British Journal of Pharmacology (2005) 146, 670–678 
www.nature.com/bjp

# Neutrophils and the kallikrein–kinin system in proteinase-activated receptor 4-mediated inflammation in rodents

[1]Steeve Houle, [1]Martin D. Papez, [1]Mara Ferazzini, [1,2]Morley D. Hollenberg & *,[1]Nathalie Vergnolle

[1]Department of Pharmacology and Therapeutics, Faculty of Medicine, Canadian Institutes of Health Proteinases and Inflammation Network, Mucosal Inflammation Research Group, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1 and [2]Department of Medicine, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

**1** We evaluated a potential role for proteinase-activated receptor 4 ($PAR_4$) in a rodent paw inflammation model, with a focus on two main features of inflammation: (1) oedema and (2) granulocyte recruitment.

**2** A $PAR_4$ antagonist (Pepducin P4pal-10; palmitoyl-SGRRYGHALR-$NH_2$) reduced both the oedema and granulocyte recruitment induced by a localized administration of carrageenan in the rat hind paw, pointing to a key role for $PAR_4$ in this inflammation model.

**3** Further, intraplantar injection in the mouse hind paw of a $PAR_4$ agonist (AYPGKF-$NH_2$), but not its standard $PAR_4$-inactive peptide control (YAPGKF-$NH_2$), caused an inflammatory reaction characterized by oedema (increased paw thickness) and granulocyte recruitment (increased paw myeloperoxidase activity). The $PAR_4$ agonist-induced effects were inhibited in mice pretreated with pepducin P4pal10.

**4** These $PAR_4$ agonist-mediated effects were not affected by pretreatment with inhibitors of either NO production or prostaglandin release (L-NAME and indomethacin, respectively).

**5** However, selective immuno-depletion of neutrophils significantly reduced $PAR_4$ agonist-induced oedema formation.

**6** Moreover, AYPGKF-$NH_2$-induced oedema was also reduced by pretreatment with either a kinin $B_2$ receptor antagonist (icatibant) or a tissue or plasma kallikrein inhibitor (FE999024 and FE999026, respectively), but not with a kinin $B_1$ receptor antagonist (SSR240612).

**7** We conclude: (1) that $PAR_4$ plays an important role in the inflammatory response as it mediates some of the hallmarks of inflammation and (2) that $PAR_4$-mediated oedema is dependent on the recruitment of neutrophils and components of the kallikrein–kinin system.
*British Journal of Pharmacology* (2005) **146,** 670–678. doi:10.1038/sj.bjp.0706371;
published online 15 August 2005

**Keywords:** Proteinase-activated receptors; inflammation; oedema; neutrophils; kallikrein; kinin receptors; mouse

**Abbreviations:** AYP, AYPGKF-$NH_2$; FE999024, ((2*S*, 2*R*)-2-(2′-amino-3′-(4″chlorophenyl)propanoyl-amino-*N*-(3-guanidinopropyl)-3-(1-naphthyl)propanoamide; FE999026, ((2′*S*, 2″*R*)-4-(2′(2″-(carboxymethylamino)-3″-cyclohexyl-propanoylamino)-3′-phenyl-propanoylamino)piperidine-1-carboxyamidine); i.pl., intraplantar; KNRK, Kirsten virus sarcoma-transformed rat kidney epithelial cell; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; PAR, proteinase-activated receptor; PAR-AP, proteinase-activated receptor-activating peptide; pepducin P4pal10, palmitoyl-SGRRYGHALR-$NH_2$; SSR240612, [(2*R*)-2-[((3*R*)-3-(1,3-benzodioxol-5-yl)-3-{[(6-methoxy-2-naphthyl)sulfonyl]-amino}propanoyl)amino]-3-(4-{[(2*R*,6*S*)-2,6-dimethylpiperidinyl]methyl}phenyl)-*N*-isopropyl-*N*-methylpropanamide hydrochloride]

## Introduction

Proteinase-activated receptors (PARs) are members of the G protein-coupled receptor superfamily. They are characterized by their unique mechanism of activation involving serine proteinases such as thrombin and trypsin. A specific proteolytic cleavage of the amino terminal sequence unmasks a new amino terminal sequence consisting of a tethered ligand that binds to and activates the receptor (Hollenberg & Compton, 2002). To date, four PARs have been cloned. They are numbered 1–4 according to their order of discovery, with $PAR_4$ being the most recent member added to the PAR family (Vu *et al*., 1991; Nystedt *et al*., 1995; Ishihara *et al*., 1997; Kahn *et al*., 1998; Xu *et al*., 1998). PARs 1, 3 and 4 were first recognized as targets for thrombin whereas $PAR_2$ was seen as a trypsin receptor. However, this view has changed in the past years with the demonstration that PARs can be activated by other serine proteinases and that the same proteinase can activate more than one PAR (Ossovskaya & Bunnett, 2004). For example, $PAR_4$ is now known to be activated by thrombin, trypsin, the activated factor X of the coagulation cascade, cathepsin G and trypsin IV (Kahn *et al*., 1998; Xu *et al*., 1998; Camerer *et al*., 2000; Sambrano *et al*., 2000;

*Author for correspondence; E-mail: nvergnol@ucalgary.ca

Cottrell *et al*., 2004). Interestingly, with the exception of $PAR_3$, PARs can also be activated by short synthetic peptides of 5 or 6 amino acids that mimic the tethered ligand (Hollenberg & Compton, 2002). Receptor-selective PAR-activating peptides (PAR-APs) have proved of considerable value to uncover the physiological and pathophysiological roles played by these novel receptors. The $PAR_4$-AP, AYPGKF-$NH_2$ (AYP), which was designed based on the mouse $PAR_4$-tethered ligand sequence, is a good example of such experimentally useful synthetic peptides (Faruqi *et al*., 2000; Hollenberg *et al*., 2004). This selective $PAR_4$ agonist does not affect either $PAR_1$ or $PAR_2$ (Hollenberg & Compton, 2002).

Besides its role in thrombin-induced platelet aggregation, the potential physiological or pathophysiological roles for $PAR_4$ are still essentially unknown. $PAR_4$ was first described as an important thrombin receptor for the aggregation of both human and mouse platelets (Kahn *et al*., 1998). Mice lacking the $PAR_4$ gene show an increased bleeding time, emphasizing the importance of $PAR_4$ signalling for coagulation events *in vivo* (Sambrano *et al*., 2001). Studies carried out *in vitro* suggest a role for $PAR_4$ in gut motor function or as a signal for the release of inflammatory mediators such as cytokines or prostaglandins (Asokananthan *et al*., 2002; Mule *et al*., 2004). It has been proposed that $PAR_4$ is the likely candidate to mediate thrombin-induced leukocyte rolling and adherence in rat mesenteric venules, as monitored by intravital microscopy (Vergnolle *et al*., 2002). In that work, the selective $PAR_4$-AP, AYPGKF-$NH_2$, but not the selective $PAR_1$-AP, reproduced the proinflammatory effects of thrombin (Vergnolle *et al*., 2002). The mechanism(s) by which $PAR_4$ recruits leukocytes to the endothelium is unknown, but $PAR_4$ has been detected by immunohistochemistry at the surface of rat neutrophils as well as in the endothelial and smooth muscle cells of the aorta (Vergnolle *et al*., 2002). Further evidence for the presence of functional $PAR_4$ on endothelial cells of different origins has also been reported (Kataoka *et al*., 2003). Moreover, recently published preliminary results show that the $PAR_4$-AP, AYPGKF-$NH_2$, can mediate the formation of oedema in a rat paw, independent of neuropeptide release from sensory neurons and of mast cell degranulation (Hollenberg *et al*., 2004).

