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# Thrombin induces DNA synthesis and phosphoinositide hydrolysis in airway smooth muscle by activation of distinct receptors

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**Abstract**

Chronic airway inflammation induces numerous structural changes of the airways involving hypertrophy and hyperplasia of airway smooth muscle (ASM). Thrombin has been identified in the bronchoalveolar lavage fluid of asthmatic subjects and displays potent bronchoconstrictor and mitogenic activity towards ASM. This study has addressed which proteinase-activated receptors (PARs) and signalling pathways are involved in mediating distinct effects of thrombin. Using cultured bovine tracheal smooth muscle (BTSM) cells as a model system, thrombin stimulated a marked increase in [$^3$H]inositol phosphate ([$^3$H]InsPs) accumulation, which was fully mimicked by a selective PAR1 activating peptide. In contrast, PAR1, PAR2, PAR3 and PAR4 activating peptides were unable to replicate the ability of thrombin to stimulate DNA synthesis as assessed by [$^3$H]thymidine incorporation. Further investigation demonstrated that the mitogenic effect of thrombin did not involve stimulation of PDGF secretion but did involve activation of PDGF or EGF receptors and a $G_{i/o}$-dependent activation of phosphoinositide 3-kinase. Thrombin, but not the PAR1, PAR2, PAR3 or PAR4 activating peptides was able to stimulate PtdIns(3,4,5)$P_3$ mass accumulation. PAR3 antisense oligonucleotides substantially inhibit thrombin-stimulated [$^3$H]thymidine incorporation and PtdIns(3,4,5)$P_3$ generation but had no effect on thrombin-induced phosphoinositide hydrolysis. These data indicate that while PI hydrolysis and $Ca^{2+}$ mobilisation induced by thrombin operates via PAR1-dependent activation of phospholipase C, phosphoinositide 3-kinase activation and DNA synthesis occurs via a distinct proteinase-activated receptor pathway, possibly involving PAR3.


*Keywords:* Thrombin; Proteinase-activated receptors; PAR; Airway smooth muscle

## 1. Introduction

Chronic airway inflammation results in structural changes in the bronchial epithelium and subepithelial structures and hyperplasia of the underlying smooth muscle layer. Such changes ultimately result in 'fixed' airway narrowing and a loss of efficacy of bronchodilating agents [1]. Inflammation in the airways initiates the release of various cytokines and growth factors that result in neutrophil, eosinophil and monocyte recruitment, altered microvasculature permeability and both hypertrophy and hyperplasia of airways smooth muscle [1–3]. Thrombin, a serine proteinase, becomes activated as a component of this inflammatory response and elicits a number of additional cellular responses including platelet aggregation, prostaglandin production from vascular endothelium and proliferation of both vascular and adventitial fibroblasts and airway myocytes [2].

Thrombin and related serine proteinases mediate their biological effects by cleaving a set of specific 'proteinase-activated receptors' (PARs) to reveal a tethered ligand that triggers receptor activation. To date, four receptor subtypes have been identified and designated PAR1, PAR2, PAR3

*Abbreviations:* ASM, airway smooth muscle; BTSM, bovine tracheal smooth muscle; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; InsPs, inositol phosphates; PAGE, polyacrylamide gel electrophoresis; PAR, proteinase-activated receptor; PBS, phosphate buffered saline; PDGFRα, platelet-derived growth factor receptor α; PDGFRβ, platelet-derived growth factor receptor β; PMSF, phenylmethylsulfonyl fluoride; PPACK, D-phenylalanyl-L-prolyly-L-arginine chloro-methyl ketone; PVDF, polyvinylidene difluoride; RTK, receptor tyrosine kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; TLC, thin layer chromatography

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and PAR4 [4,5]. PAR1 and PAR4 are known to mediate thrombin-induced platelet aggregation in humans whereas PAR3 and PAR4 mediate this response in the mouse [6,7]. Thrombin is known to cleave PAR1, PAR3 and PAR4 at the appropriate site to induce receptor activation, whereas trypsin cleaves and activates PAR2 [4,5]. Synthetic peptides corresponding to the tethered-ligand sequences of PAR1, PAR2 and PAR4 function as specific ligands for their respective G-protein coupled PARs [4,5]. However, the peptide derived from PAR3 is unable to act as a selective agonist, suggesting that other structural or receptor interactions are required for PAR3 activation [7]; as a consequence, it is not possible to induce selective activation of endogenous PAR3 using peptide ligands. Thrombin has been previously demonstrated to be mitogenic for bovine tracheal smooth muscle (BTSM) and involve a PtdIns 3-kinase-dependent mechanism [8]. In addition, thrombin induces an acute spasmogenic effect in airways smooth muscle that is mediated through activation of phospholipase C and subsequent $Ca^{2+}$ mobilisation [9]. Other spasmogens, such as bradykinin also induce $Ca^{2+}$ mobilisation but are unable to induce cellular proliferation. Importantly, inhibition of $Ca^{2+}$ mobilisation, while abolishing contraction, fails to influence the mitogenic capacity of agonists such as thrombin [9], indicating that these processes have discrete signalling requirements. In this study, we have sought to determine how the mitogenic and contractile properties of thrombin on airway smooth muscle are mediated. In particular, to address whether these two effects occur as a result of activation of multiple signalling pathways by one receptor or via activation of two discrete PAR-subtypes.

