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Trypsin stimulates proteinase-activated receptor-2-dependent and -independent activation of mitogen-activated protein kinases

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We have examined protease-mediated activation of the mitogen-activated protein (MAP) kinase cascade in rat aortic smooth-muscle cells and bovine pulmonary arterial fibroblasts. Exposure of smooth-muscle cells to trypsin evoked rapid and transient activation of c-Raf-1, MAP kinase kinase 1 and 2 and MAP kinase that was sensitive to inhibition by soybean trypsin inhibitor. The actions of trypsin were closely mimicked by the proteinase-activated receptor 2 (PAR-2)-activating peptide sequence SLIGRL but not LSIGRL. Peak MAP kinase activation in response to both trypsin and SLIGRL was also dependent on concentration, with EC_{50} values of 12.1 ± 3.4 nM and 62.5 ± 4.5 μM respectively. Under conditions where MAP kinase activation by SLIGRL was completely desensitized by prior exposure of smooth-muscle cells to the peptide, trypsin-stimulated MAP kinase activity was markedly attenuated (78.9 ± 15.1% desensitization), whereas the response to thrombin was only marginally affected (16.6 ± 12.1% desensitization). Trypsin and SLIGRL also weakly stimulated the activation of the MAP kinase homologue p38 in smooth-muscle cells without any detectable activation of c-Jun N-terminal kinase. Strong activation of the MAP kinase cascade and modest activation of p38 by trypsin were also observed in fibroblasts, although in this cell type these effects were not mimicked by SLIGRL nor by the thrombin receptor-activating peptide SFLLRNPNDKYEPF. Reverse transcriptase–PCR analysis confirmed the presence of PAR-2 mRNA in smooth-muscle cells but not fibroblasts. Our results suggest that in vascular smooth-muscle cells, trypsin stimulates the activation of the MAP kinase cascade relatively selectively, in a manner consistent with an interaction with the recently described PAR-2. Activation of MAP kinase by trypsin in vascular fibroblasts, however, seems to be independent of PAR-2 and occurs by an undefined mechanism possibly involving novel receptor species.

INTRODUCTION

It is now established that proteolytic enzymes exert actions on cells in addition to their degradative roles in food digestion, the formation of biologically active mediators from inactive precursors and the destruction of foreign bodies by lymphocytes. The best-characterized mediator acting in this way is the serine protease α-thrombin (reviewed in [1]). As well as being involved in the blood-clotting cascade, thrombin elicits a host of cellular effects such as the aggregation of platelets, the regulation of vascular tone and the stimulation of growth in certain cell types, notably fibroblasts and vascular smooth-muscle cells [2,3].

The cellular effects of thrombin seem to be transmitted largely, if not exclusively, via a thrombin receptor expressed in several mammalian species and cell types [4–6]. It now seems that this receptor might serve as a prototype for a family of receptors activated by other proteases (reviewed in [7,8]). Recently, a distinct thrombin-like receptor, designated proteinase-activated receptor 2 (PAR-2), has been cloned from a mouse genomic library [9,10]. A highly similar receptor has now been identified in humans [11]. Both the thrombin receptor and PAR-2 belong to the superfamily of receptors possessing seven transmembrane spanning domains and coupled to heterotrimeric G-proteins, and are believed to be activated by a unique mechanism. Proteolytic cleavage of an extracellular N-terminal exodomain is required to reveal a new N-terminus containing a receptor-activating sequence that serves as a 'tethered ligand', interacting with other as yet undefined sites of the receptor. Although the endogenous activator of PAR-2 *in vivo* has not been established, this receptor is activated by trypsin at nanomolar concentrations in a relatively selective manner. In addition, the synthetic agonist peptide SLIGRL, representing the first six amino acid residues adjacent to the putative trypsin cleavage site, has been shown to mimic the actions of trypsin [9,10]. PAR-2 has been detected in highly vascularized tissues [9,11] where roles in endothelial cell mitogenesis [12], smooth-muscle cell contraction [13], tissue wound healing [14], small-intestine function and pancreatic exocrine secretion [11] have been implied.

The intracellular consequences of PAR-2 activation are not well characterized and are at present limited to studies demonstrating intracellular calcium mobilization and inositol phosphate formation in a number of cell types [10,11,14]. One signal transduction pathway that has been implicated in the mitogenic effect of thrombin [15,16] and the propagation of signals gener-

Abbreviations used: BPAF, bovine pulmonary arterial fibroblast; EGFR, epidermal growth factor receptor; ERK, extracellular-regulated kinase; GSH, glutathione; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MAPKAP, MAP kinase-activated protein; MEK, mitogen-activated protein kinase or extracellular signal-regulated kinase kinase; PAR-2, proteinase-activated receptor 2; PKC, protein kinase C; RASMC, rat aortic smooth-muscle cell; RT–PCR, reverse transcriptase–PCR; SAPK, stress-activated protein kinase.

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ated by numerous other mediators acting via both G-protein-coupled and growth factor receptors is the mitogen-activated protein (MAP) kinase cascade (reviewed in [17]). MAP kinases comprise members of a family of serine/threonine-specific protein kinases believed to be involved in a number of cellular responses including the early events initiating cell proliferation [18] and regulation of smooth-muscle contractility [19]. Activation of the highly similar 42 and 44 kDa mammalian MAP kinase isoforms [also described as extracellular-regulated kinase (ERK) 1 and 2], requires phosphorylation on both tyrosine and threonine residues within a specific TEY motif [20,21] brought about by the dual-specificity kinases of the MEK (MAP kinase or ERK kinase) family [22,23]. MEK activation, in turn, is regulated by serine phosphorylation catalysed by MEK-activating kinases, including members of the Raf family of proto-oncogene products [24,25]. It is now established that novel mammalian protein kinases distantly related to MAP kinases, including the c-Jun N-terminal kinases (JNKs) [26,27] and p38 [28,29] stress-activated protein kinases (SAPKs), are elements of parallel signalling cascades. These kinases can be distinguished from the ERK group of MAP kinases by distinct dual-phosphorylation motifs and substrate specificities and by the different range of stimuli reported to cause their activation (reviewed in [30]). These include pro-inflammatory cytokines and several forms of environmental stress such as UV irradiation, high osmolarity and heat shock [30].