Taken together, these observations strongly support a possible involvement of $PAR_4$ in inflammation. Therefore, we sought to investigate the role and mechanisms of action of $PAR_4$ in the inflammatory response by using a rodent paw inflammation model. This model, which has been extensively used to characterize the inflammatory response mediated by the activation of either $PAR_1$ or $PAR_2$ (Vergnolle *et al*., 1999a, b; Steinhoff *et al*., 2000; de Garavilla *et al*., 2001), allowed us to focus on two hallmarks of inflammation at the same time: oedema and granulocyte recruitment. Here, we investigated (1) the potential role for $PAR_4$ activation in the generation of carrageenan-induced inflammation, and (2) the involvement of nitric oxide, prostaglandins, neutrophils and the kallikrein–kinin system in $PAR_4$-mediated inflammation.

## Methods

### *Animals*

Male C57bl/6 mice (4–6 weeks old; 20–25 g) and male Wistar rats (200–250 g) were obtained from Charles River Laboratories (Montréal, Québec, Canada). The rodents had free access to food and water and were housed under constant temperature (22°C) and photoperiod (12 h light–dark cycle). All experimental procedures were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

### *Paw oedema assay*

All paw oedema assays reported in this paper were performed by following the general guidelines described below. Prior to the administration of the $PAR_4$-AP, a basal measurement of the paw thickness of each mouse was recorded using an electronic calliper (Fisher Scientific, Hampton, NH, U.S.A.). For the carrageenan-induced inflammation, the rat paw volume, rather than its thickness, was measured by using a hydroplethismometer (Ugo Basile, Milan, Italy). The compounds to be tested or the vehicle were then administered by intraplantar (i.pl.) injection ($n = 5$–9 rodent per group for all experiments). These injections were performed under light halothane anaesthesia for the rats. Each compound injected into the paw, in a final volume of 10 $\mu$l per mouse paw or 100 $\mu$l per rat paw, was diluted in sterile saline (0.9% NaCl). As an index of oedema formation, paw thickness or volume was then measured every hour for 6 h after the injection.

Experiments were designed to determine the involvement and potential mechanisms of action of $PAR_4$ in inflammation. In the first experimental series, rats were pretreated or not with the $PAR_4$ antagonist, pepducin palmitoyl-SGRRYGHALR-$NH_2$ (P4pal10; (Covic *et al*., 2002); 0.5 mg kg$^{-1}$, i.p.), 1 h prior to the i.pl. injection of carrageenan (2% in saline). Carrageenan was used to induce a general inflammatory reaction. In the second experimental series, mice received i.pl. administration of the $PAR_4$-AP (AYPGKF-$NH_2$; 50 $\mu$g), the standard $PAR_4$-inactive control peptide (YAPGKF-$NH_2$; 50 $\mu$g) or the vehicle (saline) to determine the inflammatory effects that could be mediated by a direct activation of $PAR_4$. In some groups, mice were pretreated with pepducin P4pal10 (same as above), $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 25 mg kg$^{-1}$ diluted in saline, i.p. injection 1 h before i.pl. injections), indomethacin (5 mg kg$^{-1}$ diluted in 2% carboxymethylcellulose, given orally 1 h before i.pl. injections), rat anti-mouse Ly-6G antibody clone RB6-8C5 or rat IgG2b$\kappa$ control antibody (Hestdal *et al*., 1991; 125 $\mu$g diluted in saline, i.p. injection 18 h before i.pl. injections), FE999024 or FE999026 (((2*S*,2*R*)-2-(2′-amino-3′-(4″chlorophenyl)propanoyl-amino-*N*-(3-guanidinopropyl)-3-(1-naphthyl)propanoamide or ((2′*S*,2″*R*)-4-(2′(2″-(carboxymethylamino)-3″-cyclohexyl-propanoylamino)-3′-phenyl-propanoylamino)piperidine-1-carboxyamidine), respectively; (Evans *et al*., 1996a, b); 60 $\mu$mol kg$^{-1}$ diluted in saline, i.p. injection 1 h before i.pl. injections), [(2*R*)-2-[((3*R*)-3-(1,3-benzodioxol-5-yl)-3-{[(6-methoxy-2-naphthyl)sulfonyl]amino}propanoyl)amino]-3-(4-{[(2*R*,6*S*)-2,6-dimethylpiperidinyl]methyl}phenyl)-*N*-isopropyl-*N*-methylpropanamide hydrochloride] (SSR240612); (Gougat *et al*., 2004); 10 mg kg$^{-1}$ diluted in saline, i.p. injection 1 h before i.pl. injections) or icatibant (Hock *et al*., 1991; 50 $\mu$g kg$^{-1}$ diluted in saline, coinjection in the paw at $t = 0$ h). Except for icatibant, which was injected in the mouse paws in a final volume of 10 $\mu$l at the same time as AYPGKF-$NH_2$, all other inhibitors or antagonists were administered in a final

volume of 200 $\mu$l. The control groups always received a similar pretreatment with the corresponding vehicle.

### *Myeloperoxidase activity assay*

Tissue myeloperoxidase activity assay was performed as an index of granulocyte recruitment at the end of each experiment, as previously described (Steinhoff *et al*., 2000; Asfaha *et al*., 2002). Briefly, the injected paws were cut and weighed prior to their homogenization in a 0.5% hexadecyltrimethylammonium bromide phosphate buffered (pH 6.0) solution using a polytron PT10-35 homogenizer (Kinematica, Lucerne, Switzerland). The homogenates were then centrifuged at 13,000 $\times$ *g* for 3 min at 4°C in a microcentrifuge. Five aliquots of each supernatant were then transferred into 96-well plates before the addition of a solution containing 3,3′-dimethoxybenzidine and 1% hydrogen peroxide. In parallel, a number of standard dilutions of pure myeloperoxidase were also tested for their activity to construct a standard curve (OD as a function of units of enzyme activity). Optical density readings at 450 nm were taken at 1 min (which corresponds to the linear portion of the enzymatic reaction) using a Spectra Max Plus plate reader linked to the SOFTmax Pro 3.0 software (Molecular Devices Corp., Sunnyvale, CA, U.S.A.). The myeloperoxidase activity found in the paws was expressed as units of enzyme per milligrams of tissue.

### *Calcium-signalling assay*

Calcium signalling was measured as described previously (Compton *et al*., 2001). Kirsten virus sarcoma-transformed rat kidney cells (KNRK cells) were grown to near confluence in 75 $cm^2$ culture flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 1% antibiotics. Cells were harvested using a nonenzymatic dissociation buffer. Harvested KNRK cells were then incubated in 1 ml of DMEM containing 10% FBS, 0.25 mM sulphinpyrazone and 22 $\mu$M Fluo-3 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR, U.S.A.) for 25 min at room temperature with gentle shaking. Cells were then washed and resuspended in calcium assay buffer (mM: NaCl 150, KCl 3, $CaCl_2$ 1.5, glucose 10, HEPES 20, sulphinpyrazone 0.25, pH 7.4). Fluorescence measurements were performed on a Perkin-Elmer fluorescence spectrometer 650-10S, with an excitation wavelength of 480 nm and emission recorded at 530 nm. Cell suspensions (2 ml) in 4 ml cuvettes were stirred with a magnetic flea bar and maintained at 24°C. The signal produced ($E_{530}$) by the addition of a test agonist was measured as a percentage of the fluorescence peak height yielded by the addition of 2 $\mu$M calcium ionophore (A23187).

