BJP British Journal of Pharmacology

DOI:10.1111/bph.12593
www.brjpharmacol.org

RESEARCH PAPER

Proteinase-activated receptors 1 and 2 and the regulation of porcine coronary artery contractility: a role for distinct tyrosine kinase pathways

Mahmoud El-Daly[1,2], Mahmoud Saifeddine[1,2], Koichiro Mihara[1,2], Rithwik Ramachandran[1,2], Christopher R Triggle[1,2,3] and Morley D Hollenberg[1,2,4]

[1]*Libin Cardiovascular Institute of Alberta and the Snyder Institute for Chronic Diseases, Calgary, AB, Canada,* [2]*Department of Physiology and Pharmacology, The University of Calgary Faculty of Medicine, Calgary, AB, Canada,* [3]*Departments of Pharmacology and Medical Education, Weill Cornell Medical College in Qatar, Doha, Qatar, and* [4]*Department of Medicine, The University of Calgary Faculty of Medicine, Calgary, AB, Canada*

Correspondence
Professor Morley D Hollenberg, Department of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1.
E-mail: mhollenb@ucalgary.ca

Keywords
angiotensin-II; coronary contraction; cyclooxygenase; EGF; MAPKinase; $PGF_{2\alpha}$; PARs; protease; proteinase-activated receptors; tyrosine kinase signalling pathways

Received
17 June 2013
Revised
23 December 2013
Accepted
17 January 2014

BACKGROUND AND PURPOSE
Because angiotensin-II-mediated porcine coronary artery (PCA) vasoconstriction is blocked by protein tyrosine kinase (PYK) inhibitors, we hypothesized that proteinase-activated receptors (PARs), known to regulate vascular tension, like angiotensin-II, would also cause PCA contractions via PYK-dependent signalling pathways.

EXPERIMENTAL APPROACH
Contractions of intact and endothelium-free isolated PCA rings, stimulated by PAR_1/PAR_2-activating peptides, angiotensin-II, $PGF_{2\alpha}$, EGF, PDGF and KCl, were monitored with/without multiple signalling pathway inhibitors, including AG-tyrphostins AG18 (non-specific PYKs), AG1478 (EGF-receptor kinase), AG1296 (PDGF receptor kinase), PP1 (Src kinase), U0126 and PD98059 (MEK/MAPKinase kinase), indomethacin/SC-560/NS-398 (COX-1/2) and L-NAME (NOS).

KEY RESULTS
AG18 inhibited the contractions induced by all the agonists except KCl, whereas U0126 attenuated contractions induced by PAR_1/PAR_2 agonists, EGF and angiotensin-II, but not by $PGF_{2\alpha}$, the COX-produced metabolites of arachidonate and KCl. PP1 only affected the responses to PAR_1/PAR_2-activating peptides and angiotensin-II. The EGF-kinase inhibitor, AG1478, attenuated contractions initiated by the PARs ($PAR_2 >> PAR_1$) and EGF itself, but not by angiotensin-II, $PGF_{2\alpha}$ or KCl. COX-1/2 inhibitors blocked the contractions induced by all the agonists, except KCl and $PGF_{2\alpha}$.

CONCLUSION AND IMPLICATIONS
$PAR_{1/2}$-mediated contractions of the PCA are dependent on Src and MAPKinase and, in part, involve EGF-receptor-kinase transactivation and the generation of a COX-derived contractile agonist. However, the PYK signalling pathways used by PARs are distinct from each other and from those triggered by angiotensin-II and EGF. These signalling pathways may be therapeutic targets for managing coagulation-proteinase-induced coronary vasospasm.

Abbreviations
Ang-II, angiotensin-II; ISO, isoprenaline; MEK, MAPKinase-kinase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PAR, proteinase-activated receptor; PAR-AP, PAR-activating peptide; PCA, porcine coronary artery; PDGFR, platelet-derived growth factor receptor; PYK, protein tyrosine kinase

Introduction

Proteinase-activated receptors (PARs; Alexander *et al.*, 2013a) are unusual members of the GPCR superfamily. Unique among other GPCRs, PARs have a built-in activation ligand [so-called, tethered ligand (TL)] that is unmasked by the action of certain proteinases. A serine proteinase (e.g. thrombin for PAR_1 and PAR_4 or trypsin for PAR_2) cleaves the extracellular N-terminus of the receptor and reveals a cryptic ligand sequence that has the ability to bind and activate its own receptor (Coughlin, 2000; Hollenberg and Compton, 2002; Ramachandran and Hollenberg, 2008; Adams *et al.*, 2011). Depending on the source of enzyme agonist, signalling via the PARs can be triggered by proteinases acting in an endocrine or paracrine fashion. Many of the members of the serine proteinase family, including thrombin, trypsin and kallikrein-related peptidases, as well as members of the MMP family, are potential PAR modulators. Interestingly, these proteinases are known to play roles in vascular biology and pathology, apart from regulating vascular tension. In addition, PARs have been shown to regulate vascular function in both normal and pathological conditions, including inflammation (Hollenberg and Houle, 2005; Steinhoff *et al.*, 2005; Hansen *et al.*, 2008; Ramachandran and Hollenberg, 2008; Adams *et al.*, 2011). Of importance, proteinases can modulate PAR function by cleaving at a site downstream from the 'canonical' TL sequence. This cleavage can either (i) 'silence' signalling by preventing receptor activation via a proteinase that would reveal the TL (Oikonomopoulou *et al.*, 2006a,b; Hollenberg *et al.*, 2008; Ramachandran and Hollenberg, 2008; Adams *et al.*, 2011) or alternatively (ii) stimulate 'biased signalling' by unmasking a 'non-canonical' TL sequence that selectively activates only a subset of signalling pathways (Ramachandran *et al.*, 2011; Mihara *et al.*, 2013). Thus, the vascular responses to PAR activation can be expected to differ, depending on the enzyme to which the vessel is exposed and the type of vessel in which the PARs are expressed.

In addition to the endogenous enzyme modulators, synthetic peptide ligands that mimic the exposed N-terminal amino acid TL sequences of these receptors can also activate PARs without the requirement of receptor proteolysis (Al-Ani *et al.*, 2002; Hollenberg and Compton, 2002; Hollenberg, 2003; Shpacovitch *et al.*, 2007; Alier *et al.*, 2008; Adams *et al.*, 2011). These PAR-selective agonist peptides (PAR-APs; e.g. TFLLR-NH_2, a PAR_1-selective receptor-activating peptide and 2-furoyl-LIGRLO-NH_2, a PAR_2-activating peptide) have proved to be valuable pharmacological tools to assess the potential effects of PAR activation without the other complicating effects that proteinases can cause (Barry *et al.*, 2006).

Activation of PARs on endothelial and/or smooth muscle cells leads to the modulation of vessel tone in various vascular beds. The classical signalling paradigm initiated by $PAR_{1/2}$ activation involves stimulation of Gq with a subsequent activation of phospholipase Cβ, resulting in increased cytoplasmic inositol trisphosphate, which, in turn mobilizes and increases cytosolic calcium concentrations. The increased cytosolic calcium induces vascular smooth muscle contraction through a calcium/calmodulin-regulated process. In contrast, in endothelial cells, increased intracellular calcium stimulated via $PAR_{1/2}$ activation routinely leads to the release of vasorelaxant agonists, including NO, prostanoids and endothelial-derived hyperpolarizing factor(s) (McGuire *et al.*, 2001; 2002; 2004a; Ramachandran and Hollenberg, 2008; Feletou and Vanhoutte, 2009). Importantly, thrombin, which causes a PAR_1-mediated relaxation in porcine coronary artery (PCA) preparations, can also cause relaxation via an apparently PAR_1-independent mechanism (Hamilton and Cocks, 2000).

The potential role of receptor (e.g. EGF receptor kinase) and non-receptor protein tyrosine kinases (PYKs) in GPCR-mediated smooth muscle contractile responses has been known for some time (Hollenberg, 1994; 1995; Laniyonu *et al.*, 1994; Touyz and Schiffrin, 2000). In particular, we observed that non-receptor tyrosine kinases can play a role in angiotensin-II-mediated contraction of porcine coronary tissue, which also contracts in response to EGF (Saifeddine *et al.*, 1992; Laniyonu *et al.*, 1994). Comparable non-receptor tyrosine kinases blocked by genistein and tryphostins are also involved in thrombin/PAR_1-mediated gastric smooth muscle contraction (Yang *et al.*, 1993; Zheng and Hollenberg, 1997; Zheng *et al.*, 1998). In some systems, GPCR-triggered effects have been found to involve the transactivation of the EGF receptor via a MMP-mediated process (Prenzel *et al.*, 1999; Gschwind *et al.*, 2001). This extracellular mechanism of EGF receptor transactivation (involving MMP activity) has been shown to be activated by PARs in some systems (Kalmes *et al.*, 2000; Sekiguchi *et al.*, 2007). In other situations, non-MMP intracellular, rather than extracellular pathways, are involved in EGF receptor transactivation (Gschwind *et al.*, 2001; Kawao *et al.*, 2005; Caruso *et al.*, 2006). However, it is not yet known if these mechanisms are involved in PAR-mediated vascular effects. Since we previously found that non-receptor tyrosine kinase inhibitors can block the contractile actions of angiotensin-II and EGF in porcine coronary preparations (Saifeddine *et al.*, 1992; Laniyonu *et al.*, 1994), we hypothesized that tyrosine kinase signalling pathways (see Alexander *et al.*, 2013b) would also be involved in the contractile activities of PARs in this tissue. To test this hypothesis, contractions of isolated PCA rings with and without a functional endothelium in response to PAR_1- and PAR_2-activating peptide agonists as well as to $PGF_{2\alpha}$, EGF and KCl were monitored in the absence and presence of several signalling pathway inhibitors, including PP1 (a Src family selective kinase inhibitor), tyrphostins AG18 (a non-selective tyrosine kinase inhibitor), AG23, AG1478 (a selective EGF receptor kinase inhibitor), AG-1478, AG1296 [platelet-derived growth factor receptor (PDGFR)-selective inhibitor], AG-1296, and L-NAME (N^{ω}-nitro-L-arginine methyl ester; NOS inhibitor). In addition, signalling pathway inhibitors of MEK/MAPKinase-kinase, PD98059 and U0126, as well as inhibitors of COXs-1/2 (indomethacin/SC560/NS398) were used to test the mechanisms of PAR-mediated contractions. Our aim was mainly to identify signalling pathways involved in PAR-mediated contractions and to establish whether or not these same pathways might affect endothelium-dependent relaxation. We also sought to determine the possible differences in these pathways in the coronary artery preparation: (i) between those triggered by activation of PAR_1 compared to PAR_2 and (ii) between those mediated by activation of the PARs and those triggered by angiotensin-II and $PGF_{2\alpha}$.