We have demonstrated previously that activation of the prototype PAR by thrombin stimulates the activation of MAP kinase in cultured rat aortic smooth-muscle cells (RASMCs) [31]. Although trypsin has been shown to cause calcium mobilization in RASMCs [32], and PAR-2 has been shown to be highly expressed in rat aortic tissue [13], no studies so far have addressed the role of PAR-2 in signalling events in this or other vascular cell types. We have therefore investigated protease-mediated activation of MAP kinases in RASMCs and bovine pulmonary arterial fibroblasts (BPAFs).

MATERIALS AND METHODS

Materials

Bovine trypsin [10300 N^{α}-benzoyl-L-arginine ethyl ester (BAEE) units/mg of protein], bovine α-thrombin (600 NIH units/mg) and the thrombin receptor-activating peptide SFLLRNPNDK-YEPF were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). The PAR-2-activating peptide SLIGRL was synthesized by Genosys Europe (Cambridge, U.K.) and was more than 95% pure by chromatographic and mass spectral analysis. LSIGRL was kindly donated by W. F. Bahou (Stony Brook Health Sciences Center, State University of New York, Stony Brook, NY, U.S.A.). Plasmid vectors encoding histidine-tagged wild-type MAP kinase and MEK-B, glutathione S-transferase (GST)–MAP kinase-activated protein (MAPKAP) kinase-2 and GST–Jun_{5-89} were gifts from G. L. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO, U.S.A.), C. J. Marshall (Chester Beatty Laboratories, Institute of Cancer Research, London, U.K.) and J. R. Woodgett (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada) respectively. PAR-2 cDNA was kindly supplied by J. Sundelin (Division of Molecular Neurobiology, Lund University, Sweden). Antibodies to MEK-1/2 and MAP kinase were purchased from Affiniti Research Products Ltd. (Exeter, Devon, U.K.). The c-Raf-1 antibody was supplied by Santa Cruz Biotechnology (Wembley, Middlesex, U.K.). Forskolin and Pansorbin were obtained from Calbiochem (Nottingham, U.K.). The BIOTRAK® MAP kinase assay kit, secondary antibodies and ECL detection reagents were purchased from Amersham International (Little Chalfont, Bucks., U.K.). [γ-^{32}P]ATP (3000 Ci/mmol) was from NEN–Dupont (Stevenage, Hertfordshire, U.K.). Custom-designed oligonucleotide primers PF1, PR1, PF2 and PR2 were synthesized at the Molecular Biology Laboratory, University of Strathclyde, Glasgow, U.K. All other reagents were supplied either by Sigma Chemical Co. or by BDH Chemicals Ltd (Poole, Dorset, U.K.) and were of the highest commercial purity available.

Cell culture

Smooth-muscle cells were isolated from the thoracic aortae of 180–200 g male Sprague–Dawley rats by digestion with collagenase and elastase as described previously [31]. Fibroblast cultures were isolated from pulmonary arteries of adult cows by a primary explant procedure essentially outlined in [33]. Both RASMCs and BPAFs were maintained in 10% (v/v) foetal calf serum and used routinely for experiments between passages 4 and 10. Before addition of agonists, cells were rendered quiescent by serum deprivation for 48 h.

SDS/PAGE and immunoblotting

Growth-arrested cells in six-well plates were stimulated with agonists for the times indicated and rinsed in ice-cold PBS. Cells were lysed in hot (70 °C) Laemmli sample buffer [63 mM Tris/HCl (pH 6.8)/2 mM $Na_4P_2O_7$/5 mM EDTA/10% (v/v) glycerol/2% (w/v) SDS/0.007% Bromophenol Blue/50 mM dithiothreitol] and boiled for 5 min. Equal amounts of protein (25 μg) were resolved by SDS/PAGE [10% (w/v) gel] and transferred to nitrocellulose membranes (Costar, High Wycombe, Bucks., U.K.). Membranes were blocked for 3 h with 3% (w/v) BSA and probed overnight with monoclonal anti-MAP kinase antibody at 0.01 μg/ml. Blots were further incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody and immunoreactivity was detected by enhanced chemiluminescence.

MAP kinase activity assay

Cells were stimulated as before, rinsed in PBS and solubilized in lysis buffer [150 mM NaCl/10 mM Tris (pH 7.5)/1 mM EDTA/1 mM EGTA/0.2 mM Na_3VO_4/0.2 mM PMSF/0.5 mM leupeptin/0.5 mg/ml aprotinin/1% (v/v) Triton X-100/0.5% NP40]. Lysates were placed on ice for 30 min with periodic vortexing before centrifugation at 13000 $\boldsymbol{g}$ for 5 min at 4 °C. To equal quantities (approx. 5 μg) of supernatant was added 600 μM of MAP kinase substrate peptide (KRELVEPLT^{669}PAGEAPNALLR) derived from a portion of the epidermal growth factor receptor ($EGFR^{661-680}$) (BIOTRAK® MAP kinase assay kit). The reactions were initiated by the addition of [γ-^{32}P]ATP (1 μCi, 50 μM) in 75 mM Hepes buffer, pH 7.4, containing 1.2 mM $MgCl_2$, and incubated at 37 °C for 15 min. Reactions were stopped by the addition of 300 mM H_3PO_4 and mixtures were spotted on P-81 phosphocellulose ion-exchange paper (Whatman, Maidstone, Kent, U.K.). Filters were washed for 5 min twice with 75 mM H_3PO_4 and twice with distilled water; [^{32}P]phosphate incorporation into peptide was quantified by scintillation counting.