## 2. Materials and methods

### 2.1. Bovine tracheal smooth muscle cell culture

Bovine trachea was obtained from the local abattoir and tracheal smooth muscle cells isolated as described previously [8]. Cells were plated out and cultured in DMEM containing penicillin/streptomycin (5 units/ml and 5 μg/ml), amphotericin B (2.5 μg/ml) and foetal calf serum (10%, v/v). Cells from passages 3–9 were used for all experiments. Cells were allowed to grow to confluence and then quiesced in DMEM containing 0.5% (v/v) foetal calf serum for 48 h prior to experimentation. The identity of tracheal smooth muscle cells was confirmed by immunocytochemistry using an anti-bovine smooth muscle-specific α-actin monoclonal antibody.

### 2.2. [$^3$H]Thymidine incorporation

Confluent BTSM cells in six well plates were made quiescent by being washed twice in serum-free DMEM, inhibitors and mitogens added as detailed, and cells incubated for a further 24 h. [$^3$H]Thymidine (0.1 μCi/ml) was added for the final 4 h of the incubation. Cells were washed (250 μl per wash) twice in PBS, twice in trichloroacetic acid (5%, w/v), twice with ethanol and finally solubilised with NaOH (0.3 M). [$^3$H]Thymidine incorporation was determined by liquid scintillation counting.

### 2.3. PPACK treatment of thrombin

Thrombin was treated with PPACK as detailed previously [10]. Briefly, thrombin was incubated with a 10-fold molar excess of PPACK for 1 h at room temperature and unbound PPACK dialysed against PBS for 24 h. Control thrombin was treated with PBS and dialysed as for PPACK-treated thrombin. PPACK-treated thrombin was inactive when tested for its ability to cause platelet aggregation in a stirred platelet suspension at maximally effective concentrations (data not shown).

### 2.4. Measurement of total [$^3$H]inositol phosphate ([$^3$H]InsPs) accumulation

BTSM cells were grown to confluence in inositol-free DMEM supplemented with inositol-free FCS as appropriate. Cells were made quiescent for 48 h in media containing 0.5% (v/v) FCS and labelled with [$^3$H]inositol (1 μCi per well) for 18 h, washed twice with PBS and incubated with LiCl (20 mM) for 20 min prior to cell stimulation. Cells were stimulated for 30 min at 37 °C with thrombin (1 U/ml) or PAR activating peptides (100 μM) in a reaction volume of 1 ml and incubations terminated by removal of pre-treatment medium and addition of TCA (0.5 M). Cell suspensions were vortex mixed, spun for 5 min at 13,000 × *g* and the supernatants neutralised with a 1:1 mixture of trichlorotrifluoroethane/tri-*n*-octylamine. Total [$^3$H]InsPs were resolved by anion exchange chromatography using mini-columns of BioRad AG-1 (200–400 mesh in the formate form) [11].

### 2.5. Cell stimulation, immunoprecipitation, Western blotting and assay of PtdIns 3-kinase activity

BTSM cells were incubated in serum-free DMEM in six well plates, prior to addition of inhibitors and agonists as detailed in the Figure legends. Reactions were terminated by rapid aspiration of the media followed by two washes with PBS and addition of 1 ml of ice-cold lysis buffer (Hepes 50 mM, pH7.5, NaCl 150 mM, glycerol 10% (v/v), Triton X-100 1% (v/v), $MgCl_2$ 1.5 mM, EGTA 1 mM, leupeptin 10 μg/ml, aprotonin 10 μg/ml, PMSF 1 mM, $Na_3VO_4$ 200 μM, sodium pyrophosphate 10 mM and NaF 100 mM). Immunoprecipitation was carried out overnight with primary antibody (anti-p85 regulatory subunit of PtdIns 3-kinase), followed by incubation with Protein G-sepharose for 1 h. For Western blotting, samples were subjected to 10% PAGE and transferred to PVDF mem-

brane, probed with primary antibody for 1 h, and imaged with ECL following incubation with a HRP-secondary antibody.

PtdIns 3-kinase activity in anti-p85 (regulatory subunit of PtdIns 3-kinase) immunoprecipitates was assayed as described previously [12] using phosphatidylinositol/phosphatidylserine vesicles (3:1, v/v, 0.2 mg/ml) and [$\gamma$-$^{32}$P]ATP (50 $\mu$M, 10 $\mu$Ci) as substrates. [$^{32}$P]-PtdIns 3-P was resolved by TLC in a solvent system containing chloroform/methanol/ammonia/water (20/14/3/5, v/v/v/v), detected by autoradiography and $^{32}$P incorporation determined by liquid scintillation counting.