Methods

Tissue collection

Hearts of locally slaughtered pigs were collected directly after they had been killed, immersed in isotonic ice-cold physiological Krebs–Henseleit solution, which was used as a transfer buffer, with the following composition (in mM): NaCl, 115; KC1, 4.7; $CaCl_2$, 2.5; $MgC1_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; and dextrose, 10.0, previously equilibrated with 95% O_2 and 5% CO_2 (Carbogen gas) and adjusted to pH 7.4, and transported directly to the laboratory within 2 h of tissue collection. Immediately thereafter, the left anterior descending coronary arteries were dissected and carefully trimmed free of fat and adventitial tissue. The vascular tissues were either used immediately or were stored for one to two nights at 4°C in a 25 mM HEPES-fortified isotonic Krebs–Henseleit buffer adjusted to pH 7.4. Arterial sections from the middle part of the PCA, with an outer diameter between ~2.5 and 3.5 mm were cut into rings (~5 mm long) and used for the bioassay experiments as previously described (Hamilton and Cocks, 2000; Glusa and Adam, 2001).

Bioassay procedures

Monitoring vascular contractions and relaxations. The PCA rings were vertically mounted between two triangular stainless steel hooks in organ baths containing 4 mL of the above-mentioned Krebs–Henseleit buffer (pH 7.4) at 37°C and continuously aerated with Carbogen gas. All vascular preparations were equilibrated for at least 60 min before the start of the experiments under a resting tension of 3 g; this was determined to be optimum for contractility studies for this preparation in our laboratory. The physiological solution was changed at 20 min intervals and tension was re-adjusted to 3.0 g when necessary. Isometric force-displacement transducers (MLT0201/D; ADInstruments, Sydney, NSW, Australia) connected to a Powerlab/8S data acquisition system and Chart® software (Chart 5 for Windows©, ADInstruments Pty Ltd, Sydney, NSW, Australia) were used to measure changes in tension (g).

Tissue functionality was verified by monitoring the contractile response stimulated by 80 mM KCl. Tissues were used only when a consistent and reproducible contractile response was obtained over a 1 h period. After each experimental step, coronary artery rings were washed three times with physiological Krebs–Henseleit buffer and allowed to re-equilibrate for at least 20 min under a resting tension of 3 g before starting the next step. The integrity of the endothelial layer, or its absence as in endothelium-denuded rings, was tested by exposing tissues that had been constricted with 2 μM $PGF_{2\alpha}$, to substance P (SP; 20 nM) and monitoring a maximal relaxant effect. Similarly, the relaxation responses induced by the PAR-APs were monitored in rings that were first constricted with 2 μM of $PGF_{2\alpha}$. PAR-APs were applied only once (to avoid desensitization) to each constricted tissue sample in concentrations ranging from 10 nM to 10 μM. Relaxant responses for each peptide were calculated as a percentage (% SP) of the maximal relaxation induced by 20 nM of SP in each PCA preparation.

Contractile responses to the various GPCR agonists used in this study were expressed as a percentage (% KCl) of the contraction induced by 80 mM KCl in each preparation. Since contractile responses were shown to be independent of the endothelium, we minimized tissue damage in our study by conducting most experiments, where we were determining agonist-induced contractions, in endothelium-intact preparations treated with 100 μM L-NAME. Tissues at baseline tension (3 g) were exposed to different concentrations of the PAR-APs. In a preliminary study, we found that PAR-mediated contractile responses exhibited desensitization upon multiple exposures of the tissues to the PAR-APs. In particular, the contractile response to the PAR_2-activating peptides (SLIGRL-NH_2; 2-furoyl-LIGRLO-NH_2) was almost totally desensitized, not returning even after more than 2 h of equilibration. The response to the PAR_1-activating peptide also desensitized, but did recover almost completely after an hour of re-equilibration. Thus, concentration–effect curves were obtained as previously described (Laniyonu and Hollenberg, 1995); a single PAR-AP-induced contractile response was measured after monitoring a response to 80 mM KCl. Data obtained for different concentrations of the PAR-APs were obtained from at least three independently prepared coronary arteries and were pooled to establish concentration–effect curves for PAR-APs.

To assess the effects of various signalling pathway inhibitors on the contractile responses to the GPCR agonists used in this study, the corresponding inhibitor was added to each tissue preparation after washing, followed by pre-incubation with the inhibitor for 20 min before an agonist was added. Equivalent volumes of the vehicles used to dissolve each pathway inhibitor were added to organ baths that were not treated with the inhibitor. All responses were calculated as % KCl, as previously mentioned; and the effects of an agonist in the presence of a certain inhibitor were expressed as a percentage (% control) of the contractile response observed in the vehicle-treated preparations. The concentrations of the pathway inhibitors used were selected by verifying that they did not affect KCl-induced contractions (see Saifeddine *et al.*, 1992; Laniyonu *et al.*, 1994), in keeping with concentrations of the inhibitors that are selective for the targeted enzymes, as employed routinely in the published literature. Furthermore, we established that except for AG18, the inhibitors did not affect contractions induced by 1 μM $PGF_{2\alpha}$. The caution with which the data were obtained for the inhibitors that we used is much appreciated, and was taken into account for our work (Bain *et al.*, 2007; Cohen, 2010).

In all cases, after the tension had reached a plateau subsequent to agonist addition, a wash procedure was applied three times to remove all agonists from the tissue bath and the tissues were re-equilibrated for 20 min in fresh physiological Krebs–Henseleit buffer (pH 7.4) before exposure to other agonists. Amastatin (5 μM) was added to the organ bath when evaluating the effects of peptides, to inhibit any possible breakdown of PAR-APs by tissue aminopeptidase activity.

Measuring activation of MAPKinase in intact tissues stimulated by PAR-APs and EGF by Western blot analysis of increases in phospho-MAPKinase

Coronary tissue was dissected and cut into rings, as for the bioassay and placed in the organ bath, but not under tension. Agonists (EGF, TFLLR-NH_2) and inhibitor compounds (U0126, AG1478) were then added, as per the bioassay

protocol. Thus, rings were pre-incubated for 20 min in the absence or presence of inhibitor and were then exposed to agonists (TFLLR-NH_2, 5 μM; EGF, 17 nM) for 5 min, at which time samples were quick-frozen in liquid nitrogen and stored at –80°C for further processing. To extract protein, rings were quick-thawed in a 2 mL capped microfuge tube containing 50 μL of hypotonic lysis buffer containing phosphatase and proteinase inhibitors (Mihara *et al.*, 2013) along with 0.2 mm stainless steel beads for percussion extraction using a BBX24 Bullet Blender homogenizer (2 × 5 min homogenization cycles at 4°C; Next Advance Inc., Averill Park, NY, USA). Tissue extracts were clarified by centrifugation in a microfuge, and 2 μL aliquots were analysed for protein content (Pierce© Bicinchoninic Acid Protein Assy Kit, Thermo Scientific, Rockford, IL, USA). Immediately thereafter, aliquots of 2X-concentrated Laemmli electrophoresis buffer (of volume equal to the extracted protein aliquots) were added to the protein extracts and boiled for 3 min. Equal volumes (10 μL) of the resultant denatured protein samples were loaded for gel electrophoresis and Western blot detection of phospho-MAPKinase and GAPDH, essentially as described previously (Mihara *et al.*, 2013), using antisera obtained from Cell Signaling Technology (Danvers, MA, USA). To compare MAPKinase activation between samples, the p42-p44 MAPKinase signal for agonist-treated tissues (with or without added inhibitor), normalized to the GAPDH blot signal, was expressed as a percentage (% Control; Figure 9) of the GAPDH-normalized MAPKinase signal observed for untreated tissues used in parallel with the treated samples. The open software ImageJ (version 1.48f, obtained from http://imagej.nih.gov/ij/download/) was used for densitometry.