MEK activity assay

Equal quantities (150 μg) of pre-cleared lysates prepared in an identical manner to the MAP kinase assay were incubated with

a rabbit polyclonal antibody (2 μg/ml) recognizing shared epitopes on both MEK-1 and MEK-2 (MEK-1/2) for 2 h at 4 °C with mixing. MEK-1/2-bound antibody complexes were coupled to Pansorbin for a further 2 h and collected by centrifugation. Immunoprecipitates were washed once with lysis buffer, twice in the same buffer without detergents and twice in a MEK kinase buffer [50 mM Hepes (pH 7.2)/0.5 mM EDTA/0.1 mM EGTA/ 25 mM β-glycerophosphate/0.1 mM Na_3VO_4/5 mM $MgCl_2$]. MEK activity was measured *in vitro* by a coupled assay with purified recombinant wild-type MAP kinase and $EGFR^{661-680}$ as sequential substrates. Immunocomplexes were resuspended in 30 μl of kinase buffer containing 1 μg of recombinant wild-type MAP kinase, 200 μM $EGFR^{661-680}$ peptide and [γ-^{32}P]ATP (2 μCi, 50 μM) and incubated at 30 °C for 15 minutes. Reactions were terminated on ice and 300 mM H_3PO_4 was added to an aliquot of each sample. Incorporation of [^{32}P]phosphate into $EGFR^{661-680}$ by recombinant MAP kinase activated by MEK-1/2 was quantified as described for the MAP kinase activity assay. The remainder of each sample was solubilized in 4 × Laemmli sample buffer and resolved by SDS/PAGE [10 % (w/v) gel]. Gels were dried under vacuum and the incorporation of [^{32}P]phosphate into MAP kinase was detected by autoradiography.

c-Raf-1 kinase activity assay

c-Raf-1 was immunoprecipitated as described for MEK except that the lysis buffer was supplemented with 40 mM β-glycerophosphate and 10 % (v/v) glycerol, and precleared lysates were incubated with c-Raf-1 antibody (2 μg/ml). Raf immunoprecipitates were resuspended in a kinase buffer containing 100 mM NaCl, 10 mM Pipes, 10 mM $MnCl_2$ and 10 μM aprotinin with or without 1 μg of recombinant kinase-inactive MEK-1 (MEK-B) and [γ-^{32}P]ATP (10 μCi, 25 μM). The reaction was stopped by adding 4 × Laemmli sample buffer, MEK-B was resolved by SDS/PAGE and the extent of [^{32}P]phosphate incorporation was assessed by autoradiography as described for the MEK assay.

p38 and JNK activity assay

Protein kinase activity of p38 was measured in affinity precipitates of p38 bound to recombinant GST–MAPKAP kinase-2 immobilized on glutathione (GSH)–Sepharose beads [34]. Stimulated cells were lysed in solubilization buffer [20 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 % (v/v) Triton X-100/10 % (v/v) glycerol/2 mM EDTA/20 mM NaF/2.5 mM β-glycerophosphate/0.2 mM PMSF/0.5 mg/ml leupeptin/0.5 mg/ml aprotinin]. Aliquots (150 μg) of solubilized cell extracts, clarified by centrifugation, were added to 1 μg of GST–MAPKAP kinase-2/GSH–Sepharose beads and mixed for 3 h at 4 °C. The Sepharose beads were collected by centrifugation and washed three times in solubilization buffer and once in kinase buffer [25 mM Hepes (pH 7.5)/20 mM $MgCl_2$/5 mM β-glycerophosphate/0.1 mM Na_3VO_4/2 mM dithiothreitol]. The beads were resuspended in 25 μl of kinase buffer and the reaction was started by the addition of [γ-^{32}P]ATP (5 μCi, 50 mM) and incubated for 30 min at 30 °C. The reaction was terminated by adding 10 μl of 4 × Laemmli sample buffer. Samples were boiled for 5 min and resolved by SDS/PAGE [11 % (w/v) gel]. Gels were dried and the incorporation of [^{32}P]phosphate into GST–MAPKAP kinase-2 was detected by autoradiography. The activity *in vitro* of c-Jun N-terminal kinase was quantified as for p38 activity except that cell extracts were prepared in a solubilization buffer containing 20 mM Hepes, pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 0.2 mM PMSF, 0.5 mg/ml leupeptin, 0.5 mg/ml aprotinin and 1 % (v/v) Triton X-100, and incubated with a recombinant GST-tagged truncated N-terminus of c-Jun (GST–c-Jun_{5-89}) immobilized on GSH–Sepharose [26]. The kinase reaction was performed in the same buffer as that used for the p38 activity assay at pH 7.6.

Reverse transcriptase–PCR (RT–PCR) detection of PAR-2 mRNA

Cells or finely homogenized tissue were solubilized in a denaturing solution containing 4 M guanidium isothiocyanate, 1 M sodium citrate, pH 7.0, 10 % (w/v) sarcosyl and 0.7 % (v/v) 2-mercaptoethanol. Total cellular RNA was isolated from cells and tissue by acidic phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) extraction followed by serial RNA precipitation with isopropanol and ethanol. First-strand cDNA was synthesized from RNA with RNAse H–reverse transcriptase by using oligo$(dT)_{12-18}$ primer (SUPERSCRIPT II kit, Gibco Life Technologies Ltd, Paisley, Scotland, U.K.) in accordance with the manufacturer's recommendations. Aliquots (2 μl) of cDNA were used as templates for amplification by PCR with DNA polymerase derived from *Thermus brockianus* (DYNAZYME II, Flowgen Instruments Ltd., Sittingbourne, Kent, U.K.) and primer pairs specific for the published mouse PAR-2 sequence [10]. Thermal cycling was performed under the conditions for PCR detection of PAR-2 by using PF1/PR1 primer pairs (see below) described in [13]. The PCR products were separated by electrophoresis through a 2 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and detected under UV illumination. The PAR-2 primers for PCR were as follows: PAR-2 forward primers PF1 (5′-CAC CAC CTG TCA CGA TGT GCT-3′) and PF2 (5′-TGG CTG CTG GGA GGA GGT ATC AC-3′), and PAR-2 reverse primers PR1 (5′-CCC GGG CTC AGT AGG AGG TTT TAA CAC-3′) and PR2 (5′-TGC CAT GTA GGT GGT AGG AGA TC-3′). The forward primers were targeted to sequences encoding amino acid residues within the second extracellular loop of the mouse PAR-2 receptor (PF1) and towards the N-terminus from the putative trypsin cleavage site on the extracellular domain (PF2). The reverse primers were directed against a sequence encoding the mouse PAR-2 C-terminus (PR1) and residues located within the first extracellular loop of the mouse PAR-2 receptor (PR2). Signals yielded by the PAR-2 primer pairs PF1/PR1 and PF2/PR2 were normalized to the PCR product generated by an intron-spanning actin primer pair [35]: actin forward primer AF1 (5′-CGT GGG CCG CCC TAG GCA CCA-3′) and actin reverse primer AR1 (5′-TTG GCC TTA GGG TTC AGG GGG-3′). Both strands of PCR products were subjected to chain-terminator cycle sequencing with fluorescent dye-labelled dideoxynucleotides (PRISM® kit, PE-Applied Biosystems Division, Warrington, Cheshire, U.K.) and the order of nucleotides was determined by an Applied Biosystems 373A DNA sequencer.