### 2.6. Measurement of PtdIns(3,4,5)$P_3$ mass

Confluent, quiescent BTSM cells were stimulated for 5 min with thrombin (1 U/ml) or PAR activating peptides (100 $\mu$M) and lysed by the addition of TCA (10% w/v). Lipids were extracted using acidified chloroform-methanol and subjected to alkaline hydrolysis to cleave the polar head group of PtdIns(3,4,5)$P_3$ to generate Ins(1,3,4,5)$P_4$; the resulting Ins(1,3,4,5)$P_4$ was measured using a [$^3$H]Ins(1,3,4,5)$P_4$ radioreceptor displacement assay as previously detailed [13]. Results were calculated from a standard displacement curve constructed using authentic Ins(1,3,4,5)$P_4$ and expressed as picomole PtdIns(3,4,5)$P_3$ per milligram protein.

### 2.7. PCR amplification of PAR mRNA

Total RNA was isolated by lysis of adherent cells with Trizol reagent and extracted according to the manufacturer's instructions. RT-PCR amplifications were carried out following DNase treatment of RNA using a one step RT-PCR system (Life Technologies), utilising primers specific for PAR3 and β-actin. Primer sequences were PAR3: 5′-CCTGCCATCTATATCCTGCTGTTT-3′ and 5′-TGGAATTAAGAATCCAAAGAATGCCAA-3′, and β-actin: 5′-CCACCAACTGGGACGACATG-3′ and 5′-GTCTCAAACATGATCTGGGTCATC-3′. Amplified products were visualised using an UV transilluminator. DNA sequencing of the resulting PCR product was carried out by Genetix Limited (New Milton, UK).

### 2.8. PAR3 antisense oligonucleotide incorporation

BTSM cells were grown in DMEM as before to approximately 60% confluency. Following replacement of media with fresh DMEM, antisense and sense oligonucleotides (5 $\mu$M) were transfected into BTSM cells using Lipofectamine Plus (1.8 $\mu$g/ml, Life Technologies) as a transfection reagent [14]. After 20 h, the transfection media was removed and replaced with normal supplemented DMEM for a further 24 h after which cells were quiesced and DNA synthesis assessed by [$^3$H]thymidine incorporation as detailed above. The antisense oligonucleotides sequence used was 5′-gcCCAGCCATATCCGATCCtt-3′ and control sense oligonucleotide was 5′-aaGGATCGGATATGGCTGGgc-3′; each oligonucleotide contained two phosphorthioate-backbone nucleotides at either end of each sequence [15].

### 2.9. Materials

PAR1-derived peptide (Ser-Phe-Leu-Leu-Arg-Asn), PAR2-derived peptide (Ser-Leu-Ile-Gly-Arg-Leu), PAR3-derived peptide (Thr-Phe-Arg-Gly-Ala-Pro) and PAR4-derived peptide (Gly-Tyr-Pro-Gly-Lys-Phe) were purchased from Bachem (UK) Ltd. (St. Helens, UK). [$\gamma$-$^{32}$P]-ATP, [$^3$H]thymidine and [$^3$H]inositol were purchased from Amersham International (Amersham, Bucks) and [$^3$H]inositol 1,3,4,5-tetrakisphosphate from NEN Life Science Products (Boston, MA). Antibodies to the p85 regulatory subunit of PtdIns 3-kinase were purchased from TCS Biologicals (Claydon Botolph, UK); secondary antibodies were obtained from SAPU (Carluke, Scotland); smooth muscle α-actin antibody was purchased from DAKO Cytomation (Cambridge, UK), PAR3 (H-103) antibody was purchased from Santa Cruz Biotechnology Inc. (California, USA). Trizol reagent, Lipofectamine Plus and One-Step RT-PCR System were purchased from Life Technologies (Paisley, Scotland) and primers and antisense oligonucleotides were synthesised by MWG Biotech (UK) Ltd. (Milton Keynes, UK). All other reagents were of the highest commercial purity available.

### 2.10. Statistical analysis

Statistical analysis was carried out using the one-way ANOVA test with a Newman–Keuls Multiple Comparison post-test analysis, with statistical significance being achieved when $p < 0.05$.

## 3. Results

### 3.1. Thrombin-induced DNA synthesis in BTSM cells

In primary cultures of BTSM cells, mitogen-induced DNA synthesis was measured using [$^3$H]thymidine incorporation. Fig. 1A shows that at maximal concentrations, PDGF-BB (20 ng/ml) induced a 29.5 ± 1.7-fold increase in [$^3$H]thymidine incorporation whereas thrombin (1 U/ml) induced a 15.7 ± 0.9-fold increase. Thrombin has been shown to induce expression and secretion of PDGF from airway epithelial cells, leading to lung fibroblast and smooth muscle proliferation [16]. To examine whether thrombin acts to cause PDGF secretion and allowing it to act in an autocrine manner, BTSM cells were pre-incubated with a PDGF receptor-blocking antibody prior to addition of mitogens. PDGF-BB-induced [$^3$H]thymidine incorporation was inhibited by 94.7 ± 0.1% by this anti-