Chemicals and other reagents

The PAR-APs TFLLR-NH_2 2-furoyl-LIGRLO-NH_2, AYPGKF-NH_2 and SLIGRL-NH_2, as well as the reverse-sequence, PAR-inactive peptides RLLFT-NH_2, LSIGRL-NH_2 and 2-furoyl-OLRGIL-NH_2 were synthesized at the University of Calgary, Health Sciences Centre peptide synthesis unit (peplab@ucalgary.ca). SP was from Sigma (St Louis, MO, USA). All peptides were dissolved in 25 mM HEPES buffer (pH 7.4). All other chemicals were dissolved in manufacturer's recommended solvents. Ang-II, $PGF_{2\alpha}$, HEPES, L-NAME, indomethacin, PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; (5Z,9α,11α,13E,15S)-9,11,15-trihydroxyprosta-5,13-dienoic acid], arachidonic acid, amastatin, isoprenaline-HCl, $CaCl_2$ and $NaHCO_2$ were from Sigma. The Src-selective tyrosine kinase inhibitor, PP1 (3-(4-methylphenyl)-1-(2-methyl-2-propanyl)-1*H*-pyrazolo [3,4-d]pyrimidin-4-amine), was purchased from Biomol (Enzo Life Sciences Inc., Farmingdale, NY, USA). AG18 [(3,4-dihydroxybenzylidene)malononitrile], AG1478 (N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine hydrochloride), AG1296 (6,7-dimethoxy-2-phenyl-quinoxaline) and human recombinant EGF were from Calbiochem (Calbiochem© of EMD Millipore, Merck KGaA, Darmstadt, Germany). U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene), BB94 (Batimastat; (2*R*,3*S*)-N4-hydroxy-N1-[(1*S*)-2-(methylamino)-2-oxo-1-(phenylmethyl)ethyl]-2-(2-methylpropyl)-3-[(2-thienylthio)methyl]butanediamide), SC-560 [a highly selective COX-1 inhibitor, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole] and NS-398 [a selective COX-2 inhibitor, *N*-[2-cyclohexyloxy-4-nitrophenyl]methane-sulfonamide] were from Tocris (Cedarlane Corp., Burlington, ON, Canada). The concentrations of enzyme inhibitors used were in keeping with those used for other published studies and in accord with the recommendations of Cohen and colleagues for optimizing enzyme selectivity, as mentioned previously (Bain *et al.*, 2007; Cohen, 2010). Anhydrous dextrose was purchased from EMD Chemicals (EMD Millipore, Merck KGaA); magnesium chloride was from Anachemia Sciences (Vancouver, BC, Canada). Sodium chloride, potassium chloride and potassium phosphate monobasic were purchased from BDH (Toronto, ON, Canada).

Statistical analysis

Data points represent the mean ± SEM of data pooled from at least three independent tissue preparations. Concentration–response curves were obtained by fitting the data into sigmoidal curves using the GraphPad Prism (version 6.03 for Windows; GraphPad Software, San Diego, CA, USA; http://www.graphpad.com). Statistical comparisons between data points were made using either Student's unpaired *t*-test when comparing two groups or a one-way ANOVA, with Tukey's multiple comparison test when comparing multiple groups. In all cases, a significant difference was considered at $P < 0.05$.

Results

PAR-APs regulate vascular function of the PCA

Activation of PAR_1 and PAR_2 induces endothelium-dependent relaxations. In the preliminary study, we verified the ability of PAR activation to cause a relaxant response in $PGF_{2\alpha}$-constricted tissues, in keeping with previous observations (Hamilton and Cocks, 2000). As shown in Figure 1, both the PAR_2-AP, SLIGRL-NH_2 (tracing A, Figure 1) and the PAR_1-AP, TFLLR-NH_2 (tracing B, Figure 1) caused a prompt relaxation. Unlike the PAR-AP used previously by Hamilton and Cocks (2000) (SFLLRN), TFLLR-NH_2 is selective for PAR_1 and does not activate PAR_2 at the concentrations we used (Kawabata *et al.*, 1999). Of note, desensitization of the PAR_2 response by repeated exposure of the tissue to SLIGRL-NH_2 did not desensitize the tissue to the action of the PAR_1 agonist, TFLLR-NH_2 (right-hand portion of Figure 1A), which relaxed the preparation, as did SP (20 nM); and similarly, desensitization of the tissue by repeated exposure to the PAR_1 agonist, TFLLR-NH_2, did not desensitize the tissue to the action of the PAR_2 agonist, SLIGRL-NH_2 (right-hand portion of Figure 1B). The relaxant responses to the PAR-APs were not observed in endothelium-denuded preparations that did not relax in response to SP (e.g. Figure 2C,D and not shown). Further, the relaxations induced by PAR-APs, as for SP, were significantly diminished (more than 50%) in the presence of the NOS inhibitor, L-NAME (100 μM; not shown). Importantly, indomethacin did not affect the relaxation responses induced by either SP or the PAR-APs, indicating that COX products were not involved (not shown).

Exposure of $PGF_{2\alpha}$-contracted, endothelium-intact PCA rings to the PAR_1 and PAR_2-APs resulted in concentration-dependent relaxation (Figure 1C). For the PAR_1-AP, TFLLR-NH_2 the EC_{50} for the relaxation was approximately 1 μM, with a maximal relaxant effect equivalent to that of SP (Figure 1C,

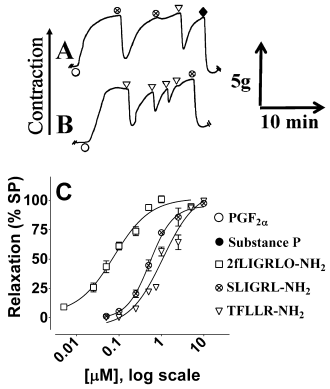

Figure 1

Relaxant responses to PAR-APs: representative tracings, lack of PAR_1/PAR_2 cross-desensitization and concentration–effect curves. (A) SLIGRL-NH_2 at 2 μM induced a rapidly desensitized endothelium-dependent relaxation without affecting TFLLR-NH_2 (1 μM)-induced relaxation in a $PGF_{2\alpha}$-constricted PCA ring that also relaxed in response to SP (20 nM). (B) Repeated exposure of the tissue to TFLLR-NH_2 (1 μM) leads to homologous desensitization of the PAR_1-mediated response without affecting relaxation induced by the PAR_2 agonist, SLIGRL-NH_2. (C) Concentration–effect curves for PAR-AP induced relaxations. Both of the PAR_2-APs 2fLIGRLO-NH_2 and SLIGRL-NH_2 and the PAR_1-AP, TFLLR-NH_2, induced concentration dependent relaxations. Data points were calculated as a percentage of SP (% SP)-induced relaxations. Data points represent mean ± SEM (bars) of at least three independent experiments.

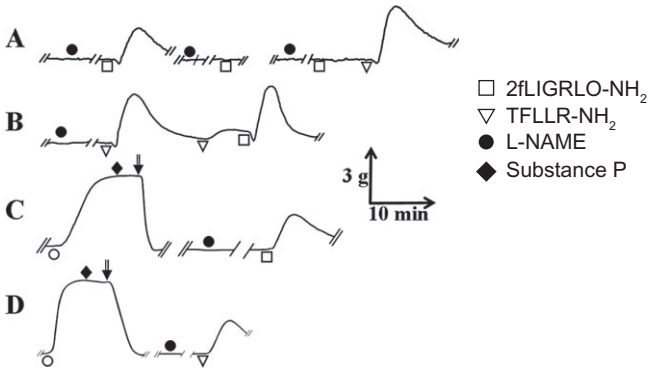

Figure 2

Contractile responses to PAR-APs: representative tracings, lack of PAR_1/PAR_2 cross-desensitization and endothelium independence. Trace A: 2fLIGRLO-NH_2 (10 μM) caused an intensely desensitizing contractile response, but did not affect the contractile response to TFLLR-NH_2 (5 μM) in the same tissue (right hand of trace A). Trace B: similarly, the desensitizing contractile response to TFLLR-NH_2 (5 μM) did not affect the subsequent contractile response to 2fLIGRLO-NH_2 (10 μM, right hand of trace B). Traces C and D: equivalent contractile responses were observed in endothelium-free tissues (no response to SP, 20 nM) treated with L-NAME (100 μM). However, the relaxation response induced by isoprenaline (ISO: 1 μM: heavy arrow) was not affected, indicating a fully functional tissue.

right-hand curve). The PAR_2-AP 2fLIGRLO-NH_2 was the most potent of the three PAR-APs evaluated, with an EC_{50} of 88 nM and a maximal effect also equivalent to that of SP (Figure 1C, left-hand curve). As expected from previous studies, where SLIGRL-NH_2 was found to be less potent than 2-furoyl-LIGRLO-NH_2 at activating PAR_2 (McGuire *et al.*, 2004b), SLIGRL-NH_2 was about 6.5-fold less potent than 2fLIGRLO-NH_2 at inducing relaxation in the PCA preparation, with an EC_{50} of 540 nM (Figure 1C, middle curve). Again, like the other PAR-APs, the maximum relaxant action of SLIGRL-NH_2 was equivalent to that of SP. Significantly, none of the reverse-sequence PAR-inactive peptides caused a response (not shown). This lack of response to reverse-sequence peptides (RLLFT-NH_2 for PAR_1; LSIGRL-NH_2 or 2-furoyl-OLRGIL-NH_2 for PAR_2) and the expected order of potency for PAR_2 activation by 2fLIGRLO-NH_2 >> SLIGRL-NH_2 indicated that PAR_1 and PAR_2 were responsible for the relaxant effects of the PAR-APs.

Surprisingly, we found that after completely desensitizing the relaxant response to the PAR_1 agonist, TFLLR-NH_2, thrombin was still able to cause a relaxation in a $PGF_{2\alpha}$-constricted tissue, presumably via a non-PAR_1-mediated mechanism (not shown). We thus concluded that the best approach to assess the responses of the tissue to PAR activation was to use the PAR-selective activating peptides since the responses to enzyme activation (thrombin; trypsin) could be misleading. Thus, to assess the actions of PAR_1 and PAR_2 in the coronary preparation further, all continuing work was performed with the PAR-selective activating peptides, TFLLR-NH_2 for PAR_1 and both 2-furoyl-LIGRLO-NH_2 and SLIGRL-NH_2 for PAR_2.

