-NH_2 or SFLLR-NH_2) (Faruqi *et al*., 2000; Covic *et al*., 2002). In contrast, the structure–activity profiles for the same peptides in the RA and LM assays differed not only from each other, but were also completely different from the peptide structure–activity relationships for the platelet aggregation assay. According to principles developed some time ago for characterizing alpha- and beta-adrenergic receptors (Ahlquist, 1948), the receptor responsible for PAR_4-AP-mediated platelet aggregation was therefore pharmacologically distinct from the ones causing relaxation of the RA tissue and contraction of the LM tissue. The differences between the platelet PAR_4 receptor and the one(s) present in the RA and LM preparations were highlighted by the actions of Tc-YPGKF-NH_2, which was an antagonist of PAR_4-AP and thrombin activation of platelets (Hollenberg & Saifeddine, 2001), but an agonist in the LM and RA assays. To our surprise, P4pal-10, which as expected was a thrombin and AYPGKF-NH_2 antagonist in the platelet aggregation assay, also showed relaxant activity in the RA assay and contractile activity in the LM assay (Figure 3), as did rev-P4pal-10. It can be noted that for some receptor systems, a compound that acts as an agonist in one bioassay may potentially act as an antagonist in a distinct assay system (Feletou *et al*., 1994; Marceau *et al*., 1994). However, while this property might apply to the N-acylated PAR_4 antagonists, the very distinct structure–activity profiles of the other peptide agonists in the three bioassay systems argue strongly for the presence of multiple receptors accounting for the responses of the tissues to the peptides. Further, the crossdesensitization studies with the LM preparation showed that once desensitized, the unknown receptor(s) responsible for the contractile action of Tc-YPGKF-NH_2 could not account for the persistent contractile activity of AYPGKF-NH_2 (and *vice versa*). It can be noted that while all peptides caused relaxation in the RA assay *via* a common endothelium-dependent, nitric oxide-mediated mechanism, the response of the LM assay depended, at least in part, on a cyclooxygenase product, with the participation of an unknown tyrosine kinase signal pathway. Thus, in the LM assay, contractions caused by the PAR_4-derived peptides appeared to be mediated by a common signalling pathway (blocked by genestein, partially resistant to indomethacin and completely resistant to PP1) distinct from that in the RA tissue and also distinct from that triggered by the PAR_1-selective agonist TFLLR-NH_2 (completely blocked by indomethacin and PP1; Zheng *et al*., 1998). Given that previous work has established that the PAR_4-derived peptides do not activate other members of the PAR family, it will be a significant challenge to identify the receptor(s) responsible for the contractile actions of these peptides in the LM preparation (possibly PAR_4 itself plus other inputs).

What our study illustrates is the complexity of using any single PAR_4-AP agonist or antagonist alone as a molecular tool to investigate the potential function of PAR_4 in any given bioassay system either *in vitro* or *in vivo*. Our data also show that PAR_4-derived 'scrambled' sequences, previously believed to be adequate PAR_4-inactive standard peptides for assessing PAR_4 function in tissues (e.g. GYPGFK-NH_2; Lan *et al*., 2000), can indeed be PAR_4 agonists, whereas other peptides, like the reverse human PAR_4-AP sequence VQGPYG-NH_2, that cannot activate PAR_4 can be as active in a bioassay system (RA) as is a 'bonafide' standard PAR_4-AP (AYPGKF-NH_2; Figures 6 and 8). Thus, studies carried out to date with PAR_4-APs in pulmonary or other response systems that did not use appropriate standard inactive PAR_4-derived peptides (e.g. Chow *et al*., 2000; Lan *et al*., 2000; Mule *et al*., 2004) must be interpreted with considerable caution, since the responses observed may not have been due uniquely to PAR_4.

The *in vivo* rat paw oedema model illustrated the challenge in distinguishing the proinflammatory properties of the standard PAR_4 agonist AYPGKF-NH_2 from that of a reverse PAR_4 sequence peptide that cannot activate PAR_4 (FKGPYA-NH_2), but which also causes inflammation, albeit with a time course distinct from that of the standard PAR_4 agonist AYPGKF-NH_2. The inflammatory target for FKGPYA-NH_2, which is evidently not activated by the partial reverse peptide YAPGKF-NH_2 and is not present on the mast cell, remains to be determined.