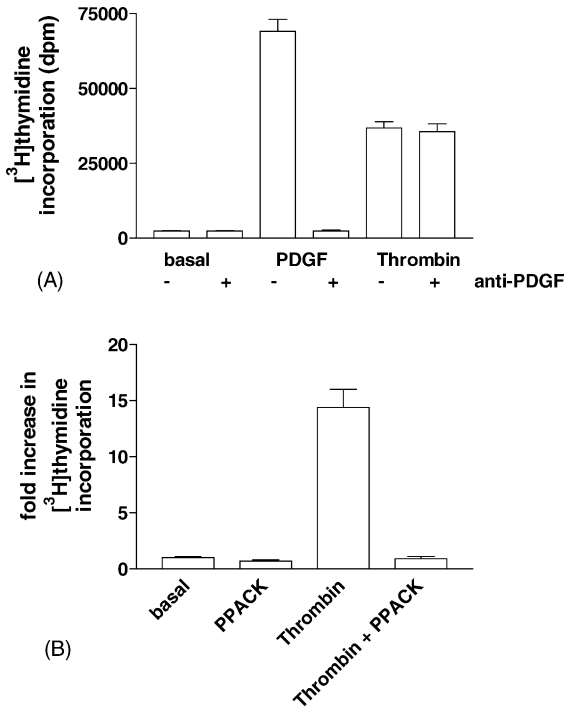


Fig. 1. (A) Effect of PDGF-receptor inhibition on DNA synthesis. BTSM cells were incubated with PDGF-receptor blocking antibodies for 1 h prior to addition of PDGF-BB (20 ng/m) or thrombin (1 U/ml) as indicated. [$^3$H]thymidine incorporation was assessed after 24 h and results are expressed as mean ± S.E.M. from three experiments each performed in triplicate. (B) Effect of PPACK on thrombin-induced DNA synthesis. Thrombin (1 U/ml) was pre-treated with PPACK as detailed in Section 2 and BTSM cells were stimulated as indicated. Results are expressed as mean ± S.E.M. from three experiments each performed in quadruplicate.

body (Fig. 1A). In contrast, thrombin-induced DNA synthesis was unaffected by this receptor-blocking antibody (Fig. 1A), indicating that the effect of thrombin is not mediated by the autocrine actions of PDGF. Importantly, PPACK-treated thrombin, which was catalytically inactive, was unable to induce [$^3$H]thymidine incorporation confirming that proteolytic activity of thrombin is required to mediate this response (Fig. 1B).

To determine whether thrombin could act to transactivate growth factor receptors, BTSM cells were treated with AG1296 (selective inhibitor of PDGF receptor kinase, $IC_{50}$ 1 μM), AG17 (selective inhibitor of PDGF receptor kinase, $IC_{50}$ 500 nM) or AG1478 (selective inhibitor of EGF receptor kinase, $IC_{50}$ 3 nM) prior to addition of thrombin. Fig. 2 shows that each of these compounds inhibited significantly thrombin-induced DNA synthesis to 11.5 ± 0.8, 18.0 ± 5.8 and 5.6 ± 3.3%, respectively, of thrombin control levels. This would indicate that thrombin may act through a transactivation mechanism to activate growth factor receptors, which may in turn mediate either $Ca^{2+}$ mobilisation or DNA synthesis in airway smooth muscle.

Thrombin induced a concentration-dependent increase in DNA synthesis ($EC_{50}$ 0.32 U/ml) (Fig. 3). However, synthetic peptides derived from PARs, which act as selective agonists (with the exception of the PAR3-derived peptide), had no effect on [$^3$H]thymidine incorporation in BTSM cells (Fig. 3). Pre-treatment of BTSM cells with the selective PAR-activating peptides for 10 min also had no effect on thrombin-induced DNA synthesis (data not shown) suggesting that these peptides do not act as antagonists at these receptors.

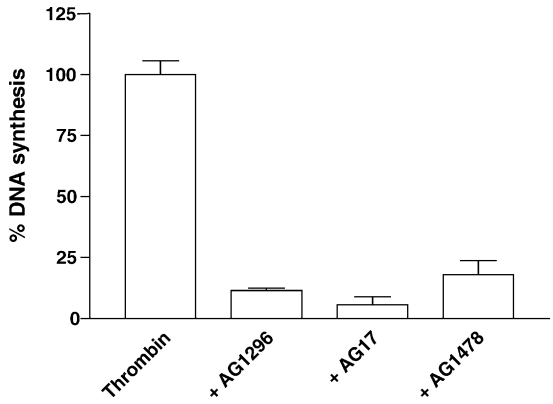


Fig. 2. Transactivation of growth factor receptors by thrombin in BTSM cells. BTSM cells were pre-treated with AG1296 (selective PDGF receptor tyrosine kinase inhibitor, 10 μM), AG17 (selective PDGF receptor tyrosine kinase inhibitor, 1 μM) or AG1478 (selective EGF receptor tyrosine kinase inhibitor, 100 nM) for 20 min prior to addition of thrombin (1 U/ml). [$^3$H]Thymidine incorporation was assessed after 24 h and results are expressed as mean % of thrombin-induced DNA synthesis ±S.E.M. from four experiments each performed in triplicate.