Lack of effects of kinase inhibitors on endothelium-dependent relaxations. As described below, we wished to evaluate the effect of a number of signalling pathway protein kinase inhibitors on the contractile responses stimulated by PAR activation, including the Src-selective tyrosine kinase inhibitor, PP1, the EGF receptor-kinase-selective inhibitor, AG1478, and the MEK inhibitor, U0126. As will be demonstrated in the following sections, all these inhibitors affected the PAR-triggered contractile responses, but none of them affected the endothelium-dependent relaxations caused either by the PAR-selective agonists (2-furoyl-LIGRLO-NH_2 for PAR_2; TFLLR-NH_2 for PAR_1) or by SP (not shown). The relaxant responses were essentially as shown in tracings A and B in Figure 1 either in the absence or presence of inhibitors. Given the lack of effect of these compounds on the relaxant responses, we then concentrated our work completely on the pharmacology of the contractile responses induced by the PAR-APs.

Activation of PAR_1 and PAR_2 induces endothelium-independent contractions. Both the PAR_1- and PAR_2-APs induced contractile responses that did not cross-desensitize (Figure 2A,B). The contractile response to the PAR_2 agonist was intensely desensitized in that the response did not return even after a 2 h re-equilibration period; at that time, the tissue did, nonetheless, contract in response to the PAR_1 agonist, TFLLR-NH_2 (Figure 2A). The response to PAR_1 activation did partially return after 30 min and was almost completely restored after 1 h (not shown). Nonetheless, its desensitization did not prevent a contraction stimulated by the PAR_2 peptide agonist (Figure 2B). Contractions induced by the PAR_1 and PAR_2 peptide agonists were independent of the endothelium since comparable contractions were observed in the PCA preparation either in endothelium-intact preparations treated with L-NAME (100 μM) or in the absence of a functional endothelium (Figure 2C,D). Thus, for efficiency, the

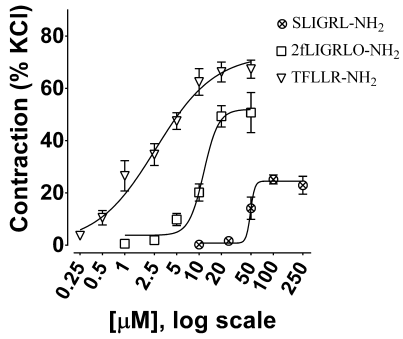

Figure 3

Concentration–effect curves for contractile responses to PAR_1- and PAR_2-activating peptides. Both of the PAR_2-APs, 2fLIGRLO-NH_2 and SLIGRL-NH_2, as well as the PAR_1-AP, TFLLR-NH_2, induced concentration-dependent contractions. Data points are calculated as a percentage of the contractile response to 80 mM KCl (% KCl) in the same tissue. Data points represent mean ± SEM of at least three independent experiments.

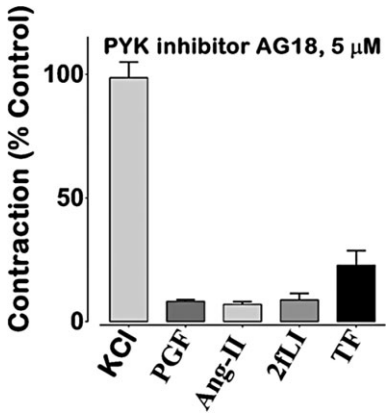

Figure 4

Effect of the non-selective tyrosine kinase (PYK) inhibitor AG18 on contractions induced by GPCR agonists in the PCA preparation. Contractile effects of all GPCR agonists used (PGF: $PGF_{2\alpha}$, 1 μM; Ang-II: angiotensin-II, 1 μM; 2fLI: 2fLIGRLO-NH_2, 10 μM; TF: TFLLR-NH_2, 5 μM) were significantly inhibited ($P < 0.01$) in tissues treated with 5 μM AG18 when compared with control-untreated tissues. All tissues were pretreated with L-NAME (100 μM). Histograms represent mean ± SEM (bars) of at least three independent experiments.

concentration–effect curves for the contractile actions of the PAR-activating peptides were obtained in endothelium-intact, L-NAME-treated preparations (Figure 3). The relative potencies of these peptides to induce contractile responses (Figure 3) were as follows: TFLLR-NH_2 (EC_{50} = 2.6 ± 1.4 μM) > 2fLIGRLO-NH_2 (EC_{50} = 12 ± 1 μM) > SLIGRL-NH_2 (46 ± 1 μM). As expected for a PAR_2-mediated response in this assay, 2fLIGRLO-NH_2 was four to five times more potent than SLIGRL-NH_2. Relative to 2fLIGRLO-NH_2, SLIGRL-NH_2 was a partial agonist in the contraction assay (Figure 3, right-hand curve), although both were full agonists in the relaxation assay (Figure 1C). Thus, 2fLIGRLO-NH_2 was used as a contractile PAR_2 agonist for all subsequent experiments. Furthermore, as for the relaxation responses, treating the PCA rings with any of the reverse-sequence, PAR-inactive peptides did not induce a contractile response (not shown). Additionally, the PAR_4-AP AYPGKF-NH_2 (up to 200 μM) had no effect on vascular contractility (data not shown). We thus concluded that in addition to causing a relaxant response in the porcine coronary preparation, as found previously, PAR_1 and PAR_2 peptide agonists also trigger a receptor-selective contractile response akin to responses induced by angotensin-II and $PGF_{2\alpha}$. Our attention thus turned to studying the signalling pathways responsible for the PAR-stimulated contractile responses, in comparison with those caused by angiotensin-II and $PGF_{2\alpha}$. We focused on tyrosine kinase-MAPKinase signalling and on the possible involvement of COX pathways that we had previously found to be involved in EGF, Ang-II and PAR-triggered contractile responses in gastric smooth muscle preparations (Yang *et al.*, 1993; Zheng and Hollenberg, 1997; Zheng *et al.*, 1998).

PAR-mediated contractions are blocked by tyrosine kinase inhibitors

The non-specific tyrosine kinase inhibitor, AG18, inhibits contractions induced by all agonists, without affecting KCl-stimulated contractions. To test the hypothesis that, as for Ang-II, the PAR-induced contractions were dependent on protein tyrosine kinases in the isolated PCA, tissues were pretreated with the non-selective tyrosine kinase inhibitor, tyrphostin AG18 (5 μM), in the organ bath for 20 min. This compound, originally designed as a pseudo-substrate tyrosine kinase inhibitor, non-selectively blocks a number of non-receptor and receptor tyrosine kinases (RTKs; Levitzki and Gazit, 1995; Levitzki and Mishani, 2006). AG18, at 5 μM, a concentration much lower than that found to block RTKs, significantly inhibited the contractile effects induced by all GPCR agonists including $PGF_{2\alpha}$, Ang-II and PAR-activating peptides in L-NAME-treated (100 μM) rings when compared with control untreated tissues (Figure 4). This result indicates the involvement of a number of non-receptor tyrosine kinases. To focus, we next used a selective inhibitor of the Src-family, PP1, that we had used in previous work (Hanke *et al.*, 1996; Zheng *et al.*, 1998).

The Src family-selective kinase inhibitor, PP1, attenuated contractions induced by PAR_1 and PAR_2 agonists as well as by Ang-II, without affecting $PGF_{2\alpha}$ contractions. Pre-equilibration of the L-NAME-treated (100 μM) tissues for 20 min with the selective Src kinase inhibitor PP1 showed differential effects on the contractile agonists used in this study (Figure 5). Responses mediated by PAR_1, PAR_2 and Ang-II were sensitive to PP1 at 1 μM, with inhibition of 50% or more, when compared with control untreated responses (Figure 5). However, at the same concentration of PP1, the contractile responses to EGF (100 ng·mL^{-1}; 17 nM) or $PGF_{2\alpha}$ (1 μM) were not significantly affected. The effects of PP1 were selective for signalling by the PAR-APs and angiotensin-II since this concentration did not affect contractions induced by 80 mM KCl, $PGF_{2\alpha}$ or EGF (Figure 5).

The EGF-receptor-kinase-targeted inhibitor, AG1478, partially blocks PAR-mediated contractions but not those induced by $PGF_{2\alpha}$ and Ang-II. Because GPCR activation can possibly trigger transactivation of the EGF receptor kinase (Daub *et al.*, 1996),

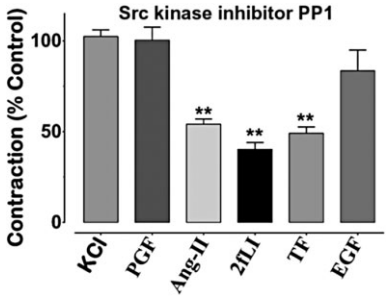

Figure 5

Effect of the Src-selective tyrosine kinase inhibitor PP1 (1 μM) on contractions induced by GPCR agonists and EGF in the PCA preparation. Only contractions induced by the PAR-APs and Ang-II were significantly inhibited ($P < 0.01$) in tissues treated with 1 μM of PP1 when compared to control untreated tissues. (PGF: $PGF_{2\alpha}$, 1 μM; Ang-II: angiotensin-II, 1 μM; 2fLI: 2fLIGRLO-NH_2, 10 μM; TF: TFLLR-NH_2, 5 μM; EGF, 17 nM/100 ng·mL^{-1}). $^{**}P < 0.01$ compared with controls. All tissues were pretreated with L-NAME (100 μM). Histograms represent mean ± SEM (bars) of at least three independent experiments.