### *Chemicals*

All peptides (AYPGKF-$NH_2$; YAPGKF-$NH_2$; P4pal10, pepducin P4pal10, (Covic *et al*., 2002)) were obtained from the Peptide Synthesis Facility of the University of Calgary (Calgary, Alberta, Canada; peplab@ucalgary.ca, Dr Dennis McMaster, Director). The antibody used to deplete the mice of their granulocytes (anti-mouse Ly-6G antibody clone RB6-8C5; (Hestdal *et al*., 1991)) and the control antibody (rat IgG2b$\kappa$ antibody) were purchased from eBioscience (San Diego, CA, U.S.A.). The tissue and plasma kallikrein inhibitors (FE999024 and FE999026, respectively; also known as CH-2856 and CH-4215, respectively; Evans *et al*., 1996a, b) were a kind gift from Dr D. Michael Evans (Ferring Research Ltd, Southampton, U.K.) and the nonpeptidic kinin $B_1$ receptor antagonist, SSR240612 (Gougat *et al*., 2004), was a kind gift from Dr Denis Riochet (Sanofi-Synthelabo Recherche, France). The myeloperoxidase, isolated from human neutrophils and used as a standard, was obtained from EMD Biosciences Inc. (San Diego, CA, U.S.A.). All other drugs and reagents were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.), most notably L-NAME (the nonselective nitric oxide synthase inhibitor), indomethacin (the nonselective cyclooxygenase inhibitor), icatibant (the specific $B_2$ kinin receptor antagonist; (Hock *et al*., 1991)), carrageenan and calcium ionophore (A23187).

### *Statistical analysis*

All results are reported as mean $\pm$ s.e.m. They were analyzed using the Instat 3.0 statistics software (GraphPad Software, San Diego, CA, U.S.A.). Comparisons among groups were performed using the paired ANOVA test for repeated measures followed by the parametric Dunnett's test. For all statistical analysis, an associated probability (*P*-value) of less than 5% was considered significant.

## Results

### *Role for $PAR_4$ activation in inflammatory processes*

In order to determine if $PAR_4$ participates in the inflammatory response, we studied the effects of the $PAR_4$ antagonist, pepducin P4pal10 (0.5 mg $kg^{-1}$), in a rat model of paw inflammation (i.pl. injection of 2% carrageenan in saline) classically used to test anti-inflammatory drugs. It has previously been demonstrated that this dose of pepducin P4pal10 can prevent the thrombin-mediated aggregation of mouse platelets *in vivo* (Covic *et al*., 2002). As expected (Levy, 1969), carrageenan provoked the formation of a substantial oedema over a 4 h period as well as the recruitment of granulocytes (Figure 1). Interestingly, the pepducin P4pal10 treatment significantly reduced both the oedema (Figure 1a) and the granulocyte infiltration (Figure 1b) induced by carrageenan. These results pointed strongly to the involvement of $PAR_4$ in the carrageenan-induced inflammatory response.

### *Inflammation induced by $PAR_4$ activation*

Next, we evaluated the ability of a direct activation of $PAR_4$ to induce an inflammatory response. In this experiment, we used AYPGKF-$NH_2$ as the $PAR_4$ agonist and YAPGKF-$NH_2$ as a standard $PAR_4$-inactive control peptide. This choice of peptides was based on a structure–activity relationship study showing that this combination of receptor probes is optimal to study the functions of $PAR_4$ *in vivo* (Hollenberg *et al*., 2004). Here, injection of AYPGKF-$NH_2$ (50 $\mu$g) into the mouse paw caused a rapid oedema (statistically significant at less than 1 h) that lasted for 6 h (Figure 2a and c) and a substantial granulocyte recruitment (Figure 2b and d) when compared to the control peptide YAPGKF-$NH_2$ (50 $\mu$g) or the saline vehicle. These two inflammatory events were markedly

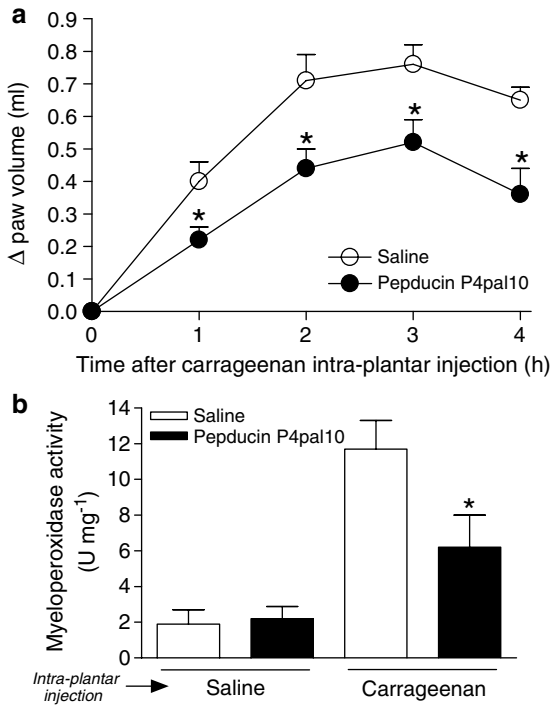


**Figure 1** Effect of a $PAR_4$ antagonist on a general inflammatory reaction induced by carrageenan in the rat paw. Pepducin P4pal10 ($0.5\,mg\,kg^{-1}$)-dependent reduction of the carrageenan ($200\,\mu g$)-mediated oedema formation (a) and granulocyte recruitment (b). Saline was used as a control for both the pepducin P4pal10 and carrageenan injections. Values are mean ± s.e.m. of $n=8$ per group. *Significantly different from the vehicle + carrageenan group, $P<0.05$.

attenuated in mice pretreated with the $PAR_4$ antagonist, pepducin P4pal10, supporting the hypothesis that the peptide AYPGKF-$NH_2$ acts through $PAR_4$. For both parameters (oedema and granulocyte recruitment), the standard $PAR_4$-inactive control peptide had little or no effect when compared to the saline vehicle (Figure 2a and b). On occasion, the control peptide YAPGKF-$NH_2$ induced a slightly greater response than saline, a response which appeared to be independent of $PAR_4$ in view of the lack of effect of pepducin P4pal10 pretreatment (Figure 2c and d). These observations showed that the $PAR_4$-AP induces two important hallmarks of inflammation: oedema and granulocyte recruitment.

### *Nitric oxide and prostanoids are not involved in $PAR_4$-induced inflammation*

Since NO and prostaglandins are two major inflammatory mediators (Ushikubi *et al*., 2000; Guzik *et al*., 2003), we evaluated their respective contribution to the $PAR_4$-mediated proinflammatory effects. Mice were pretreated with either L-NAME (a nonselective inhibitor of nitric oxide synthases; $25\,mg\,kg^{-1}$; Vergnolle *et al*., 1999a, b) or indomethacin (a non-specific inhibitor of cyclooxygenases; $5\,mg\,kg^{-1}$; Vergnolle *et al*., 1999a, b) prior to the i.pl. administration of AYPGKF-$NH_2$. Neither of these two inhibitors affected oedema formation (Figure 3a) or granulocyte recruitment (Figure 3b) induced by $PAR_4$ activation.

### *Contribution of neutrophils to $PAR_4$-induced oedema*

Neutrophils are considered a major component of the innate immune system due to their scavenging roles and because of the many proinflammatory mediators they can release (Scapini *et al*., 2000). In order to determine if neutrophil recruitment following the activation of $PAR_4$ might participate in oedema formation, we pretreated groups of mice with a granulocyte-depleting antibody (rat anti-mouse Ly-6G antibody clone RB6-8C5, $125\,\mu g$; a dose that has previously shown to deplete mice from their circulating neutrophils by 95%; (Bonder *et al*., 2004)), a control antibody (rat IgG2b$\kappa$ control antibody; $125\,\mu g$) or the vehicle before the i.pl. administration of AYPGKF-$NH_2$. The marked decrease in myeloperoxidase activity reflected a dramatic reduction in the migration of granulocytes into the paw (Figure 4b). Further, neutrophil-depleted mice showed a significant reduction of the $PAR_4$-induced oedema throughout the first 5 h compared to control antibody-injected mice (Figure 4a). These results suggest that $PAR_4$-recruited neutrophils play an important role in the formation of oedema, possibly by releasing or generating inflammatory factors.