### 3.2. Thrombin activates phospholipase C and PtdIns 3-kinase

It has been previously demonstrated that thrombin-induced $Ca^{2+}$ mobilisation in human airway smooth muscle cells was mediated by the activation of phospholipase C activation and the generation of inositol 1,4,5-trisphosphate [9]. In agreement, this study found that thrombin induced an increase in [$^3$H]InsP accumulation in BTSM cells by 3.4 ± 0.1-fold (Fig. 4A). Interestingly, the PAR1-activating peptide (100 μM), shown above to be unable to stimulate [$^3$H]thymidine incorporation, induced a similar

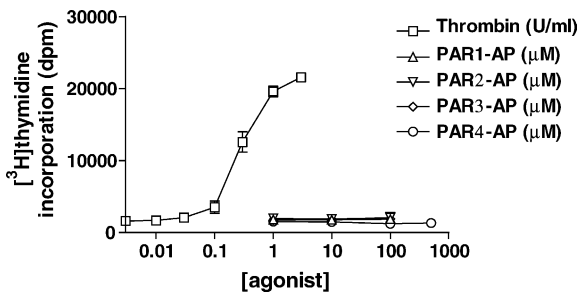


Fig. 3. Role for PARs in activating BTSM cells. Quiescent BTSM cells were exposed to thrombin (0.1–3 U/ml), and PAR1, PAR2, PAR3 and PAR4 activating peptides (1–500 μM) as indicated for 24 h and [$^3$H]thymidine incorporation assessed. Results are expressed as mean ± S.E.M. of incorporation from three experiments performed in triplicate.

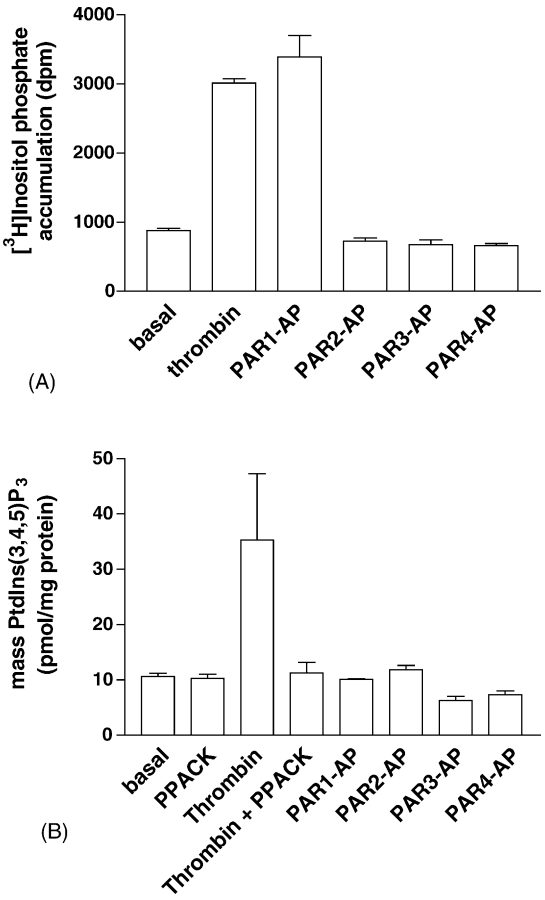


Fig. 4. (A) Activation of phospholipase C in BTSM cells. BTSM cells were pre-labelled with [$^3$H]inositol and stimulated with agonists (thrombin 1 U/ml, PPACK-treated thrombin 1 U/ml, PAR1-AP 100 μM, PAR2-AP 100 μM, PAR3-AP 100 μM, PAR4-AP 500 μM) in the presence of LiCl (20 mM) for 30 min as indicated; cells were then lysed and [$^3$H]InsPs extracted and analysed as detailed in Section 2. Results are expressed as mean dpm ± S.E.M. from three independent experiments each performed in triplicate. (B) Effect of thrombin and PAR-activating peptides on PtdIns(3,4,5)$P_3$ mass. BTSM cells were stimulated with agonists (thrombin 1 U/ml, PPACK-treated thrombin 1 U/ml, PAR1-AP 100 μM, PAR2-AP 100 μM, PAR3-AP 100 μM, PAR4-AP 500 μM) for 5 min as indicated, phosphoinositide lipids were extracted following cell lysis and mass PtdIns(3,4,5)$P_3$ measured using a [$^3$H]Ins(1,3,4,5)$P_4$ radioreceptor displacement assay. Results are expressed as mean ± S.D. from two separate experiments performed in duplicate.

increase in [$^3$H]InsP accumulation to 3.9 ± 0.4-fold above control values (Fig. 4A). However, neither the PAR2 (100 μM), PAR3 (100 μM) or PAR4-activating peptides (500 μM) had any significant effect on [$^3$H]InsP generation (Fig. 4A). These data suggest that thrombin-induced airway smooth muscle contraction is mediated at least in part by PAR1.