RESULTS

Trypsin and PAR-2-derived activating peptide stimulate components of the MAP kinase signalling cascade in rat aortic smooth-muscle cells

Exposure of RASMCs to trypsin evoked a time- and concentration-dependent activation of MAP kinase as assessed by kinase activity *in vitro* and by retardation of MAP kinase electrophoretic mobility indicative of enzyme phosphorylation. Stimulation of kinase activity was evident as early as 2 min, maximal at 5 min (approx. 4-fold) and returned to basal levels by 30 min (Figures 1B and 1C). Activation of MAP kinase by trypsin was abrogated when the protease was treated with

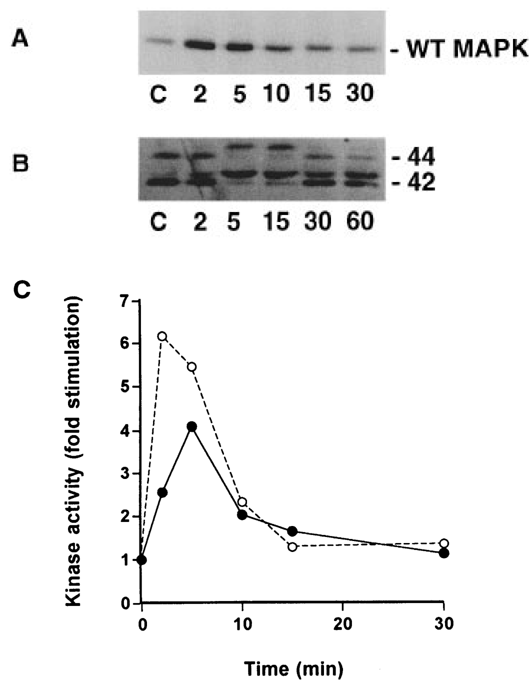

Figure 1 Activation of MAP kinase and MEK by trypsin in RASMCs

Cells were exposed to trypsin (30 nM) for the indicated times in minutes or with vehicle for 5 min (C). Lysates were assayed *in vitro* for MAP kinase activity as assessed by the incorporation of [^{32}P]phosphate into EGFR$^{661-680}$ or resolved by SDS/PAGE and immunoblotted for MAP kinase. Immunoprecipitates of MEK-1/2 were prepared from cell lysates and MEK activity was measured *in vitro* by a coupled kinase assay with recombinant wild-type MAP kinase (WT MAPK) and EGFR$^{661-680}$ as sequential substrates as outlined in the Materials and methods section. (**A**) Autoradiograph showing [^{32}P]phosphorylation of WT MAPK by MEK-1/2; (**B**) immunoblot showing electrophoretic shifts associated with the 42 and 44 kDa MAP kinase isoforms; (**C**) MAP kinase (●) and MEK-1/2 (○) activities expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from one time course representative of three separate experiments (controls: 36.2 pmol of P_i/min per mg of protein (MAPK); 0.48 pmol of P_i/min per mg of protein (MEK-1/2).

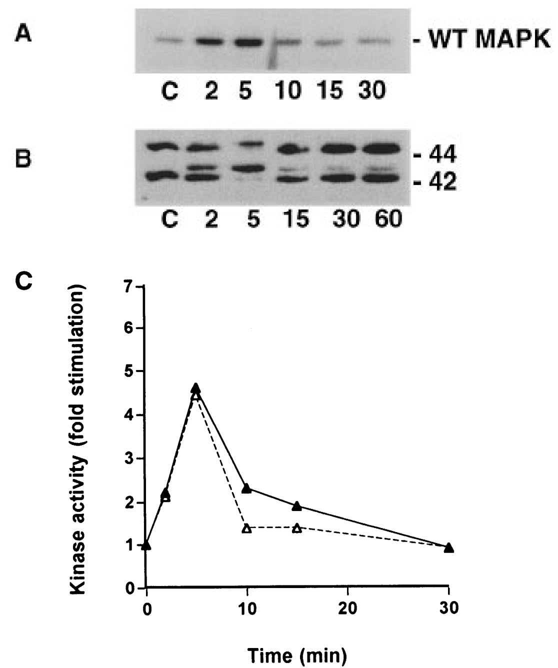

Figure 2 Activation of MAP kinase and MEK by PAR-2-activating peptide in RASMCs

Cells were exposed to SLIGRL (300 μM) for the indicated times in minutes or with vehicle for 5 min (C). Lysates were assayed *in vitro* for MAP kinase activity as assessed by the incorporation of [^{32}P]phosphate into EGFR$^{661-680}$ or resolved by SDS/PAGE and immunoblotted for MAP kinase. Immunoprecipitates of MEK-1/2 were prepared from cell lysates and MEK activity was measured *in vitro* by a coupled kinase assay with recombinant wild-type MAP kinase (WT MAPK) and EGFR$^{661-680}$ as sequential substrates as outlined in the Materials and methods section. (**A**) Autoradiograph showing [^{32}P]phosphorylation of WT MAPK by MEK-1/2; (**B**) immunoblot showing electrophoretic shifts associated with the 42 and 44 kDa MAP kinase isoforms; (**C**) MAP kinase (▲) and MEK-1/2 (△) activities expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from one time course representative of three separate experiments (controls: 36.2 pmol of P_i/min per mg of protein (MAPK); 0.48 pmol of P_i/min per mg of protein (MEK-1/2).