### *Contribution of the kallikrein–kinin system in $PAR_4$-induced oedema*

To study further the mechanisms of $PAR_4$-induced inflammation, we hypothesized that $PAR_4$ activation can trigger kallikrein activity, which in turn might participate in the formation of oedema by producing active kinins. To test this hypothesis, we pretreated groups of mice with inhibitors of either tissue or plasma kallikreins (FE999024 and FE999026, respectively; $60\,\mu mol\,kg^{-1}$) before the i.pl. administration of AYPGKF-$NH_2$. The doses administered of these inhibitors have been shown to be highly selective for their respective kallikrein target *in vitro* as well as *in vivo* in a rat model of acute pancreatitis (Griesbacher *et al*., 2002). The tissue kallikrein inhibitor, FE999024, significantly reduced the $PAR_4$-AP-induced oedema throughout the first 5 h, whereas the plasma kallikrein inhibitor, FE999026, was effective in reducing oedema for only the first 3 h (Figure 5a). The data suggest that both kallikreins can play distinct roles in $PAR_4$-induced oedema.

Since kallikreins are responsible for the release of active kinins, we next investigated a possible role for activation of the two known kinin receptors (the inducible $B_1$ and the constitutive $B_2$; (Marceau *et al*., 1998) in $PAR_4$-induced oedema. We observed that pretreatment of mice with the kinin $B_1$ receptor antagonist (SSR240612; $10\,mg\,kg^{-1}$; a concentration known to inhibit the kinin $B_1$ receptor-mediated oedema in the mouse paw; (Gougat *et al*., 2004) had no effect on $PAR_4$-induced oedema (Figure 6a). In contrast, mice pretreated with the $B_2$ receptor antagonist (icatibant; $50\,\mu g\,kg^{-1}$; (Decarie *et al*., 1996) showed a reduced oedema in response to the $PAR_4$ agonist at all of the observed time points (Figure 6b). These results demonstrated the involvement of the kinin $B_2$ (but not the $B_1$) receptor in $PAR_4$-induced oedema.

We have also evaluated the possibility that the $PAR_4$-AP, AYPGKF-$NH_2$, could activate directly the $B_2$ receptor. To test this hypothesis, we have performed a calcium-signalling assay using a KNRK cell line that possesses functional $B_2$ receptors

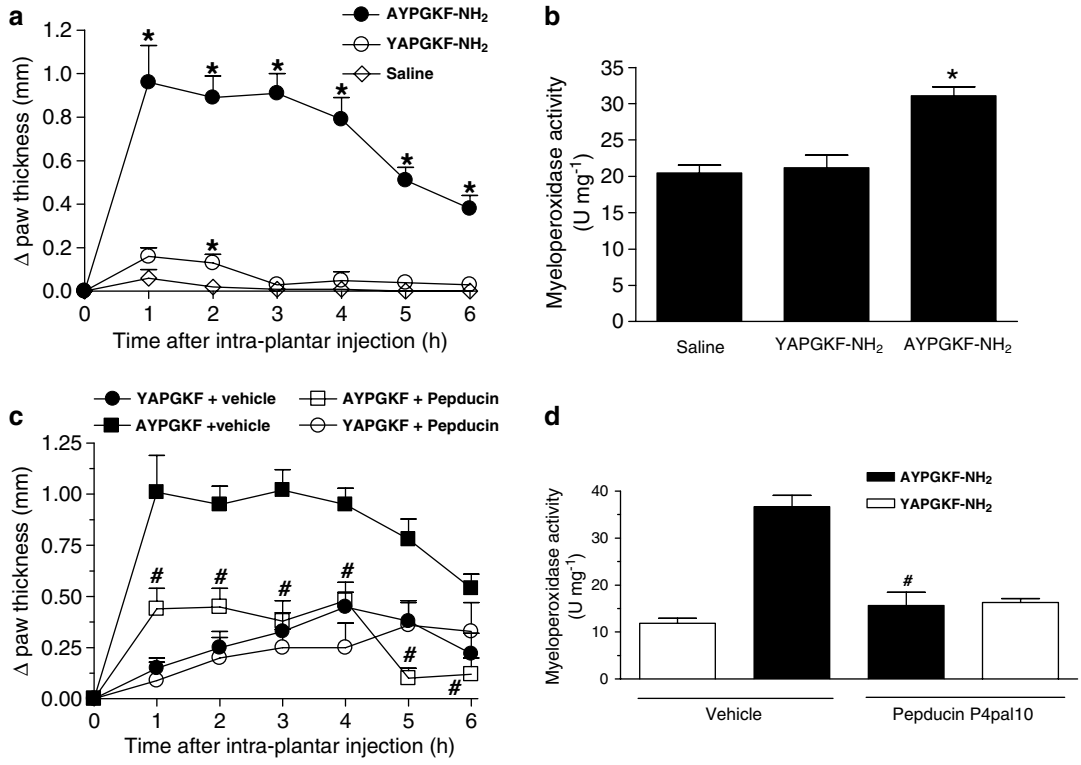


**Figure 2** Direct inflammatory effects of $PAR_4$ activation in the mouse paw. The oedema formation (a) and granulocyte recruitment (b) were evaluated following an intraplantar injection of the $PAR_4$-AP, AYPGKF-$NH_2$ (50 $\mu$g), the control inactive peptide, YAPGKF-$NH_2$ (50 $\mu$g), or the saline vehicle. The effect of a pepducin P4pal10 (0.25 mg kg$^{-1}$) pretreatment was also evaluated against the oedema formation (c) and granulocyte recruitment (d) induced by AYPGKF-$NH_2$ (50 $\mu$g) and YAPGKF-$NH_2$ (50 $\mu$g). Values are mean $\pm$ s.e.m. of $n = 5$–8 per group. *Significantly different from the saline vehicle or the control peptide group (a and b), $P < 0.05$; #significantly different from the vehicle + AYPGKF-$NH_2$ group (c and d), $P < 0.05$.

but not $PAR_4$. Bradykinin, at a concentration of 10 nM, induced a rapid calcium response (Figure 7). This response was clearly mediated by the $B_2$ receptor as the bradykinin-induced calcium signal was abrogated by 30 nM of the specific antagonist icatibant. The $PAR_4$-AP, AYPGKF-$NH_2$, at a concentration of 200 $\mu$M, failed to induce a calcium response in these kinin $B_2$ receptor responsive cells (Figure 7). Thus, it is unlikely that AYPGKF-$NH_2$ interacts directly with the $B_2$ receptor to activate some signalling pathways.

Although affecting the oedema response, none of the inhibitors or antagonists of the kallikrein–kinin system mentioned above had a statistically significant effect on granulocyte recruitment mediated by $PAR_4$ activation (Figures 5b and 6c).

## Discussion

A main conclusion that can be drawn from our study is that activation of $PAR_4$ can play a key role in generating two hallmarks of the inflammatory response: oedema and granulocyte infiltration. In this context, neutrophils and the kallikrein–kinin system are important contributors. Thus, $PAR_4$ can take its place alongside $PAR_1$ and $PAR_2$ as an important receptor for the regulation of the inflammatory response (Ossovskaya & Bunnett, 2004). The overall scheme we propose for this inflammatory role of $PAR_4$ is summarized in Figure 8.