Unfortunately, the complex pharmacological properties of the PAR_4 antagonists (either Tc-YPGKF-NH_2 or P4pal-10) did not permit their use for evaluating the role of PAR_4 in the *in vivo* paw oedema inflammation model, and we therefore had to rely on data obtained with the receptor agonists. The bioassay profiles obtained *in vitro* enabled a selection of an optimal standard agonist, AYPGKF-NH_2, and an optimal

partial reverse-sequence standard inactive peptide, YAPGKF-NH_2, for evaluating a potential inflammatory role for PAR_4 *in vivo*. Clearly, like PARs 1 and 2 (Vergnolle *et al*., 1999a, b; Steinhoff *et al*., 2000; de Garavilla *et al*., 2001), the presumed activation of PAR_4 by AYPGKF-NH_2 caused an inflammatory response characterized by a sustained oedema that lasted more than 5 h. The response to the standard PAR_4 agonist AYPGKF-NH_2 was readily distinguishable from the response to the partial reverse-sequence standard PAR_4-inactive peptide YAPGKF-NH_2 (Figure 10a). In contrast, the inflammatory action of the complete reverse peptide FKGPYA-NH_2 could be distinguished from the response to the standard PAR_4 agonist only by its distinct time course. For other PAR peptide agonists, mast cell activation *in vivo* can contribute to a PAR-inactive peptide response, masking what we believe to be a true PAR-mediated inflammatory response (Vergnolle *et al*., 1999a). However, a mast cell contribution appears not to be involved in the oedema responses either to the standard PAR_4 agonist AYPGKF-NH_2 or to its complete reverse-sequence peptide. Given the distinct time course and magnitude of inflammatory response caused by AYPGKF-NH_2, particularly in comparison with the partial reverse-sequence peptide YAPGKF-NH_2 and more subtly so for the complete reverse-sequence peptide FKGPYA-NH_2, the data strongly support our working hypothesis that the oedema is mediated by PAR_4. This hypothesis is substantiated further by our preliminary data in a murine paw oedema model, in which the PAR_4 antagonist (P4pal-10) inhibits the inflammatory action of carrageenan (Di Rosa *et al*., 1971) (unpublished data).

In contrast with the ability of capsaicin treatment to attenuate the inflammatory actions of agonists for PAR_1 and PAR_2, the inflammatory response caused by the PAR_4 agonist AYPGKF-NH_2 was unaffected by prior treatment of the animals with capsaicin (Figure 10c). Thus, what distinguishes the inflammatory response to PAR_4-AP from that caused by either PAR_1- or PAR_2-APs is the lack of dependence on a neurogenic mechanism (Steinhoff *et al*., 2000; De Garavilla *et al*., 2001). This distinct non-neurogenic mechanism that we hypothesize for PAR_4-induced inflammation is one that clearly merits further investigation. In this regard, work with PAR_4-deficient tissues (Kataoka *et al*., 2003) or intact $PAR_4^{-/-}$ mice will be of key importance.

In summary, our data point to receptors other than PAR_4 that in complex tissues may signal in response to a number of PAR_4-targeted peptide agonists and antagonists. Further, our work emphasizes the caution that must be used in interpreting data obtained either *in vitro* or *in vivo* with the use of PAR_4-derived peptide agonists. That said, for a specific biological response, an evaluation of two or more established PAR_4-targeted peptides (e.g. one standard peptide with and one without activity at PAR_4) and the possible use of one or more PAR_4 antagonists can provide evidence for or against a potential role for PAR_4. Based on the structure–activity data we report in this study, we would suggest in the future that the agonist AYPGKF-NH_2 and its partial reverse PAR_4-inactive peptide YAPGKF-NH_2 be employed as a standard agonist and a standard inactive peptide for evaluating PAR_4 function. With these peptides, we have obtained evidence for a non-neurogenic inflammatory role for PAR_4 that can be added to its recognized role in haemostasis and its participation in leukocyte recruitment (Vergnolle *et al*., 2002).

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