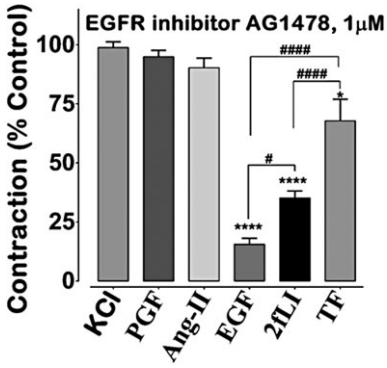

Figure 6

Effect of the selective EGF receptor kinase inhibitor, AG1478, on contractions induced by GPCR agonists and EGF in the PCA preparation. In tissues treated with 1 μM AG1478, significant inhibitions of contractions were observed in the responses to EGF (17 nM/100 ng·mL^{-1}) and 2fLIGRLO-NH_2 (10 μM) ($P < 0.0001$), as well as for TFLLR-NH_2 (5 μM) ($P < 0.05$) when compared with control untreated tissues. $^{*}P < 0.05$ and $^{****}P < 0.0001$ when compared with untreated controls. $^{\#}P < 0.05$, $^{\#\#\#\#}P < 0.0001$ denote significance level for differences between selected groups. All tissues were pretreated with L-NAME (100 μM). Histograms represent means ± SEM (bars) of three or more independent experiments carried out in different tissue preparations.

and because EGF can cause contraction of the PCA (Gan *et al.*, 1989; Saifeddine *et al.*, 1992), we evaluated a possible role for EGF receptor transactivation in triggering the PAR responses using the EGF-kinase-selective inhibitor, AG1478. In vascular rings pre-incubated with 1 μM AG1478, contractions induced by EGF were, as expected, inhibited (Figure 6). We did not wish to exceed this concentration of AG1478; at higher concentrations it will inhibit the EGF receptor kinase completely but can also affect kinases other than the EGF receptor. Thus, the contractile effect of EGF was substantially, but not completely, blocked by 1 μM AG1478. Nonetheless, for the GPCR agonists, only contractions induced by the PAR-APs were diminished, whereas responses to Ang-II and $PGF_{2\alpha}$ were not affected (Figure 6). The contractile response to EGF was significantly more inhibited (by about 88%) than that of either the PAR_2-AP (about 65%: $P < 0.0001$) or the PAR_1-AP (about 32%: $P < 0.05$), indicating that the PAR-induced responses can only be attributed in part to the involvement of the EGF receptor kinase. It should be noted that the EGF-kinase inhibitor had a much greater effect on contractions induced by 2fLIGRLO-NH_2 than those evoked by TFLLR-NH_2. As for AG18, AG1478 did not affect KCl-induced contractile responses. Furthermore, although PDGF also stimulated a small contractile response (minimal, relative to that caused by EGF: not shown), the receptor-selective PDGFR-kinase inhibitor, AG1296, had no effect on contractions caused by any of the other agonists used (also not shown). Finally, since GPCR-induced transactivation of the EGF receptor can occur either (i) via a MMP-catalysed release of a cell-surface-tethered EGF receptor agonist such as heparin-binding EGF, TGF-α or amphiregulin (Daub *et al.*, 1996; Gschwind *et al.*, 2001) or (ii) via an MMP-independent process involving intracellular mediators such as elevated calcium, PKC or Src (Tsai *et al.*, 1997; Eguchi *et al.*, 1998; Gschwind *et al.*, 2001), we evaluated the effect of the potent broad-spectrum MMP inhibitor, BB94, on contractions induced by the PAR_1- and PAR_2-APs. The MMP inhibitor had no effect (not shown) and thus we conclude that an MMP-independent, intracellular mechanism leads to the transactivation of the EGF receptor.

MEK/MAPKinase-kinase inhibitors reduce contractions induced by all agonists except for $PGF_{2\alpha}$

Differential effects of MAPKinase inhibitors on GPCR- and EGF-mediated contractions. As for our study of the contractile actions of the PAR_1 agonist, TFLLR-NH_2, and EGF in gastric smooth muscle (Zheng *et al.*, 1998), we evaluated a potential role for a MAPKinase pathway in inducing contractions in the PCA preparation, in response to all agonists. The selective and relatively potent MEK/MAPKinase-kinase inhibitor, U0126 (1 μM), inhibited the PAR_2-mediated contractile responses induced by 2fLIGRLO-NH_2 by up to 53%, and blocked the EGF-stimulated contractions by a comparable degree (60% inhibition; Figure 7A). Contractions induced by Ang-II and those induced by TFLLR-NH_2 were also diminished, but only by about 32 and 30% respectively. In contrast, contractions induced by KCl and $PGF_{2\alpha}$ were essentially unaffected in comparison with the other agonists (Figure 7A). The MEK/MAPKinase-kinase inhibitor, PD98059, at concentrations that did not significantly affect KCl-induced contractions (2 μM), had similar effects (Figure 7B). Of note, both of the MAPKinase inhibitors blocked EGF-stimulated contractions by over 60%, and the effect of PD98059 was substantially greater in blocking EGF-triggered contractions compared with its effect on the PAR-stimulated responses (Figure 7B). The inhibitory actions of both MEK/MAPKinase-kinase inhibitors indicated a role for MAPKinase in the contractile actions of the two PAR agonists as well as for Ang-II and EGF, but not for either $PGF_{2\alpha}$ or KCl. Furthermore, the data indicate that the PAR-stimulated contractile responses

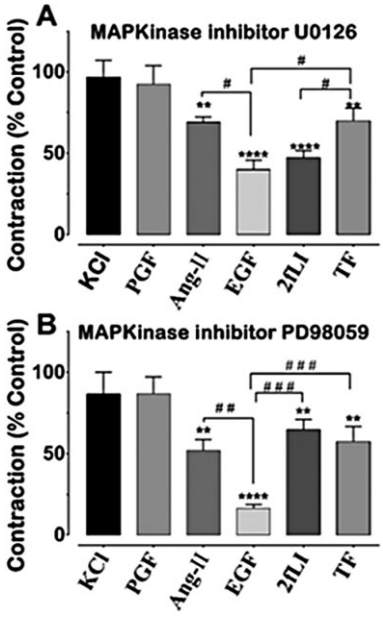

Figure 7

Effect of MEK inhibition on contractions induced by GPCR agonists and EGF in the PCA preparation. (A) Treatment of the PCA rings with U0126 (1 μM) significantly reduced (53%) the contractions induced by the PAR_2-AP, 2fLIGRLO-NH_2 (10 μM) ($P < 0.0001$) as well as by EGF ($P < 0.0001$), whereas the effects of Ang-II and TFLLR-NH_2 were reduced by 32 and 30%, respectively ($P < 0.01$), when compared with untreated control tissues. (B) Similar effects on PAR-AP-induced contractions were observed with PD98059 (2 μM), but with more pronounced effects on EGF >> Ang-II-induced contractile responses. All tissues were pretreated with L-NAME (100 μM). $^{**}P < 0.01$ and $^{****}P < 0.0001$ when compared to untreated controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ denote significance level for differences between indicated groups. Histograms represent means ± SEM (bars) for at least three independent experiments.

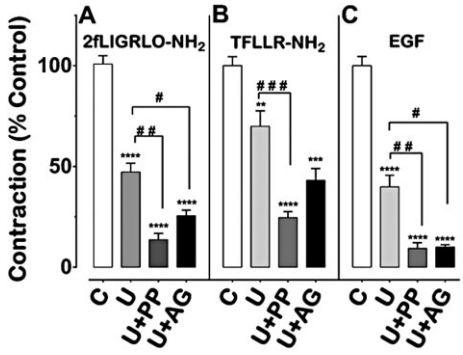

Figure 8

Effects of combined inhibitors on contractions caused by 2fLIGRLO-NH_2, TFLLR-NH_2 and EGF. The effects of the MEK inhibitor, U0126 (**U**, 1 μM) alone, or in combination with either the Src-inhibitor, PP1 (**PP**, 1 μM), or the EGF-receptor-kinase inhibitor, AG1478 (**AG**, 1 μM), were evaluated for their effects on contractions induced by 2fLIGRLO-NH_2 (10 μM, panel A), TFLLR-NH_2 (5 μM, panel B) and EGF (17 nM/100 ng·mL^{-1}, panel C). The contractile responses are expressed as a percentage (% Control) of the contractions induced by the three agonists in untreated tissues (**C**), evaluated in the same experiment. All tissues were L-NAME treated. $^{**}P < 0.05$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ when compared with untreated (no inhibitor) controls. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ denote significance level for differences between indicated groups. Histograms represent means (± SEM: bars) of measurements carried out with three or more independently prepared tissue rings from different PCA vessels.

were less affected by the MAPKinase-kinase inhibitors than the EGF-stimulated contraction.

Combined effects of MAPKinase and tyrosine kinase inhibitors on PAR- and EGF-mediated responses. Since the MAPKinase inhibitors did not completely suppress contractile responses mediated by PARs and EGF receptors (Figure 7), we assessed the effect of combining the MAPKinase inhibitor, U0126, which caused a comparable inhibition of EGF- and PAR-mediated responses (Figure 7A), with the Src-selective inhibitor, PP1, and the EGF-receptor-kinase-selective inhibitor, AG1478 (Figure 8). As shown in Figure 8, the combined action of U0126 and PP1 inhibited the PAR-induced responses more than the combination of U0126 and the EGF-kinase inhibitor, AG1578; but the combination of these compounds did not completely block PAR-mediated contractions. Furthermore, this combination of inhibitors (U0126 plus PP1) blocked the contractile action of EGF more effectively than either the Src-kinase inhibitor or the MAPKinase inhibitors acting on their own (cf. Figures 5 and 6 with Figure 8). Perhaps not surprisingly, the combination of U0126 with the EGF-kinase inhibitor did not inhibit the EGF response more than the EGF-kinase inhibitor alone (cf. Figure 6 with Figure 8C). The data indicate that for the PAR agonists, multiple kinase signalling pathways are involved and that the same combination of signalling pathways are downstream of the EGF-kinase contractile signalling pathway.