Owing to the relatively recent discovery of $PAR_4$ (Kahn *et al*., 1998; Xu *et al*., 1998), its functions are still largely unknown. There is strong evidence that this receptor is involved in platelet-mediated haemostasis in humans and rodents. However, the role that $PAR_4$ activation plays in other events related to hæmostasis and inflammation has yet to be clarified. The data we present here substantially extend our preliminary work in which we showed, without exploring the underlying mechanisms, that a selective $PAR_4$-AP can cause oedema in the rat (Hollenberg *et al*., 2004). Added to this oedema, our new data also demonstrate that AYPGKF-$NH_2$ increases the recruitment of granulocytes to the site of inflammation in the mouse paw. Moreover, the blockade of $PAR_4$ with a specific antagonist, pepducin P4pal10 (Covic *et al*., 2002), reduced both the oedema and granulocyte recruitment responses caused by the inflammatory stimulant, carrageenan, when injected in the rat paw. Previous work had singled out $PAR_4$ as the likely candidate to mediate thrombin-induced leukocyte rolling and adherence in rat mesenteric venules (Vergnolle *et al*., 2002). Our present observations demonstrate further the involvement of $PAR_4$ activation in inflammatory processes, particularly in the generation of oedema and inflammatory cell recruitment. Although oedema and granulocyte infiltration were significantly reduced by the $PAR_4$ antagonist in the carrageenan inflammation model, these parameters were not completely abolished. This result suggests that although $PAR_4$ plays a prominent role in carrageenan-

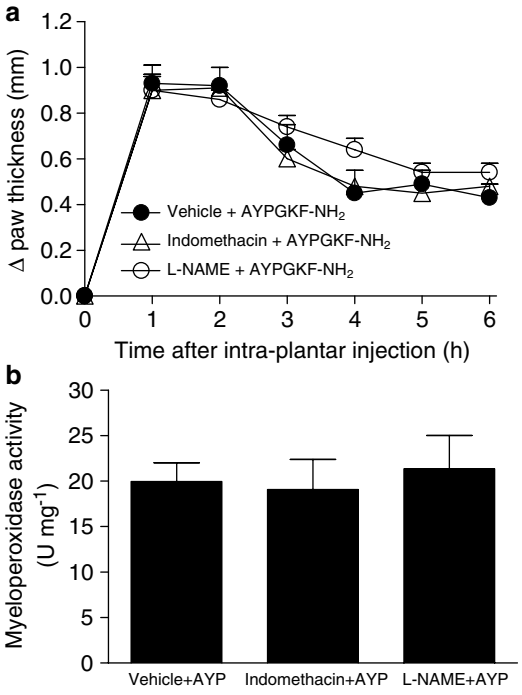


**Figure 3** Noninvolvement of NO and prostanoids in $PAR_4$-mediated oedema formation and granulocyte recruitment in the mouse paw. Effect of indomethacin (cyclooxygenases inhibitor, $5\,mg\,kg^{-1}$) or L-NAME (nitric oxide synthases inhibitor, $25\,mg\,kg^{-1}$) on oedema formation (a) and on granulocyte recruitment (b) observed following $PAR_4$ activation with AYPGKF-$NH_2$ ($50\,\mu g$). Values are mean ± s.e.m. of $n = 8$–9 per group. No statistically significant difference was observed between groups.

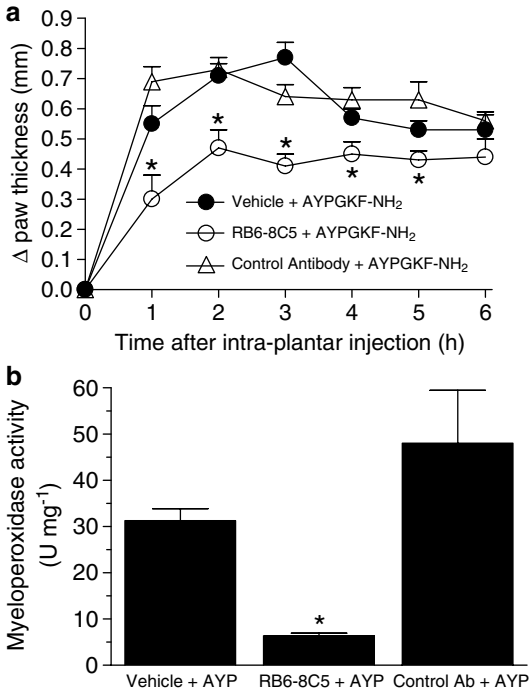


**Figure 4** Role for neutrophils in the $PAR_4$-mediated oedema formation in the mouse paw. A granulocyte-depleting antibody (anti-mouse Ly-6G antibody clone RB6-8C5, $125\,\mu g$) causes a marked reduction of myeloperoxidase, indicative of neutropenia (b) as well as reducing the oedema (a) mediated by AYPGKF-$NH_2$ ($50\,\mu g$) whereas a control antibody (rat IgG2b$\kappa$ antibody, $125\,\mu g$) does not. Values are mean ± s.e.m. of $n = 8$ per group. *Significantly different from the vehicle + AYPGKF-$NH_2$ group, $P < 0.05$.

induced inflammation, other mediators are undoubtedly also involved.

In a rat paw inflammation model, we found that oedema caused by the activation of $PAR_1$ or $PAR_2$ (Vergnolle *et al*., 1999a, b) results from the release of neuropeptides from sensory neurons (Steinhoff *et al*., 2000; de Garavilla *et al*., 2001). In contrast, the $PAR_4$-mediated oedema response differs, in that a neurogenic mechanism does not appear to be involved nor does mast cell degranulation play a role (Hollenberg *et al*., 2004). In common with the $PAR_1$-triggered inflammatory response (Vergnolle *et al*., 1999b), we found that NO production and eicosanoid synthesis do not play a major role in the inflammatory events mediated by $PAR_4$. Taken together, our observations suggest that $PAR_4$ is unique with regards to its inflammatory mechanisms of action when compared to the other PARs. Further, the $PAR_4$-mediated inflammatory mechanisms appear to differ from the classical inflammatory pathways (NO and prostaglandin generation) often triggered by other G protein-coupled receptor systems, such as $PAR_2$ (Vergnolle *et al*., 1999a).

Since $PAR_4$ agonists, including thrombin, can induce leukocyte rolling and adhesion in the vasculature (Vergnolle *et al*., 2002) and since $PAR_4$-induced oedema is not mediated by sensory neurons and mast cells (Hollenberg *et al*., 2004), a potential role for $PAR_4$ activation of leukocytes, particularly neutrophils, was hypothesized. Here, our data show (1) that the recruitment of granulocytes goes hand-in-hand with oedema formation and (2) that the presence of neutrophils *per se* is a major contributor to the development of $PAR_4$-

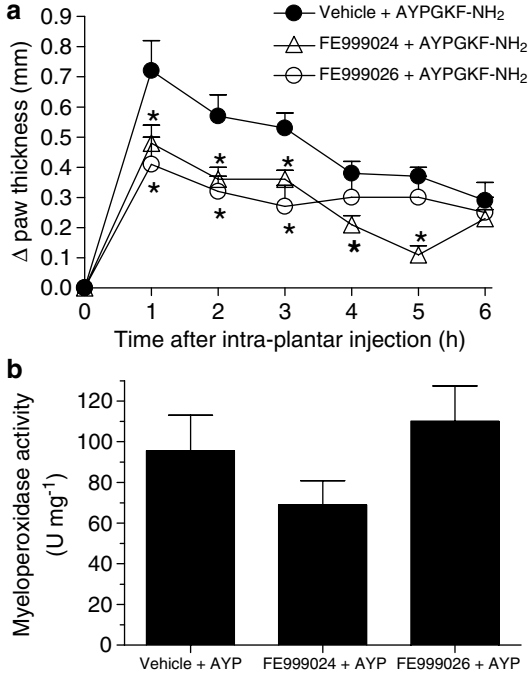


**Figure 5** Role for the tissue and plasma kallikreins in $PAR_4$-mediated oedema formation in the mouse paw. Effect of FE999024 (tissue kallikrein inhibitor, $60\,\mu mol\,kg^{-1}$) or FE999026 (plasma kallikrein inhibitor, $60\,\mu mol\,kg^{-1}$) on the oedema formation (a) and granulocyte recruitment (b) observed following $PAR_4$ activation with AYPGKF-$NH_2$ ($50\,\mu g$). Values are mean ± s.e.m. of $n = 8$ per group. *Significantly different from the vehicle + AYPGKF-$NH_2$ group, $P < 0.05$.