Table 1 Agonist-stimulated MAP kinase activity in RASMCs

Cells were treated with 30 nM trypsin, 300 μM SLIGRL, 300 μM LSIGRL or vehicle for 5 min. In some instances, trypsin or vehicle was incubated for 5 min at room temperature with 1 mg/ml soybean trypsin inhibitor (SBTI) before application to cells. Cell lysates were assayed *in vitro* for MAP kinase activity as outlined in the Materials and methods section. Activities are expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into peptide and are means ± S.E.M. for three separate experiments (control: 36.9 ± 7.4 pmol of P_i/min per mg of protein).

Treatment	MAPK activity (fold stimulation)
Vehicle	1.00
SBTI	1.02 ± 0.01
Trypsin	7.00 ± 0.57
Trypsin + SBTI	0.94 ± 0.02
SLIGRL	5.17 ± 0.58
LSIGRL	1.13 ± 0.01

1 mg/ml soybean trypsin inhibitor for 5 min before application to cells (Table 1). Trypsin also stimulated time-dependent activation of the MAP kinase activator MEK-1/2 (Figures 1A and 1C). Activation of MEK-1/2 by the protease preceded MAP kinase activation and was more transient, returning to basal levels at between 10 and 15 min. MEK-1/2 activation, in turn, was preceded by the activation of the canonical MEK-1/2 activator c-Raf-1 (results not shown).

As trypsin has been reported to be a potent and relatively specific activator of PAR-2 [9,10] we investigated whether the effects of trypsin could be mimicked by the PAR-2-activating peptide sequence SLIGRL. Exogenous application of SLIGRL stimulated MAP kinase (Figures 2B and 2C), MEK-1/2 (Figures 2A and 2C) and c-Raf-1 (results not shown) activities with time courses that closely resembled activation by trypsin. In contrast, at a concentration and exposure time (300 μM at 5 min) found to evoke a maximum response to SLIGRL, the effect of the hexapeptide sequence LSIGRL on MAP kinase activity was negligible (Table 1).

Both trypsin and SLIGRL responses were also dependent on concentration. Although maximal MAP kinase activities evoked by each agonist were comparable (Figures 1C and 2C), the concentration of peptide required for this was considerably greater than that for the protease (Figure 3), as has been reported previously in other cell systems [9,14]. Peak MAP kinase activation in response to trypsin occurred at 30 nM, whereas the EC_{50} was 12.1 ± 3.4 (S.E.M.) nM, comparable to that for trypsin-stimulated Ca^{2+} mobilization in lung adenocarcinoma cells [11] and inositol phosphate formation in keratinocytes [14]. Peptide-

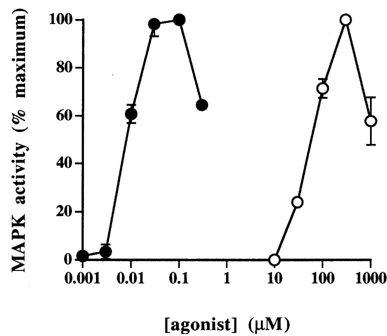

Figure 3 Activation of MAP kinase by trypsin and PAR-2-activating peptide in RASMCs

Cells were stimulated with trypsin (●) or SLIGRL (○) for 5 min at the concentrations indicated, and MAP kinase activity in lysates was determined as outlined in the Materials and methods section. Results are expressed as the percentages of maximum activity evoked by each agonist, and are means ± S.E.M. for three separate experiments (maximum activities: 216.4 ± 7.6 pmol of P_i/min per mg of protein (100 nM trypsin); 199.8 ± 1.9 pmol of P_i/min per mg of protein (300 μM SLIGRL).

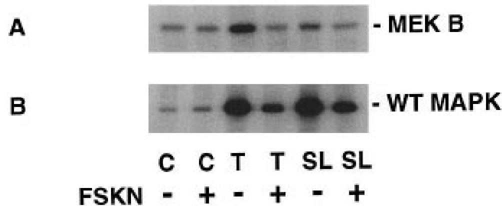

Figure 4 Effect of forskolin on agonist-stimulated c-Raf-1 and MEK activity in RASMCs

Cells were treated with vehicle (−) or 10 μM forskolin (FSKN) (+) for 30 min before exposure to 30 nM trypsin (T), 300 μM SLIGRL (SL) or vehicle (C) for 5 min. Lysates were analysed for c-Raf-1 (**A**) or MEK-1/2 (**B**) kinase activities by the incorporation of [^{32}P]phosphate into kinase-inactive MEK-1 (MEK B) or wild-type MAP kinase (WT MAPK) substrates respectively, as outlined in the Materials and methods section. Each of the autoradiographs depicted is representative of three separate experiments.

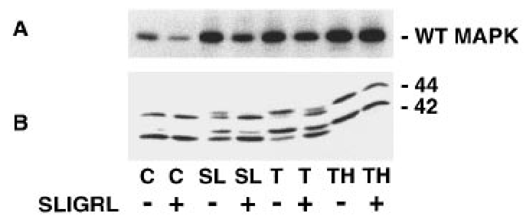

Figure 5 Desensitization of agonist-stimulated MAP kinase and MEK activation in RASMCs by PAR-2-activating peptide pretreatment

Cells were treated with vehicle (−) or 300 μM SLIGRL (+) for 20 min before a second exposure to 300 μM SLIGRL (SL), 30 nM trypsin (T), 30 nM thrombin (TH) or vehicle (C) for 5 min. Cell lysates were either assayed for MEK phosphotransferase activity by the incorporation of [^{32}P]phosphate into wild-type MAP kinase (WT MAPK) (**A**) or resolved by SDS/PAGE and immunoblotted for MAP kinase (**B**) as described in the Materials and methods section. Both the autoradiograph and the immunoblot are representative of three separate experiments.

Table 2 Desensitization of agonist-stimulated MAP kinase and MEK activation in RASMCs by PAR-2-activating peptide pretreatment

Cells were treated with 300 μM SLIGRL or vehicle for 20 min before a second exposure to SLIGRL (300 μM), trypsin (30 nM), thrombin (30 nM) or vehicle for 5 min. MAP kinase and MEK phosphotransferase activities were assayed as described in the Materials and methods section. Desensitization was determined by expressing agonist-evoked increases in kinase activity after SLIGRL pretreatment as the percentage inhibition of kinase stimulation by the same agonist in cells pretreated with vehicle. Results are presented as means ± S.E.M. for four separate experiments (MAP kinase activities: 181.3 ± 25.1, 217.2 ± 9.7 and 303.6 ± 87.8 pmol of P_i/min per mg of protein for SLIGRL, trypsin and thrombin respectively).