PAR_1-AP and EGF activate MAPKinase in coronary tissue: inhibition by U0126. Since the contractile effects of all the agonists studied except for KCl and $PGF_{2\alpha}$ were diminished by the MAPKinase inhibitors, we assessed the activation of MAPKinase directly in the tissues, as indicated by the increase in abundance of phospho-MAPKinase (Figure 9). For this purpose, we used EGF and the PAR_1-AP as representative agonists. As shown in Figure 9, both agonists caused an activation of MAPKinase (increased phospho-MAPKinase) that was attenuated by the MEK/MAPKinase-kinase inhibitor, U0126. Based on these data, we hypothesized that the activation of MAPKinase might lead to the phosphorylation and activation of PLA_2, which, in turn, could result in the release of arachidonate to enable the production of prostanoids via COX activity. We therefore used COX inhibitors to assess the role of COX products for triggering the contractile responses.

COX inhibitors inhibit contractions induced by all agonists except for $PGF_{2\alpha}$

In keeping with our previous observations that indomethacin is able to block the contractile actions of Ang-II, TFLLR-NH_2 and EGF in gastric smooth muscle (Yang *et al.*, 1993; Zheng *et al.*, 1998), and to block EGF contractions in the PCA (Gan *et al.*, 1989), we found that the dual COX1/2 inhibitor, indo-

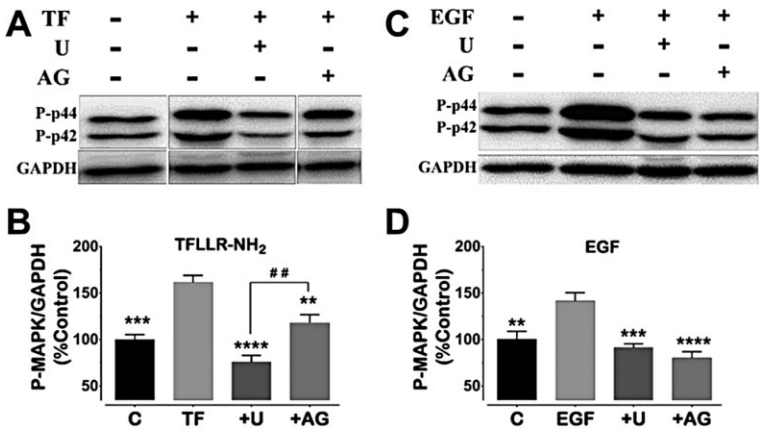

Figure 9

Activation of MAPKinase by PAR_1-activating peptide (TF) and EGF in intact tissues: inhibition by the MAPKinase-kinase inhibitor, U0126 (**U**), and the EGF-receptor kinase inhibitor, AG1478 (**AG**). The representative Western blots (panels A and C) show the increases in phospho-p42/p44-MAPKinase (P-MAPK) over the control levels (**C**), stimulated by the PAR_1-AP, TFLLR-NH_2 (TF, 5 μM) and by EGF (100 ng·mL^{-1}; 17 nM) as well as the inhibition of those increases by the two kinase inhibitors. The lower panels (B and D) show the quantified blot densitometry-based increases in activated MAPKinase (P-MAPK) shown as means (± SEM, bars), calculated as a percentage (% Control) of the normalized blot signal (phospho-p-42/p-44 MAPK relative to GAPDH) observed in the absence of agonists or kinase inhibitors. Experiments were repeated at least three times. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ denote significance levels when compared with agonist treated tissues (TF or EGF); $^{\#\#}P < 0.01$ denotes significance from indicated group.

methacin, blocked the contractile responses induced by all the agonists tested (Ang-II, 2fLIGRLO-NH_2, TFLLR-NH_2 and EGF) in the PCA preparation by more than 50%, except those induced by $PGF_{2\alpha}$ (Figure 10A) or KCl (not shown). This result supports our hypothesis that MAPKinase activation by these receptor agonists could stimulate PLA_2 to release arachidonate, which, in turn, could be metabolized to a contractile COX-derived agonist. To test the contribution of COX-1 versus COX-2 enzymes to the contractile response of agonists affected by indomethacin, selective COX inhibitors were used. Comparable inhibitory effects were observed with the COX-1 inhibitor, SC-560 (0.1 μM), and the COX-2 inhibitor, NS-398 (2 μM), although the relative ability of the two inhibitors to diminish the contractile response differed somewhat for the different agonists (Figure 10B,C).

To verify that an arachidonic acid metabolite generated by the tissue could be responsible for the contractile responses, we assessed the action of adding arachidonate alone to the organ bath. As for the agonist-induced contractile responses, contractions induced by the addition of arachidonate were blocked by pretreatment of the tissue with indomethacin, with the COX-1 selective inhibitor, SC-560, or with the COX-2-selective inhibitor, NS-398, or a combination of SC-560 and NS-398 (Figure 10D). However, the inhibition of the arachidonate-mediated response caused by the COX-2 inhibitor was significantly lower than that observed with the combination of COX-1 and COX-2 inhibitors ($P < 0.001$), indomethacin ($P < 0.01$) or the COX-1 inhibitor alone ($P < 0.05$). Importantly, the contractile effects of arachidonate were not affected by MEK/MAPKinase-kinase inhibition (U0126; Figure 10D). Thus, the role of MAPKinase in the PAR-mediated contractions are likely to be 'upstream' of the response induced by the as-yet-unknown arachidonate-derived COX-generated agonist, as suggested by the summary scheme in Figure 11.

Discussion

Our data show that in addition to causing an endothelium-dependent relaxant response, activation of PAR_1 and PAR_2 with receptor-selective agonist peptides stimulates endothelium-independent contractions in the PCA. The PAR-selective activating peptides that we used (TFLLR-NH_2, 2fLIGRLO-NH_2 and SLIGRL-NH_2) provide more definitive results for the distinct actions of PAR_1 versus PAR_2 in the coronary preparation, compared with the PAR-non-selective agonist, SFLLRN, used in previous studies (Hwa *et al.*, 1996; Hamilton and Cocks, 2000). The use of the non-selective PAR-AP in the previous work (SFLLRN) would have simultaneously activated both PAR_1 and PAR_2. Thus, our results provide a new perspective for understanding the 'dual' effect that activation of both PARs may have on coronary artery function (either relaxation or contraction), depending on the functionality of the endothelium. *In vivo*, this kind of PAR-mediated effect might result either from activation of the coagulation-cascade proteinases (thrombin; activated protein C) in the coronary circulation or from the release of tissue proteinases induced by vascular inflammation, atherosclerosis or ischaemia-reperfusion. Thus, PAR activation could have a bidirectional effect on coronary artery tension. On balance, in the context of atherosclerotic coronary artery disease where endothelial function would be compromised, this activation could in principle trigger vasoconstriction.

The PAR-dependent contractions were attenuated by both non-selective and Src-selective tyrosine kinase inhibitors (AG18; PP1) as well as by inhibitors of MEK/MAPKinase-kinase (U0126; PD98059) and the EGF receptor kinase (AG1478). Furthermore, the contractile responses mediated by PAR and EGF receptors were significantly diminished by both COX-1- and COX-2-selective inhibitors, although the actions of $PGF_{2\alpha}$ were not affected. While recognizing that the

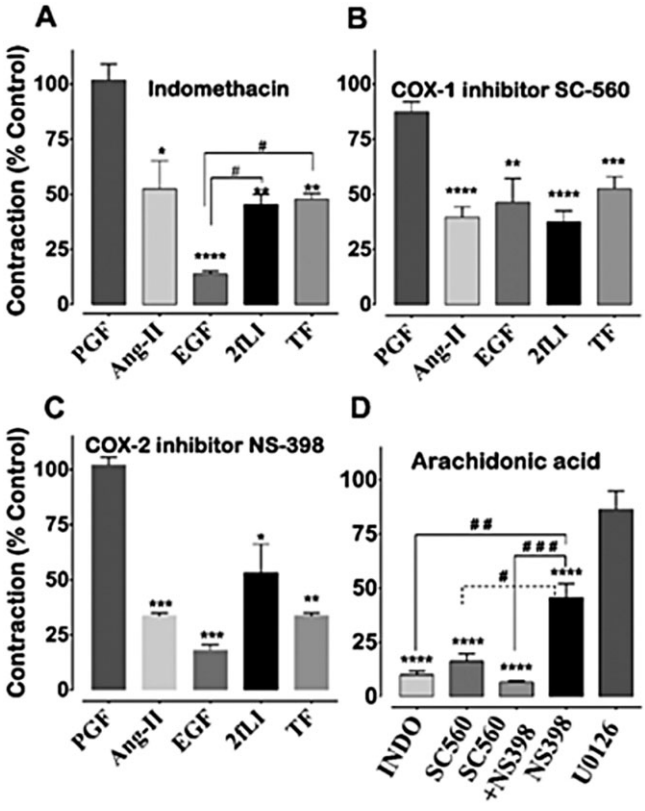

Figure 10

Effect of COX inhibition on agonist-induced contractility in the PCA preparation. (A) Indomethacin (3 μM) caused significant inhibition of contractile responses induced by PAR-APs, Ang-II and EGF when compared with untreated controls. (B) Effect of tissue treatment with the COX-1 inhibitor, SC-560 (0.1 μM). (C) Effect of COX-2 inhibition by NS-398 (2 μM) on agonist-induced contractions. (D) Effect of COX or MAPKinase inhibition on the contractile effect of arachidonic acid (5 μM). All tissues were pretreated with L-NAME (100 μM). Histograms represent means ± SEM (bars) of at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ compared with controls; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with the indicated group.

effects of these enzyme inhibitors should be interpreted with caution (Davies *et al.*, 2000; Bain *et al.*, 2007; Cohen, 2010), we verified that (i) KCl-stimulated contractions were not diminished by any of the inhibitors at the concentrations used; (ii) except for AG18, the inhibitors did not affect contractions induced by $PGF_{2\alpha}$ (a representative 'non-affected' GPCR); and (iii) these agents were employed at or below their enzyme-inhibitory concentrations, as deduced from previous studies. Thus, although the precise enzyme target(s) affected by the compounds could not be identified, these agents are well-established inhibitors for known kinase pathways, including Src (PP1), MEK/MAPKinase-kinase (U0126; PD98059) and the EGF-receptor kinase (AG1478). Further, the differential effects of the inhibitors on the contractile actions of the different agonists demonstrated clearly that each agonist was triggering a distinct signalling profile.