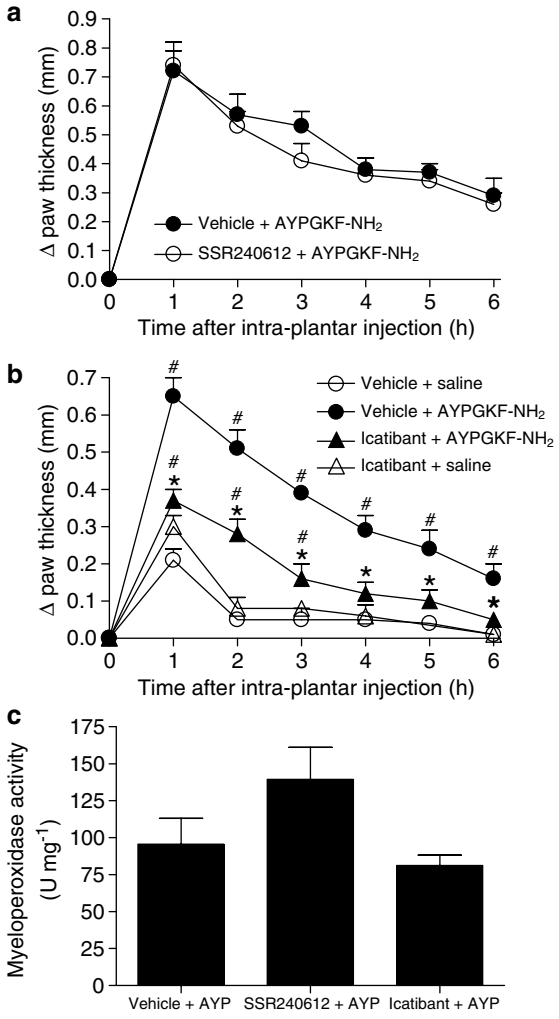


**Figure 6** Role for the kinin $B_2$ receptor, but not the $B_1$, in $PAR_4$-mediated oedema formation in the mouse paw. Effect of SSR240612 (kinin $B_1$ receptor antagonist, $10\,mg\,kg^{-1}$) or of icatibant (kinin $B_2$ receptor antagonist, $50\,\mu g\,kg^{-1}$) on the oedema formation (a and b, respectively) and granulocyte recruitment (c) observed following $PAR_4$ activation with AYPGKF-$NH_2$ ($50\,\mu g$). Values are mean ± s.e.m. of $n=8$ per group. *Significantly different from the vehicle + AYPGKF-$NH_2$ group, $P<0.05$; #significantly different from either the vehicle + saline or the icatibant + saline groups, $P<0.05$.

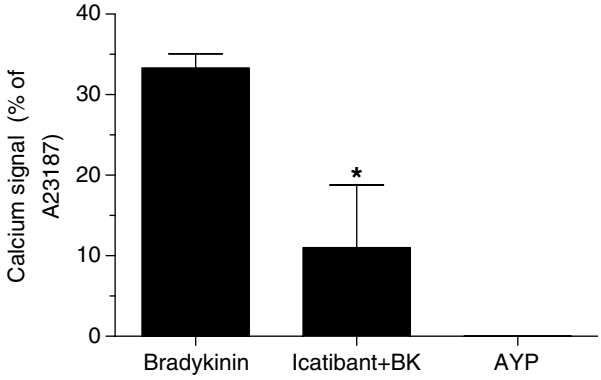


**Figure 7** The $PAR_4$-AP, AYPGKF-$NH_2$, does not activate directly the kinin $B_2$ receptor. Calcium signal emission was recorded at 530 nm in KNRK cells following stimulation with bradykinin (10 nM) or AYPGKF-$NH_2$ ($200\,\mu M$). Some cells were pretreated with icatibant (kinin $B_2$ receptor antagonist; 30 nM). Values are mean ± s.e.m. of $n=3$ experiments and are expressed as the percentage of the fluorescence peak height yielded by the addition of $2\,\mu M$ calcium ionophore (A23187). *Significantly different from the bradykinin group, $P<0.05$.

induced oedema, particularly within the first hour of the oedema response. Whether or not the $PAR_4$-triggered activation of platelets might also play some role in the neutrophil activation process represents an important topic for our work in the future. The neutrophils rapidly recruited to the site of inflammation undoubtedly release a number of inflammatory mediators that contribute to oedema (see our proposed model in Figure 8). In this regard, we identified components of the kallikrein–kinin system as the potential mediators linking neutrophil recruitment to oedema formation (Figure 8). Indeed, inhibitors of both plasma and tissue kallikreins reduced the formation of oedema to the same extent as did the depletion of neutrophils. Neutrophils are known to possess all of the components of the kallikrein–kinin system: (1) tissue and plasma kallikreins, (2) high and low molecular weight kininogens and (3) the kinin $B_1$ and $B_2$ receptors (Figueroa *et al*., 1989; Gustafson *et al*., 1989; Henderson *et al*., 1994; Rajasekariah *et al*., 1997). Since thrombin can increase the release of kallikrein activity by neutrophils (Cohen *et al*., 1991) and considering that kallikreins are involved in the oedema triggered by $PAR_4$, our results support the hypothesis that $PAR_4$ could be the target responsible for thrombin-induced kallikrein release at the site of inflammation.

Given that our work links kallikrein activity to $PAR_4$-induced oedema, we suggest that active kinins are produced locally from the cleavage of kininogens and could thereby activate local kinin receptors. In keeping with this hypothesis, blockade of the kinin $B_2$ receptor led to a reduction in oedema comparable to that caused by either neutrophil depletion or the kallikrein inhibitors. This result strongly suggests that endothelial cell kinin $B_2$ receptor activation, caused by locally produced kinins, is responsible for a large proportion of $PAR_4$-mediated oedema (Figure 8). It is now recognized that activated neutrophils are able to produce biologically active kinins from kininogens (Stuardo *et al*., 2004). Moreover, supporting this indirect activation of the $B_2$ receptor by newly produced kinins following $PAR_4$ stimulation is the observation that the $PAR_4$-AP AYPGKF-$NH_2$ failed to induce a calcium response through the $B_2$ receptor. Interestingly, the lack of effect of the $B_1$ receptor antagonist showed that the kinin $B_1$ receptor, which expression is induced by inflammatory mediators (Marceau *et al*., 1998), plays no role in the $PAR_4$-mediated oedema response. Added to the importance of the kallikrein–kinin system, the contact system (in which plasma kallikrein and high molecular weight kininogen are major components) present at the surface of endothelial cells would also be activated in the course of $PAR_4$-induced inflammation (Figure 8). This activation would occur as soon as there is a breach in the endothelium to allow plasma extravasation (Colman & Schmaier, 1997).

Since a residual oedema response is still observed after either neutrophil depletion or kallikrein–kinin system inhibition, we also propose that $PAR_4$ activation on endothelial cells could mediate signalling events increasing vascular permeability

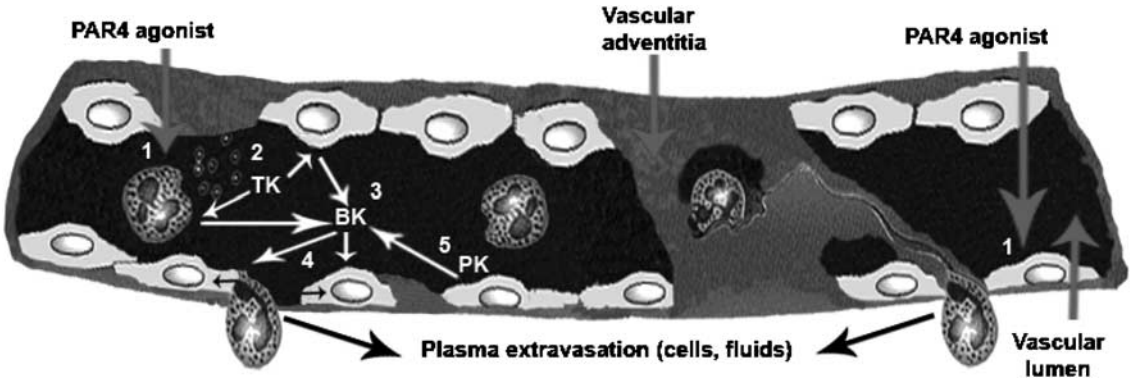


**Figure 8** Proposed model for the mechanisms of action of $PAR_4$-mediated oedema formation. In the blood vessel depicted schematically, we propose that $PAR_4$ activation could occur both on endothelial cells and neutrophils to affect vascular permeability (1). Activated neutrophils would release tissue kallikrein activity (2). Tissue kallikrein would then cleave kininogens found at the surface of neutrophils and endothelial cells to produce active kinins (3). These kinins would in turn bind to and activate endothelial $B_2$ receptors to increase vascular permeability, resulting in oedema and the migration of neutrophils into the tissue (4). With the breach of endothelial integrity, plasma kallikrein could then add to the response following activation of the contact system (5). TK, tissue kallikrein; PK, plasma kallikrein; BK, bradykinin.