PAR-activating peptides were used to investigate a potential role for PAR1 in mediating not only phospholipase C activation by thrombin but also activation of PtdIns 3-kinase. Measurement of mass PtdIns(3,4,5)$P_3$, the product of PtdIns 3-kinase, showed that thrombin (1 U/ml, 5 min) increased PtdIns(3,4,5)$P_3$ levels from 10.6 ± 0.6 pmol/mg protein to 34.0 ± 8.0 pmol/mg protein (Fig. 4B). Thrombin inactivated by PPACK treatment, was unable induce any increase in PtdIns(3,4,5)$P_3$ levels demonstrating that activation of PtdIns 3-kinase occurs through a proteinase-activated mechanism (Fig. 4B). Furthermore, no increase in PtdIns(3,4,5)$P_3$ was observed with any of the PAR-derived activating peptides, suggesting that thrombin couples to PtdIns 3-kinase via a receptor other than PAR1, which is responsible for the action of thrombin on phospholipase C. It is important to note that the PAR3-derived peptide, unlike the PAR1, PAR2 and PAR4 peptides, has no agonistic activity at PAR3 and hence this receptor cannot be ruled out as a potential candidate for mediating the mitogenic effects of thrombin.

### 3.3. Pertussis toxin inhibits mediates thrombin-induced DNA synthesis and PtdIns 3-kinase activity

Thrombin (1 U/ml) induced a 4.5 ± 0.4-fold increase in PtdIns 3-kinase activity in p85α-immunoprecipitates (Fig. 5A), which was abolished by the PtdIns 3-kinase inhibitor wortmannin (100 nM) (Fig. 5A). Pre-treatment of

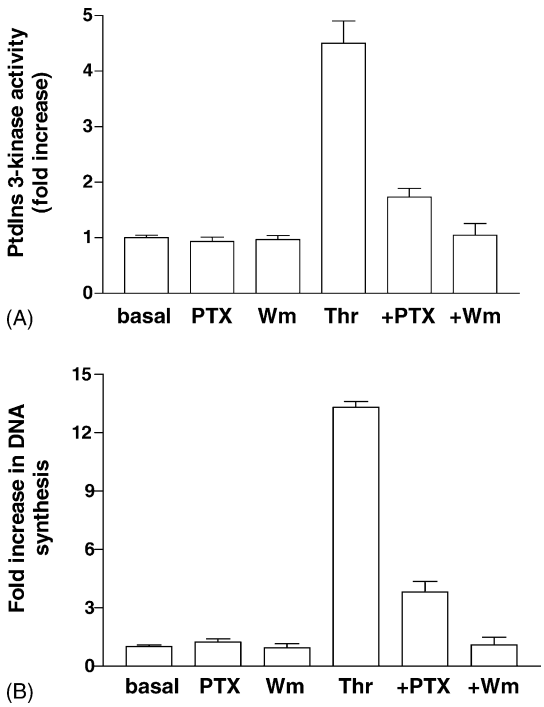


Fig. 5. (A) Effect of pertussis toxin on thrombin-induced PtdIns 3-kinase activity. BTSM cells were pre-treated with pertussis toxin (100 ng/ml, 18 h) or wortmannin (100 nM, 20 min) followed by activation with thrombin (1 U/ml) for 10 min. Cells were lysed, PtdIns 3-kinase immunoprecipitated with a specific p85 antibody and enzyme activity assayed using phosphoinositide vesicles as substrate. Results are expressed as mean fold increase over basal ±S.E.M. of thrombin-treated cells, from three independent experiments each performed in triplicate. (B) Effect of pertussis toxin on thrombin-induced proliferation. [$^3$H]Thymidine incorporation was assessed in quiescent BTSM cells following pretreatment of cells with pertussis toxin (100 ng/ml, 18 h) or wortmannin (100 nM, 20 min) followed by exposure to vehicle (PBS) or thrombin (1 U/ml) for 24 h, as indicated. Results are expressed as mean fold increase over basal ±S.E.M. of incorporation from four experiments performed in triplicate.

BTSM cells with pertussis toxin (100 ng/ml, 18 h), known to catalyse ADP-ribosylation and inhibition of $G_{i/o}$, resulted in inhibition of PtdIns 3-kinase activity induced by thrombin by 79.2 ± 4.5% indicating that $G_{i/o}$ is the predominant G protein involved in mediating this response (Fig. 5A). Pertussis toxin (100 ng/ml, 18 h) also caused a similar degree of inhibition of thrombin-stimulated DNA synthesis (69.0 ± 4.5%) (Fig. 5B). The key role of PtdIns 3-kinase in mediating DNA synthesis in BTSM cells was confirmed by demonstrating that thrombin-stimulated [$^3$H]thymidine incorporation was inhibited completely by wortmannin (100 nM) to control levels (Fig. 5B). These data demonstrate the requirement for PtdIns 3-kinase activation in DNA synthesis in BTSM cells and that the ability of thrombin to cause activation of this signalling pathway through a pertussis toxin-sensitive G protein.