The effect of these enzyme inhibitors on the PAR-induced contractile responses was in many ways similar to their effects on contractile signalling pathways we have observed previously. These include: (i) the contractile action of Ang-II on the porcine coronary, which was blocked by tyrosine kinase inhibitors (Saifeddine *et al.*, 1992; Laniyonu *et al.*, 1994); (ii) contractions induced by the selective PAR_1-AP, TFLLR-NH_2, and by EGF in gastric smooth muscle preparations (Zheng *et al.*, 1998). Thus, the contractile signalling pathways we documented in the coronary preparation employ tyrosine kinase pathways that in part involve transactivation of the EGF receptor. This transactivation of the EGF receptor was not mediated by the MMP-catalysed release of a ligand that stimulates the EGF receptor, but rather via an intracellular signalling pathway that we presume involves the activation of Src-MAPKinase. The precise signalling pathway that results in transactivation of the EGF receptor remains to be determined.

A common feature of all agonists that we evaluated, except for $PGF_{2\alpha}$, was that the signalling pathways, including those triggered by EGF, ultimately resulted in the release of a COX product that drives contraction, as outlined in the scheme shown in Figure 11. Significantly, the contractile effect of the COX-generated metabolite of the supplementary arachidonic acid was not affected by the signalling pathway inhibitors that we used. The identity of the contractile prostanoid and its target receptor (very possibly, one of the EP receptor family, e.g. EP_1 or EP_3), that like $PGF_{2\alpha}$ mediates contractions via a PYK-independent signalling pathway, remains to be determined. Notwithstanding, our data suggest that in a situation where coronary PAR activation might lead to coronary vasospasm, COX inhibitors can be considered as a therapeutic option.

Although the signalling pathways outlined earlier apply in general to the actions of both PARs, there were significant differences in the ability of the enzyme inhibitors to affect contractions caused not only by the PAR_1 and PAR_2 agonists but also by the two other GPCR agonists we evaluated, namely Ang-II and $PGF_{2\alpha}$ as well as for EGF. Except for AG18, known to target the vasculature (summarized by Levitzki and Mishani, 2006), none of the other kinase inhibitors affected the contractile actions of $PGF_{2\alpha}$, which served as a GPCR-signalling 'control' for these compounds. As already mentioned, in keeping with the data obtained for $PGF_{2\alpha}$, contractions induced by the COX metabolite generated from arachidonate were not affected by the MEK/MAPKinase-kinase inhibitor, U0126, whereas all other agonists except for $PGF_{2\alpha}$ were affected. Thus, the signalling pathways triggered by the FP ($PGF_{2\alpha}$) and possibly by the EP receptors activated by the COX product are distinct from those stimulated by the PARs and Ang-II. Like Ang-II, the contractile responses induced by the PAR agonists were diminished by both the Src-selective inhibitor, PP1, and the MEK/MAPKinase-kinase inhibitors, U0126 and PD98059; and like Ang-II, the PAR-induced contractions were blocked by COX inhibitors. Yet, PAR-mediated contractions were partially blocked by the EGF-kinase inhibitor, AG1478, as were contractions triggered by EGF, as expected; but the action of Ang-II was unaffected by AG1478. Thus, it can be concluded that the contractile actions of the PAR-APs depend on signalling pathways that are similar but distinct from those activated by Ang-II and from EGF.

Although both PARs appear to employ most contractile signalling pathways in common, contractions induced by the

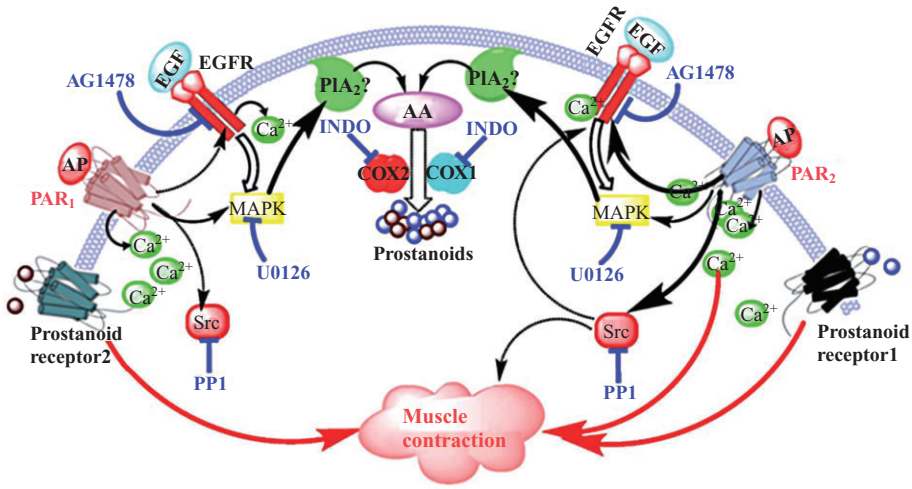

Figure 11

Summary scheme illustrating the signalling pathways activated by stimulation of PAR_1 and PAR_2 as well as by EGF to cause contraction and the sites of inhibition of the kinase and COX inhibitors. The scheme illustrates the effect of each agonist (PAR_1-AP; PAR_2-AP and EGF) to activate intracellular MAPKinase (MAPK) as well as Src and calcium signalling (Ca^{2+}). The transactivation of the EGF receptor via the PAR-activating peptides (APs) is also shown (black arrow from PAR to EGF receptor). These signals, in turn, are shown to lead, possibly via MAPKinase-mediated activation of PLA_2, to the release of arachidonate, which, in turn, is metabolized to COX products (circles) that cause a contractile response via prostanoid receptors. The potential sites of inhibition caused by the kinase (U0126; PP1; AG1478) and COX inhibitors (INDO) are shown in blue. Contraction is shown as resulting both from activation of prostaglandin receptors by the COX metabolites and by elevated intracellular calcium (Ca^{2+}), which can result from PAR-stimulated G_q.

PAR_2 agonist differed in two ways from the effects of the PAR_1 agonist. Firstly, although contractions induced by both PAR systems partly involved transactivation of the EGF receptor (blocked by AG1478), the PAR_2-mediated response was much more sensitive to the EGF-kinase inhibitor than the PAR_1-mediated response (Figure 6, right two histograms). Furthermore, the contractile response induced by the PAR_2 agonist was significantly desensitized, and did not recover after over a 2 h equilibration time, whereas the PAR_1-triggered response was much more resistant to a repeated exposure to TFLLR-NH_2.

In summary, activation of PAR_1 and PAR_2 in the coronary preparation can have both contractile and relaxant effects with the contractile actions involving tyrosine kinase signalling pathways that are distinct from those used by either Ang-II or $PGF_{2\alpha}$. In common with the other agonists, except for $PGF_{2\alpha}$, the contractile effects of the agonists depend on the generation of a COX product. One can argue that in the setting of coronary atherosclerotic artery disease with compromised endothelial function, activated coagulation proteinases or released enzymes might trigger contractile vasospasm via the signalling pathways we describe, including those stimulated by transactivation of the EGF receptor kinase. The contractile signalling pathways that we have identified can thus be considered as potential therapeutic targets for the treatment of coronary vascular disease.

Acknowledgements

M. E-D. was supported by an Egyptian Government Doctoral Scholarship. This work was made possible by grants from the Heart & Stroke Foundation of Alberta, Nunavut and Northwest Territories (to M. D. H.), the Qatar Foundation National Priorities Research Program (NPRP 08165-3-054 to C. R. T. and M. D. H., and NPRP 4-910-3-244 to C. R. T.) and the Canadian Institutes of Health Research (to M. D. H. and C. R. T.). We are grateful for the technical assistance provided by Dr Zenguo Yu for the dissection of coronary tissue.

Conflict of interest

All authors assert that they have no conflicts of interest related to the conduct of the research described in this article.

References

Adams MN, Ramachandran R, Yau MK, Suen JY, Fairlie DP, Hollenberg MD *et al.* (2011). Structure, function and pathophysiology of protease activated receptors. Pharmacol Ther 130: 248–282.

Al-Ani B, Wijesuriya SJ, Hollenberg MD (2002). Proteinase-activated receptor 2: differential activation of the receptor by tethered ligand and soluble peptide analogs. J Pharmacol Exp Ther 302: 1046–1054.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013a). The Concise Guide to PHARMACOLOGY 2013/14: G-Protein Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013b).