(Figure 8). $PAR_4$ was detected at the surface of leukocytes, endothelial cells and smooth muscle cells (Vergnolle *et al*., 2002), suggesting that $PAR_4$ could be activated both on leukocytes and endothelial cells to induce inflammatory signs. Further, multiple signalling pathways are triggered in endothelial cells of different origins following $PAR_4$ agonist exposure (Kataoka *et al*., 2003). The combined activation of $PAR_4$ and the kinin $B_2$ receptor on endothelial cells could explain the magnitude of the response observed as well as its sustained duration. Indeed, the kinetics of desensitization for both receptors could explain the time course of oedema formation. The $B_2$ receptor is a receptor known to be desensitized rapidly by G protein-coupled receptor kinases (Blaukat *et al*., 2001). $PAR_4$ appears to be slowly desensitized by a mechanism that is still unclear (Shapiro *et al*., 2000). The effects of the inhibitors of the kallikrein–kinin system are more evident within the first hour, underscoring the importance in early oedema events of the $B_2$ receptor, before its desensitization. The residual sustained response is more likely the result of a direct activation of $PAR_4$ on endothelial cells which could signal for hours.

In conclusion, our data illustrate that $PAR_4$ participates in the inflammatory response by mediating at least two of the hallmarks of inflammation: oedema and granulocyte recruitment. Both neutrophils and the kallikrein–kinin system appear to be key mediators in the oedema response mediated by $PAR_4$.

We are grateful to Dr Dennis McMaster, director of the University of Calgary Peptide Synthesis Facility (peplab@ucalgary.ca), for provision of the synthetic peptides. We thank Kevin Chapman, Laurie Cellars, Zhenguo Yu and Bernard Renaux for their expert technical assistance and Linda Nadeau for her help with the scheme describing our inflammation model (Figure 7). We also thank Dr D. Michael Evans (Ferring Research Ltd) and Dr Denis Riochet (Sanofi-Synthelabo Recherche) for their gifts of inhibitors of the kallikrein–kinin system. This work was supported by Canadian Institutes of Health Research (CIHR) Proteinases and Inflammation Group Grant, by CIHR operating grant funds (M.D.H. and N.V.) and by a Servier International Alliance Stratégique grant (to M.D.H. and N.V.). S.H. is the recipient of a postdoctoral fellowship from the Alberta Heritage Foundation for Medical Research and Neuroscience Canada. N.V. is an Alberta Heritage Foundation for Medical Research Scholar and a Canadian Institutes for Health Research Investigator.

## References

ASFAHA, S., BRUSSEE, V., CHAPMAN, K., ZOCHODNE, D.W. & VERGNOLLE, N. (2002). Proteinase-activated receptor-1 agonists attenuate nociception in response to noxious stimuli. *Br. J. Pharmacol.,* **135,** 1101–1106.

ASOKANANTHAN, N., GRAHAM, P.T., FINK, J., KNIGHT, D.A., BAKKER, A.J., MCWILLIAM, A.S., THOMPSON, P.J. & STEWART, G.A. (2002). Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. *J. Immunol.,* **168,** 3577–3585.

BLAUKAT, A., PIZARD, A., BREIT, A., WERNSTEDT, C., HENC-GELAS, F., MULLER-ESTERL, W. & DIKIC, I. (2001). Determination of bradykinin B2 receptor *in vivo* phosphorylation sites and their role in receptor function. *J. Biol. Chem.,* **276,** 40431–40440.

BONDER, C.S., AJUEBOR, M.N., ZBYTNUIK, L.D., KUBES, P. & SWAIN, M.G. (2004). Essential role for neutrophil recruitment to the liver in concanavalin A-induced hepatitis. *J. Immunol.,* **172,** 45–53.

CAMERER, E., HUANG, W. & COUGHLIN, S.R. (2000). Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 5255–5260.

COHEN, W.M., WU, H.F., FEATHERSTONE, G.L., JENZANO, J.W. & LUNDBLAD, R.L. (1991). Linkage between blood coagulation and inflammation: stimulation of neutrophil tissue kallikrein by thrombin. *Biochem. Biophys. Res. Commun.,* **176,** 315–320.

COLMAN, R.W. & SCHMAIER, A.H. (1997). Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood,* **90,** 3819–3843.

COMPTON, S.J., RENAUX, B., WIJESURIYA, S.J. & HOLLENBERG, M.D. (2001). Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. *Br. J. Pharmacol.,* **134,** 705–718.

COTTRELL, G.S., AMADESI, S., GRADY, E.F. & BUNNETT, N.W. (2004). Trypsin IV: a novel agonist of protease-activated receptors 2 and 4. *J. Biol. Chem.,* **279,** 13532–13539.

COVIC, L., MISRA, M., BADAR, J., SINGH, C. & KULIOPULOS, A. (2002). Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat. Med.,* **8,** 1161–1165.

DE GARAVILLA, L., VERGNOLLE, N., YOUNG, S.H., ENNES, H., STEINHOFF, M., OSSOVSKAYA, V.S., D'ANDREA, M.R., MAYER, E.A., WALLACE, J.L., HOLLENBERG, M.D., ANDRADE-GORDON, P. & BUNNETT, N.W. (2001). Agonists of proteinase-activated receptor 1 induce plasma extravasation by a neurogenic mechanism. *Br. J. Pharmacol.,* **133,** 975–987.

DECARIE, A., ADAM, A. & COUTURE, R. (1996). Effects of captopril and Icatibant on bradykinin (BK) and des [Arg9] BK in carrageenan-induced edema. *Peptides,* **17,** 1009–1015.

EVANS, D.M., JONES, D.M., PITT, G.R., ASHWORTH, D., DE, C.F., VERHEYEN, F. & SZELKE, M. (1996a). Synthetic inhibitors of human tissue kallikrein. *Immunopharmacology,* **32,** 117–118.

EVANS, D.M., JONES, D.M., PITT, G.R., SUEIRAS-DIAZ, J., HORTON, J., ASHWORTH, D., OLSSON, H. & SZELKE, M. (1996b). Selective inhibitors of plasma kallikrein. *Immunopharmacology,* **32,** 115–116.

FARUQI, T.R., WEISS, E.J., SHAPIRO, M.J., HUANG, W. & COUGHLIN, S.R. (2000). Structure function analysis of protease activated receptor 4 tethered ligand peptides: determinants of specificity and utility in assays of receptor function. *J. Biol. Chem.,* **275,** 19728–19734.

FIGUEROA, C.D., MACIVER, A.G. & BHOOLA, K.D. (1989). Identification of a tissue kallikrein in human polymorphonuclear leucocytes. *Br. J. Haematol.,* **72,** 321–328.