### 3.4. PAR3 antisense oligonucleotides inhibit thrombin-induced responses

Total mRNA was extracted from BTSM cells and, in RT-PCR reactions using receptor subtype-specific primers derived from murine PAR3 to generate a predicted amplicon of 531 base pairs, we obtained a product for PAR3 of the predicted size (Fig. 6A). DNA sequencing and a BLAST database search showed that this RT-PCR product showed marked homology to human PAR3 (89%), mouse PAR3 (81%) and rat PAR3 (80%) indicating that the product is most likely the bovine form of PAR3 and is not due to contamination with human DNA or is an unrelated non-PCR product. To address a potential role for PAR3 in mediating thrombin-induced DNA synthesis antisense oligonucleotides to PAR3 were introduced into cells using Lipofectamine as a transfection agent. As no bovine PARs have thus far been completely sequenced, antisense oligonucleotides to PAR3 were designed based upon a region of homology in the human and mouse PAR3 sequences and distinct from PAR1 and PAR4. Transfection efficiency, as assessed by expression of fluorescein-tagged oligonucleotides, was 69.5 ± 3.5% (data not shown). Expression of PAR3 in BTSM cells was reduced in part following transfection of PAR3 antisense oligonucleotides as determined by Western blotting using a PAR3 antibody (Fig. 6B). In antisense-transfected BTSM cells, thrombin-induced [$^3$H]thymidine incorporation was inhibited by 61.0 ± 0.2% (Fig. 6C). A complimentary sense oligonucleotide had no effect on thrombin-induced DNA synthesis (94.8 ± 22.1% of control values). Furthermore, PDGF-induced DNA synthesis was not altered in cells transfected with the PAR3 oligonucleotides (128 ± 9.4% of PDGF-stimulated untransfected controls, $n = 3$, $p > 0.05$ compared to controls).

In confirmation of the key regulatory role of PtdIns 3-kinase in mediating DNA synthesis in airway smooth muscle, PAR3 antisense oligonucleotides significantly inhibited thrombin-induced PtdIns(3,4,5)$P_3$ levels from

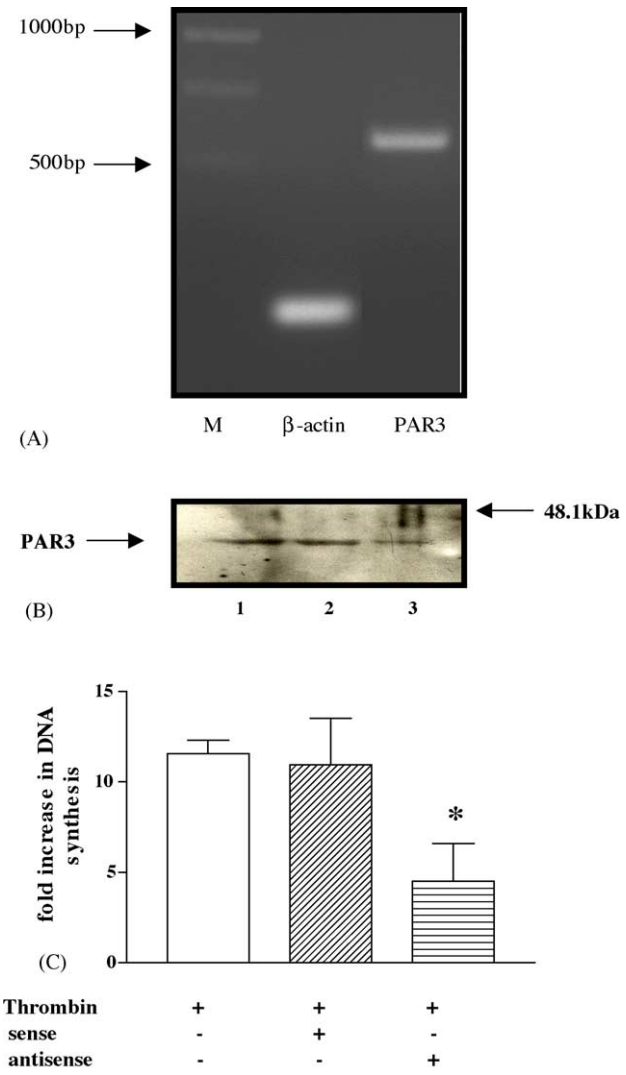


Fig. 6. (A) Expression of PARs in BTSM cells. Following RT-PCR, amplified products were separated by electrophoresis on 1.2% (w/v) agarose gels, molecular weight markers (M), β-actin control, and PAR3 (531 bp). (B) Effect of antisense oligonucleotides on PAR3 expression. Subconfluent BTSM cells were transfected with antisense and control sense oligonucleotides as detailed in Section 2, 1: untreated controls, 2: sense-transfected, 3: antisense-transfected. Whole cell lysates were subjected to PAGE and western blotting with a PAR3 (H-103) antibody (1:250), molecular weight markers and PAR3 are indicated with arrows, this is a representative blot from four experiments with similar results. (C) Effect of PAR3 antisense oligonucleotides on thrombin-induced DNA synthesis. Transfected BTSM cells were quiesced and [$^3$H]thymidine incorporation assessed following stimulation with thrombin (1 U/ml) for 24 h. Results are expressed as mean ± S.E.M. of fold increase in incorporation above basal from three experiments each performed in triplicate. *$p < 0.05$ compared to untransfected and sense-transfected cells.