Pretreatment	Agonist	Desensitization of MEK activity (%)	Desensitization of MAPK activity (%)
SLIGRL	SLIGRL	81.8 ± 10.5	98.5 ± 1.5
SLIGRL	trypsin	71.6 ± 12.7	78.9 ± 15.1
SLIGRL	thrombin	12.9 ± 3.6	16.6 ± 12.1

evoked MAP kinase activation, in contrast, was maximal at 300 μM with an EC_{50} of approx. 62.5 ± 4.5 μM.

Phosphorylation of recombinant MEK *in vitro* by c-Raf-1, and of MAP kinase by MEK-1/2 immunopurified from RASMCs after exposure to trypsin or PAR-2-derived peptide, was markedly decreased by prior treatment of cells with 10 μM forskolin (Figure 4). This correlated with approx. 95 % inhibition by forskolin of MEK activity evoked by either agonist. A similar effect was observed when RASMCs were pretreated for 24 h with 100 nM 12-phorbol 13-myristate acetate (results not shown), which we have previously shown to down-regulate protein kinase C (PKC) α and ϵ isoforms [31].

PAR-2 desensitization attenuates trypsin-stimulated responses in RASMCs

We designed a PAR-2 desensitization protocol to investigate whether trypsin signalling events were mediated by the activation of PAR-2. For the prototype PAR, the thrombin receptor, a brief exposure to thrombin or agonist peptide renders the cell refractory to a second stimulus for 10–20 min [36,37]. We chose a desensitizing stimulus of 20 min exposure to 300 μM SLIGRL, which coincided with the time taken for the transient MAP kinase signal to return to basal levels (Figure 2C), to check whether PAR-2 undergoes similar desensitization. Prior treatment of RASMCs with SLIGRL prevented the subsequent activation of MEK and MAP kinase by a second exposure to the peptide (Figure 5 and Table 2). Under these desensitization conditions, trypsin-stimulated kinase activities were attenuated by approx. 80 %. In contrast, over 85 % of the increase in MEK and MAP kinase activity evoked by thrombin was preserved after initial exposure to SLIGRL (Figure 5 and Table 2).

Bovine pulmonary arterial fibroblasts respond to trypsin by a PAR-2-independent mechanism

We investigated whether analogous protease-mediated signalling events occurred in other cell types. Trypsin (30 nM) also caused a strong activation of MAP kinase within 5 min in BPAFs, although in this cell type the activity increased further with time, to peak at 15 min (4.5-fold stimulation), and was sustained up to 1 h (Figures 6C and 7). This effect was accompanied by transient activation of c-Raf-1 and MEK-1/2 (Figures 6A and 6B). The PAR-2-derived peptide, however, was ineffective at activating MAP kinase (Figure 7) or MEK-1/2 (results not shown) in BPAFs at a concentration (300 μM) that evoked maximal kinase

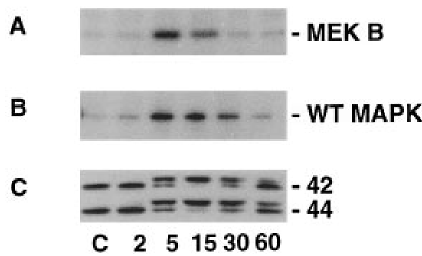

Figure 6 Effect of trypsin on MAP kinase, MEK and c-Raf-1 activities in BPAFs

Cells were stimulated with trypsin (30 nM) for the times indicated or with vehicle for 15 min (C). Lysates were analysed for c-Raf-1 (**A**) or MEK-1/2 (**B**) kinase activities by the incorporation of [^{32}P]phosphate into kinase-inactive MEK-1 (MEK B) or wild-type MAP kinase (WT MAPK) substrates respectively, or resolved by SDS/PAGE and immunoblotted for MAP kinase (**C**) as outlined in the Materials and methods section. The autoradiographs and immunoblot shown are each representative of three separate experiments.

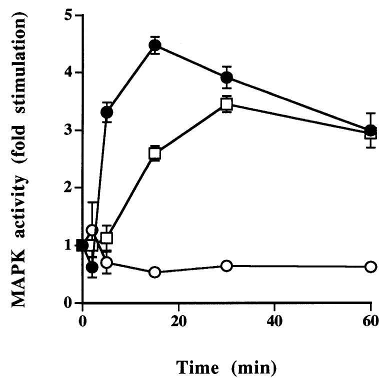

Figure 7 Agonist-stimulated MAP kinase activity in BPAFs

Cells were stimulated with 30 nM trypsin (●), 300 μM SLIGRL (○) or 300 nM thrombin (□) for the times indicated or with vehicle for 15 min, and cell lysates were assayed for MAP kinase activity as outlined in the Materials and methods section. Activity is expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into peptide substrate for each time course representative of three separate experiments (control: 44.2 ± 15.8 (S.E.M.) pmol of P_i/min per mg of protein).