GOUGAT, J., FERRARI, B., SARRAN, L., PLANCHENAULT, C., PONCELET, M., MARUANI, J., ALONSO, R., CUDENNEC, A., CROCI, T., GUAGNINI, F., URBAN-SZABO, K., MARTINOLLE, J.P., SOUBRIE, P., FINANCE, O. & LE, F.G. (2004). *SSR*240612 [(2*R*)-2-[((3*R*)-3-(1,3-benzodioxol-5-yl)-3-[[(6-methoxy-2-naphthyl)-sulfonyl]amino]propanoyl)amino]-3-(4-[[2*R*,6*S*)-2,6-dimethylpiperidinyl]methyl]phenyl)-*N*-isopropyl-*N*-methylpropanamide hydrochloride], a new nonpeptide antagonist of the bradykinin B1 receptor: biochemical and pharmacological characterization. *J. Pharmacol. Exp. Ther.,* **309,** 661–669.

GRIESBACHER, T., RAINER, I., TIRAN, B. & EVANS, D.M. (2002). Involvement of tissue kallikrein but not plasma kallikrein in the development of symptoms mediated by endogenous kinins in acute pancreatitis in rats. *Br. J. Pharmacol.,* **137,** 692–700.

GUSTAFSON, E.J., SCHMAIER, A.H., WACHTFOGEL, Y.T., KAUFMAN, N., KUCICH, U. & COLMAN, R.W. (1989). Human neutrophils contain and bind high molecular weight kininogen. *J. Clin. Invest.,* **84,** 28–35.

GUZIK, T.J., KORBUT, R. & MEK-GUZIK, T. (2003). Nitric oxide and superoxide in inflammation and immune regulation. *J. Physiol. Pharmacol.,* **54,** 469–487.

HENDERSON, L.M., FIGUEROA, C.D., MULLER-ESTERL, W. & BHOOLA, K.D. (1994). Assembly of contact-phase factors on the surface of the human neutrophil membrane. *Blood,* **84,** 474–482.

HESTDAL, K., RUSCETTI, F.W., IHLE, J.N., JACOBSEN, S.E., DUBOIS, C.M., KOPP, W.C., LONGO, D.L. & KELLER, J.R. (1991). Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J. Immunol.,* **147,** 22–28.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, S., BREIPOHL, G., KONIG, W. & KNOLLE, J. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.,* **102,** 769–773.

HOLLENBERG, M.D. & COMPTON, S.J. (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol. Rev.,* **54,** 203–217.

HOLLENBERG, M.D., SAIFEDDINE, M., SANDHU, S., HOULE, S. & VERGNOLLE, N. (2004). Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists *in vivo*. *Br. J. Pharmacol.,* **143,** 443–454.

ISHIHARA, H., CONNOLLY, A., ZENG, D., KAHN, M., ZHENG, Y., TIMMONS, C., TRAM, T. & COUGHLIN, S. (1997). Protease-activated receptor-3 is a second thrombin receptor in humans. *Nature,* **386,** 502–506.

KAHN, M., ZHENG, Y., HUANG, C., BIGORNIA, V., ZENG, D., MOFF, S., FARESE, R., TAM, C. & COUGHLIN, S. (1998). A dual thrombin receptor system for platelet activation. *Nature,* **394,** 690–694.

KATAOKA, H., HAMILTON, J.R., MCKEMY, D.D., CAMERER, E., ZHENG, Y.W., CHENG, A., GRIFFIN, C. & COUGHLIN, S.R. (2003). Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood,* **102,** 3224–3231.

LEVY, L. (1969). Carrageenan paw edema in the mouse. *Life Sci.,* **8,** 601–606.

MARCEAU, F., HESS, J.F. & BACHVAROV, D.R. (1998). The B1 receptors for kinins. *Pharmacol. Rev.,* **50,** 357–386.

MULE, F., PIZZUTI, R., CAPPARELLI, A. & VERGNOLLE, N. (2004). Evidence for the presence of functional protease activated receptor 4 (PAR(4)) in the rat colon. *Gut,* **53,** 229–234.

NYSTEDT, S., EMILSSON, K., LARSSON, A.K., STROMBECK, B. & SUNDELIN, J. (1995). Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur. J. Biochem.,* **232,** 84–89.

OSSOVSKAYA, V.S. & BUNNETT, N.W. (2004). Protease-activated receptors: contribution to physiology and disease. *Physiol. Rev.,* **84,** 579–621.

RAJASEKARIAH, P., WARLOW, R.S. & WALLS, R.S. (1997). High affinity bradykinin binding to human inflammatory cells. *Biochem. Mol. Biol. Int.,* **43,** 279–290.

SAMBRANO, G.R., HUANG, W., FARUQI, T., MAHRUS, S., CRAIK, C. & COUGHLIN, S.R. (2000). Cathepsin G activates protease-activated receptor-4 in human platelets. *J. Biol. Chem.,* **275,** 6819–6823.

SAMBRANO, G.R., WEISS, E.J., ZHENG, Y.W., HUANG, W. & COUGHLIN, S.R. (2001). Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature,* **413,** 74–78.

SCAPINI, P., LAPINET-VERA, J.A., GASPERINI, S., CALZETTI, F., BAZZONI, F. & CASSATELLA, M.A. (2000). The neutrophil as a cellular source of chemokines. *Immunol. Rev.,* **177,** 195–203.

SHAPIRO, M.J., WEISS, E.J., FARUQI, T.R. & COUGHLIN, S.R. (2000). Protease-activated receptors 1 and 4 are shutoff with distinct kinetics after activation by thrombin. *J. Biol. Chem.,* **275,** 25216–25221.

STEINHOFF, M., VERGNOLLE, N., YOUNG, S., TOGNETTO, M., AMADESI, S., ENNES, H., TREVISANI, M., HOLLENBERG, M.D., WALLACE, J.L., CAUGHEY, G., MITCHELL, S., WILLIAMS, L., GEPPETTI, P., MAYER, E. & BUNNETT, N. (2000). Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat. Med.,* **6,** 151–158.

STUARDO, M., GONZALEZ, C.B., NUALART, F., BORIC, M., CORTHORN, J., BHOOLA, K.D. & FIGUEROA, C.D. (2004). Stimulated human neutrophils form biologically active kinin peptides from high and low molecular weight kininogens. *J. Leukoc. Biol.,* **75,** 631–640.

USHIKUBI, F., SUGIMOTO, Y., ICHIKAWA, A. & NARUMIYA, S. (2000). Roles of prostanoids revealed from studies using mice lacking specific prostanoid receptors. *Jpn. J. Pharmacol.,* **83,** 279–285.

VERGNOLLE, N., DERIAN, C.K., D'ANDREA, M.R., STEINHOFF, M. & ANDRADE-GORDON, P. (2002). Characterization of thrombin-induced leukocyte rolling and adherence: a potential pro-inflammatory role for Proteinase-Activated Receptor-4 (PAR-4). *J. Immunol.,* **169,** 1467–1473.

VERGNOLLE, N., HOLLENBERG, M.D. & WALLACE, J.L. (1999a). Pro- and anti-inflammatory actions of thrombin: a distinct role for proteinase-activated receptor-1 (PAR1). *Br. J. Pharmacol.,* **126,** 1262–1268.

VERGNOLLE, N., HOLLENBERG, M.D., SHARKEY, K. & WALLACE, J.L. (1999b). Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR-2)-activating peptides in the rat paw. *Br. J. Pharmacol.,* **127,** 1083–1090.

VU, T., HUNG, D., WHEATON, V. & COUGHLIN, S. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell,* **64,** 1057–1068.

XU, W., ANDERSEN, H., WHITMORE, T., PRESNELL, S., YEE, D., CHING, A., GILBERT, T., DAVIE, E. & FOSTER, D. (1998). Cloning and characterization of human protease-activated receptor-4. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 6642–6646.

*(Received March 3, 2005*
*Revised May 26, 2005*
*Accepted July 18, 2005*
*Published online 15 August 2005)*