The Concise Guide to PHARMACOLOGY 2013/14:Catalytic Receptors. Br J Pharmacol 170: 1676–1705.

Alier KA, Endicott JA, Stemkowski PL, Cenac N, Cellars L, Chapman K *et al*. (2008). Intrathecal administration of proteinase-activated receptor-2 agonists produces hyperalgesia by exciting the cell bodies of primary sensory neurons. J Pharmacol Exp Ther 324: 224–233.

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H *et al*. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J 408: 297–315.

Barry GD, Le GT, Fairlie DP (2006). Agonists and antagonists of protease activated receptors (PARs). Curr Med Chem 13: 243–265.

Caruso R, Pallone F, Fina D, Gioia V, Peluso I, Caprioli F *et al*. (2006). Protease-activated receptor-2 activation in gastric cancer cells promotes epidermal growth factor receptor trans-activation and proliferation. Am J Pathol 169: 268–278.

Cohen P (2010). Guidelines for the effective use of chemical inhibitors of protein function to understand their roles in cell regulation. Biochem J 425: 53–54.

Coughlin SR (2000). Thrombin signalling and protease-activated receptors. Nature 407: 258–264.

Daub H, Weiss FU, Wallasch C, Ullrich A (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 379: 557–560.

Davies SP, Reddy H, Caivano M, Cohen P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95–105.

Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, Utsunomiya H *et al*. (1998). Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. J Biol Chem 273: 8890–8896.

Feletou M, Vanhoutte PM (2009). EDHF: an update. Clin Sci (Lond) 117: 139–155.

Gan BS, Hollenberg MD, Lederis K (1989). Epidermal growth factor-urogastrone (EGF-URO) causes contraction of porcine coronary arterial strips: inhibition by indomethacin. Proc West Pharmacol Soc 32: 269–271.

Glusa E, Adam C (2001). Endothelium-dependent relaxation induced by cathepsin G in porcine pulmonary arteries. Br J Pharmacol 133: 422–428.

Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A (2001). Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. Oncogene 20: 1594–1600.

Hamilton JR, Cocks TM (2000). Heterogeneous mechanisms of endothelium-dependent relaxation for thrombin and peptide activators of protease-activated receptor-1 in porcine isolated coronary artery. Br J Pharmacol 130: 181–188.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ *et al*. (1996). Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor: study of Lck- and Fynt-dependent T cell activation. J Biol Chem 271: 695–701.

Hansen KK, Oikonomopoulou K, Li Y, Hollenberg MD (2008). Proteinases, proteinase-activated receptors (PARs) and the pathophysiology of cancer and diseases of the cardiovascular, musculoskeletal, nervous and gastrointestinal systems. Naunyn Schmiedebergs Arch Pharmacol 377: 377–392.

Hollenberg M, Oikonomopoulou K, Hansen K, Saifeddine M, Ramachandran R, Diamandis E (2008). Kallikreins and proteinase-mediated signaling: proteinase-activated receptors (PARs) and the pathophysiology of inflammatory diseases and cancer. Biol Chem 389: 643–651.

Hollenberg MD (1994). Tyrosine kinase pathways and the regulation of smooth muscle contractility. Trends Pharmacol Sci 15: 108–114.

Hollenberg MD (1995). Tyrosine kinase-mediated signal transduction pathways and the actions of polypeptide growth factors and G-protein-coupled agonists in smooth muscle. Mol Cell Biochem 149–150: 77–85.

Hollenberg MD (2003). Proteinase-mediated signaling: proteinase-activated receptors (PARs) and much more. Life Sci 74: 237–246.

Hollenberg MD, Compton SJ (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol Rev 54: 203–217.

Hollenberg MD, Houle S (2005). Proteinases as hormone-like signal messengers. Swiss Med Wkly 135: 425–432.

Hwa JJ, Ghibaudi L, Williams P, Chintala M, Zhang R, Chatterjee M *et al*. (1996). Evidence for the presence of a proteinase-activated receptor distinct from the thrombin receptor in vascular endothelial cells. Circ Res 78: 581–588.

Kalmes A, Vesti BR, Daum G, Abraham JA, Clowes AW (2000). Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. Circ Res 87: 92–98.

Kawabata A, Saifeddine M, Al-Ani B, Leblond L, Hollenberg MD (1999). Evaluation of proteinase-activated receptor-1 (PAR1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR2 by PAR1-targeted ligands. J Pharmacol Exp Ther 288: 358–370.

Kawao N, Nagataki M, Nagasawa K, Kubo S, Cushing K, Wada T *et al*. (2005). Signal transduction for proteinase-activated receptor-2-triggered prostaglandin E2 formation in human lung epithelial cells. J Pharmacol Exp Ther 315: 576–589.

Laniyonu AA, Hollenberg MD (1995). Vascular actions of thrombin receptor-derived polypeptides: structure-activity profiles for contractile and relaxant effects in rat aorta. Br J Pharmacol 114: 1680–1686.

Laniyonu AA, Saifeddine M, Yang SG, Hollenberg MD (1994). Tyrosine kinase inhibitors and the contractile action of G-protein-linked vascular agonists. Can J Physiol Pharmacol 72: 1075–1085.

Levitzki A, Gazit A (1995). Tyrosine kinase inhibition: an approach to drug development. Science 267: 1782–1788.

Levitzki A, Mishani E (2006). Tyrphostins and other tyrosine kinase inhibitors. Annu Rev Biochem 75: 93–109.

McGuire JJ, Ding H, Triggle CR (2001). Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor(s). Can J Physiol Pharmacol 79: 443–470.

McGuire JJ, Hollenberg MD, Andrade-Gordon P, Triggle CR (2002). Multiple mechanisms of vascular smooth muscle relaxation by the activation of proteinase-activated receptor 2 in mouse mesenteric arterioles. Br J Pharmacol 135: 155–169.

McGuire JJ, Hollenberg MD, Bennett BM, Triggle CR (2004a). Hyperpolarization of murine small caliber mesenteric arteries by

activation of endothelial proteinase-activated receptor 2. Can J Physiol Pharmacol 82: 1103–1112.

McGuire JJ, Saifeddine M, Triggle CR, Sun K, Hollenberg MD (2004b). 2-furoyl-LIGRLO-amide: a potent and selective proteinase-activated receptor 2 agonist. J Pharmacol Exp Ther 309: 1124–1131.

Mihara K, Ramachandran R, Renaux B, Saifeddine M, Hollenberg MD (2013). Neutrophil elastase and proteinase-3 trigger G protein-biased signaling through proteinase-activated receptor-1 (PAR1). J Biol Chem 288: 32979–32990.

Oikonomopoulou K, Hansen K, Saifeddine M, Tea I, Blaber M, Blaber S *et al*. (2006a). Proteinase-activated receptors, targets for kallikrein signaling. J Biol Chem 281: 32095–32112.

Oikonomopoulou K, Hansen K, Saifeddine M, Vergnolle N, Tea I, Diamandis E *et al*. (2006b). Proteinase-mediated cell signalling: targeting proteinase-activated receptors (PARs) by kallikreins and more. Biol Chem 387: 677–685.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C *et al*. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402: 884–888.

Ramachandran R, Hollenberg M (2008). Proteinases and signalling: pathophysiological and therapeutic implications via PARs and more. Br J Pharmacol 153 (Suppl. 1): S263–S282.

Ramachandran R, Mihara K, Chung H, Renaux B, Lau CS, Muruve DA *et al*. (2011). Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2 (PAR2). J Biol Chem 286: 24638–24648.

Saifeddine M, Laniyonu A, Yang SG, Hollenberg MD (1992). Tyrosine kinase inhibitors and the contractile action of angiotensin-II in vascular tissue. Pharmacol Rev Comm 1: 177–184.

Sekiguchi F, Saito S, Takaoka K, Hayashi H, Nagataki M, Nagasawa K *et al*. (2007). Mechanisms for prostaglandin E2 formation caused by proteinase-activated receptor-1 activation in rat gastric mucosal epithelial cells. Biochem Pharmacol 73: 103–114.

Shpacovitch VM, Seeliger S, Huber-Lang M, Balkow S, Feld M, Hollenberg MD *et al*. (2007). Agonists of proteinase-activated receptor-2 affect transendothelial migration and apoptosis of human neutrophils. Exp Dermatol 16: 799–806.

Steinhoff M, Buddenkotte J, Shpacovitch V, Rattenholl A, Moormann C, Vergnolle N *et al*. (2005). Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocr Rev 26: 1–43.

Touyz RM, Schiffrin EL (2000). Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol Rev 52: 639–672.

Tsai W, Morielli AD, Peralta EG (1997). The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. EMBO J 16: 4597–4605.

Yang SG, Saifeddine M, Laniyonu A, Hollenberg MD (1993). Distinct signal transduction pathways for angiotensin-II in guinea pig gastric smooth muscle: differential blockade by indomethacin and tyrosine kinase inhibitors. J Pharmacol Exp Ther 264: 958–966.

Zheng XL, Hollenberg MD (1997). Dependence of thrombin receptor-mediated contraction of guinea pig gastric smooth muscle on growth factor signalling pathways: comparison with EGF and implication of a role for Src-family kinases. Proc West Pharmacol Soc 40: 45–48.

Zheng XL, Renaux B, Hollenberg MD (1998). Parallel contractile signal transduction pathways activated by receptors for thrombin and epidermal growth factor-urogastrone in guinea pig gastric smooth muscle: blockade by inhibitors of mitogen-activated protein kinase-kinase and phosphatidyl inositol 3′-kinase. J Pharmacol Exp Ther 285: 325–334.