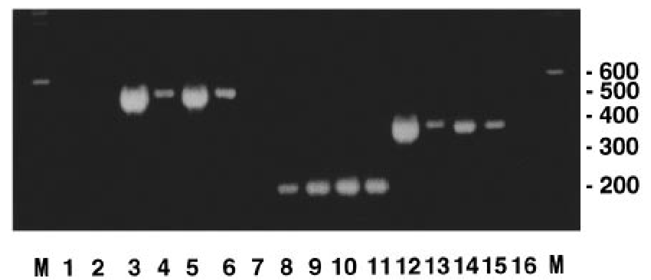

Figure 8 Detection of PAR-2 mRNA in RASMCs by RT–PCR

Total RNA extracted from cells and tissues was analysed by RT–PCR with PAR-2 primer pairs PF1/PR1 (lanes 1–7), actin primer pairs AF1/AR1 (lanes 8–11) and PAR-2 primer pairs PF2/PR2 (lanes 12–16) as outlined in the Materials and methods section. Products of PCR amplification were separated by electrophoresis through a 2% (w/v) agarose gel containing ethidium bromide and detected under UV illumination. Lane 1, PCR control (no cDNA); lane 2, reverse transcriptase control (no reverse transcriptase); lanes 3 and 12, mouse PAR-2 cDNA; lanes 4, 8 and 13, mouse intestine; lanes 5, 9 and 14, rat intestine; lanes 6, 10 and 15, RASMCs; lanes 7, 11 and 16, BPAFs. DNA size markers were run on the outside lanes (M) and the positions of bands corresponding to 200, 300, 400, 500 and 600 bp are indicated at the right. The figure depicts PCR products representative of at least three separate PCR reactions performed on three different RNA samples from each tissue or cell type.

activities in RASMCs. Although thrombin stimulated MAP kinase in this cell type, the kinetics of this response was dissimilar to that of trypsin (Figure 7). The onset of thrombin-stimulated MAP kinase activation was delayed until after 5 min, when activity increased slowly to reach a maximum at 30 min and was sustained up to 1 h. In contrast, the 14-mer thrombin receptor-activating peptide ($TRAP_{14}$) SFLLRNPNDKYEPF, derived from the human thrombin receptor, was unable to stimulate MAP kinase activity in BPAFs at a concentration (30 μM) that we found to cause strong activation of MAP kinase in RASMCs (results not shown), and has previously been reported to be active in other bovine cells [38].

Expression of PAR-2 mRNA in RASMCs

We used RT–PCR amplification to investigate whether PAR-2 mRNA was expressed in RASMCs. RT–PCR analysis of total cellular RNA extracted from RASMCs, using a primer pair specific to mouse PAR-2 (PF1/PF2) [13], yielded a single product of the predicted size (approx. 526 bp) as assessed by agarose gel electrophoresis, which co-migrated with the PCR product derived from mouse PAR-2 cDNA (Figure 8). Products of the same size were also detected in rat intestine and mouse intestine, a tissue that has been shown to express PAR-2 highly [9]. To exclude the possibility that the RT–PCR signals were derived from the amplification of contaminating genomic DNA, we used primer pairs PF2/PR2 encompassing a 710 bp region of mouse PAR-2 and spanning a 310 bp intron sequence. With this primer pair, PCR analysis of the reverse transcriptase product derived from RASMC RNA yielded a fragment of approx. 400 bp in length (Figure 8), confirming that this signal was amplified from cDNA derived from an intronless mRNA sequence. Both products of PCR amplification with primer pairs PF1/PR1 and PF2/PR2 from RASMCs were subjected to partial DNA sequencing. The order of nucleotides in 347 and 435 bp sequences within these fragments were found to show 89 % and 91 % similarity respectively to the published sequence for the cloned mouse PAR-2 [10]. Furthermore the sequence encoding the putative receptor-activating amino acid sequence SLIGRL within the extracellular N-terminus was conserved. In contrast with RASMCs, no detectable signal was generated by RT–PCR when RNA extracted from BPAFs was probed with either primer pair PF1/PR1 or PF2/PR2 (Figure 8).

Trypsin weakly stimulates p38 activation in RASMCs and BPAFs

We investigated whether trypsin was a stimulus for activating the MAP kinase homologues JNK/SAPK and p38 in the vascular cell types studied. Treatment of RASMCs with trypsin (30 nM) or SLIGRL (300 μM) caused a modest increase in p38 activity, compared with the marked level of kinase activity evoked by 0.5 M sorbitol (Figure 9B). The time course for p38 activation by these agents was rapid and transient, reaching a maximum at 5 min (3.14 ± 0.43-fold and 2.53 ± 0.56-fold increases for trypsin and SLIGRL respectively) and returning to basal levels at 15 min. Weak activation of p38 by trypsin with similar kinetics was also evident in BPAFs although, as for MAP kinase activation in this cell type, this effect was not mimicked by SLIGRL (results not shown). Neither trypsin nor SLIGRL stimulated detectable JNK activity in either RASMCs (Figure

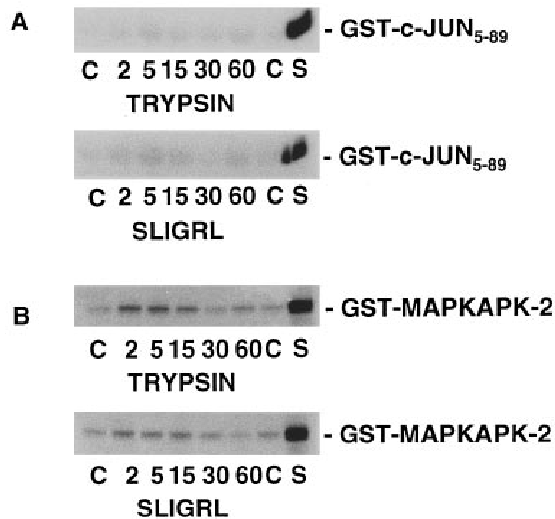

Figure 9 Effect of trypsin and SLIGRL on JNK and p38 activation in RASMCs

Cells were treated with 30 nM trypsin or 300 μM SLIGRL for the times indicated in minutes, 0.5 M sorbitol (S) for 30 min or with vehicle (C) for 2 and 60 min. Lysates were assayed *in vitro* for JNK (**A**) or p38 (**B**) activities as assessed by the incorporation of [^{32}P]phosphate into GST–c-Jun$_{5-89}$ or GST–MAPKAP kinase-2 immobilized on GSH–Sepharose respectively, as outlined in the Materials and methods section. Phosphorylation of substrates, resolved by SDS/PAGE, was detected by autoradiography. Each of the autoradiographs shown is representative of three separate experiments.

9A) or BPAFs (results not shown), whereas strong activation of JNK was evident in response to sorbitol.

DISCUSSION

The purpose of this study was to investigate protease activation of the MAP kinase signalling system in mesenchymal cells and to determine whether the characteristics of this response were indicative of the involvement of receptor-mediated signalling mechanisms. We found that trypsin stimulates MAP kinase activity in RASMCs at a concentration reported to activate the novel PAR-2 specifically [9]. To define the role of PAR-2 in the actions of trypsin we used the hexapeptide sequence SLIGRL derived from the first six amino acid residues constituting the new N-terminus after the proposed cleavage of the cloned PAR-2 exodomain. Our demonstration that in RASMCs the PAR-2-activating peptide closely mimicked the effect of trypsin, and the detection of an RT–PCR product amplified from a PAR-2-specific mRNA transcript in RASMC, strongly suggest that functional PAR-2 might mediate the cellular effects of the protease. In an analogous manner to thrombin-receptor activation, trypsin presumably cleaves the protease recognition site located in the extracellular N-terminal domain of PAR-2, revealing the peptide sequence at the new N-terminus that functions as a tethered ligand to activate the receptor. In support of this model for the activation of PARs, we have confirmed, using soybean trypsin inhibitor, the requirement of proteolytically active trypsin for these responses.

In a manner consistent with propagation of signals leading to MAP kinase activation described for other agonists that activate authentic transmembrane receptors [23,24], both trypsin and PAR-2-derived peptide also activated components of the cascade upstream of MAP kinase, including MEK-1/2 and c-Raf-1. In addition, this activation was markedy attenuated either by forskolin, an agent that elevates the intracellular cAMP concentration by stimulating adenylate cyclase, or the down-regulation of PKC by phorbol ester pretreatment. The effect of these interventions on inhibiting the activation of MAP kinase in response to various agonists has been described previously in specific cell types [39,40]. As forskolin and PKC down-regulation were effective at the level of c-Raf-1, inhibition might involve c-AMP-dependent protein kinase A-mediated phosphorylation of c-Raf-1 within the kinase domain [41] and the removal of a PKC-dependent component required for c-Raf-1 activation [42].

Further evidence in support of a role for PAR-2 in trypsin-evoked responses in RASMCs was provided by receptor desensitization studies. Our finding that trypsin-stimulated MAP kinase and MEK activation were markedly inhibited under conditions where PAR-2-activating peptide responses were refractory strongly suggests that trypsin acts largely via the same mechanism. Whether the loss of responsiveness of PAR-2-mediated signalling events on exposure to a second SLIGRL stimulus involves analogous processes of desensitization to thrombin or thrombin mimetic peptides, consisting of rapid phosphorylation, internalization and processing of the thrombin receptor [36,37], remains to be determined. Although we have shown previously that similar transient kinetics for activation of the MAP kinase cascade by trypsin are elicited by thrombin in RASMCs [31], it is unlikely that the two serine proteases act via the same mechanism. Both MAP kinase and MEK activities evoked by thrombin were only marginally affected by SLIGRL pretreatment, in marked contrast with trypsin. Despite other studies arguing in favour of trypsin and thrombin acting at the same receptor [32], this might be a consequence of the micromolar concentrations of trypsin used in such experiments. At the nanomolar concentrations used in this study, trypsin was unable to activate the human thrombin receptor expressed in *Xenopus* oocytes [4]. It was only at concentrations over 50-fold higher than the EC_{50} for the trypsin-evoked responses determined here that a significant response to the protease was recorded. Thus it is likely that the specificity of trypsin for PAR-2 depends largely on both the concentration and specific activity of trypsin used.

We found that the cellular responses evoked by trypsin in RASMCs could be reproduced in BPAFs. In this cell type, however, the effects of trypsin do not seem to be mediated by PAR-2 because the protease stimulated a potent and sustained activation of components of the MAP kinase signalling cascade that was not mimicked by SLIGRL. Furthermore we were unable to detect PAR-2 mRNA expression in BPAFs as has been reported in other fibroblast types [14]. The effects of trypsin in BPAFs are also unlikely to be mediated by the thrombin receptor because trypsin activates MAP kinase rapidly, whereas thrombin exhibits slower kinetics for activation, and the thrombin receptor-activating peptide $TRAP_{14}$ was ineffective. Thus, in BPAFs, trypsin does not seem to act via either of the two proteolytically activated receptors so far described, raising the possibility that other cell-surface proteins present in this cell type might be substrates for proteases. These targets could be novel members of the tethered-ligand receptor family with distinct peptide receptor-activating sequences.

We addressed the specificity of trypsin-evoked signalling via the MAP kinase cascade in relation to other MAP kinase-related pathways by testing for activation of the two MAP kinase homologues JNK/SAPK and p38. These protein kinases are believed to be components of parallel signalling pathways that are activated by distinct physiological or pathological stimuli [30]. In agreement with other studies [29], we found that a marked increase in p38 and JNK activities in both vascular

smooth-muscle cells and fibroblasts occurred in response to sorbitol-induced hyperosmolarity. Other cellular stresses known to strongly activate these signalling molecules include UV irradiation [26,29] and heat shock [28], as well as the pro-inflammatory cytokines interleukin 1 and tumour necrosis factor [29]. Trypsin was found to cause a small increase in p38 activity in both RASMCs and BPAFs, without any detectable increase in JNK activity, implying that there might be limited similarities in the pattern of MAP kinase and p38 regulation by the protease. However, in comparison with the magnitude of MAP kinase activity stimulated by trypsin, which was found to be comparable to platelet-derived growth factor in these cell types (results not shown), and the level of p38 activity evoked by sorbitol, activation of p38 is not the major response to this stimulus. Thus trypsin seems to activate the ERK subfamily of MAP kinases relatively selectively, as described previously for mitogenic stimuli such as EGF and phorbol ester [26,27,29].

In summary, we have shown that in vascular smooth-muscle cells trypsin activates components of the MAP kinase cascade and provided evidence to support a role for the recently described PAR-2 in signal transduction by the protease. In vascular fibroblasts, activation of the MAP kinase signalling pathway by trypsin does not seem to be a cellular consequence of PAR-2 or thrombin receptor activation, implying that in specific cell types trypsin might act via a unique mechanism, perhaps involving novel receptor species. Given the role that MAP kinase plays in a number of cellular processes, our findings imply that PAR-2 and other cleavable membrane proteins might mediate functional responses in vascular cells and turn out to be physiologically significant target substrates for protease mediators present in the extracellular milieu.

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