37.6 ± 0.1 pmol/mg protein to 18.8 ± 8.7 pmol/mg protein ($p < 0.05$) but had no significant effect on PDGF-induced increases in PtdIns(3,4,5)$P_3$ mass (Fig. 7A), demonstrating that PAR3 appears to couple to PtdIns 3-kinase in BTSM cells and this receptor mediates DNA synthesis. In contrast, PAR3 antisense oligonucleotides had no significant effect on [$^3$H]InsPs accumulation induced by thrombin (1 U/ml) or PAR1-AP (100 μM) (Fig. 7B) suggesting that this response

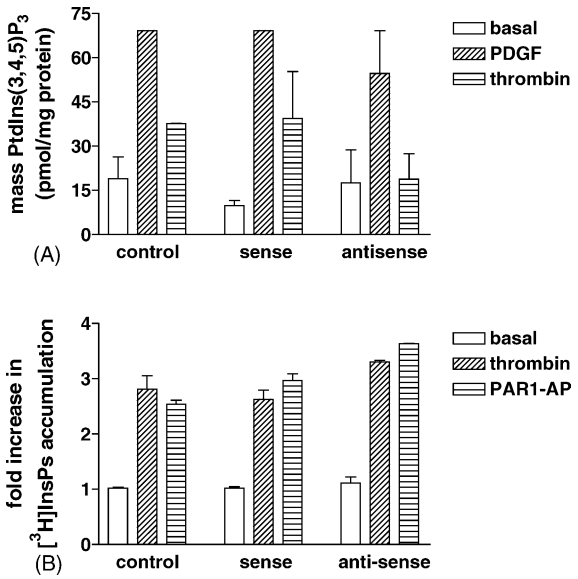


Fig. 7. (A) Effect of PAR3 antisense oligonucleotides on thrombin-induced PtdIns(3,4,5)$P_3$ mass. BTSM cells were transfected as detailed for Fig. 6B and mass PtdIns(3,4,5)$P_3$ measured as outlined in Section 2. Results are expressed as mean ± S.D. from two separate experiments performed in duplicate. (B) Effect of PAR3 antisense oligonucleotides on thrombin-induced phospholipase C activation. BTSM cells were transfected as detailed for Fig. 6B and [$^3$H]InsPs measured as outlined in Section 2. Results are expressed as mean dpm ± S.E.M. from two independent experiments each performed in triplicate.

is not mediated through PAR3 but that phospholipase C is activated solely through PAR1 in airway smooth muscle. These data also demonstrate that the PAR3 antisense oligonucleotides are not acting in a non-specific manner to ameliorate responses via other proteinase-activated receptors.

## 4. Discussion

The serine proteinase thrombin is well-recognised for its ability to activate platelets at sites of vascular injury. However, thrombin is also generated at sites of inflammation and appears to be capable of inducing smooth muscle contraction and other pro-inflammatory and tissue remodelling events. For example, thrombin activation has been demonstrated to induce cellular proliferation in both vascular and airways smooth muscle, and these responses are thought to be vital components in the formation of atherosclerotic plaques and airway remodelling [2]. Thrombin was first shown to activate its receptor by proteolytic cleavage of an amino terminal exodomain to generate a new N-terminus capable of interacting with and activating the thrombin receptor (PAR1) [17]. Subsequent research has identified three other receptors activated by a similar mechanism termed PAR2, PAR3 and PAR4 [6]. Activating peptides based upon the newly exposed N-terminus of three of these receptors have now been synthesised and established as selective agonists for their respective receptors [7]. This has yielded biologically active ligands for PAR1, PAR2 and PAR4, however, the PAR3-derived peptide (Thr-Phe-Arg-Gly-Ala-Pro) appears unable to activate its receptor, suggesting that additional structural interactions, which are achieved by thrombin but not the peptide alone, are required to induce PAR3 activation [7]. Thrombin has been shown to activate PAR1, PAR3 and PAR4 however, aside from the ability of these receptors to regulate platelet function (depending upon species), distinct functional responses have not yet been assigned to each of these receptor subtypes. Furthermore, it has been suggested that these G protein-coupled receptors may represent key pharmacological targets as they appear to be involved in mediating a range of cellular responses involved in the progression of lung disease [18].

In airways smooth muscle, thrombin has been demonstrated to activate phospholipase C, mobilise $Ca^{2+}$, generate force in bronchial rings and induce DNA synthesis in cultured airway smooth muscle cells [3,8,9,19]. In agreement with previous reports, we have demonstrated that thrombin is mitogenic for primary cultures of BTSM cells, albeit with a lower efficacy than PDGF-BB (Fig. 1) [8]. It is important to note that PDGF receptor-blocking antibodies were ineffectual in blocking thrombin-induced DNA synthesis, thus it is unlikely that PDGF is secreted from BTSM cells in response to thrombin and thereby involved in an autocrine induction of mitogenesis. The ability of PPACK to inhibit thrombin-induced DNA synthesis completely demonstrates that the proteolytic activity of thrombin is an essential requirement for its mitogenic potential and for the activation of a signalling pathway most likely involves a proteinase-activated receptor. These observations are in contrast to the effect of thrombin on human airway smooth muscle where PAR-independent growth and GM-CSF production have been reported [10].

Activation of RTKs involves tyrosine phosphorylation of key residues, which interact with and activate downstream signalling enzymes and adaptor proteins [20]. As DNA synthesis induced by thrombin was blocked by selective inhibitors of both PDGF receptor kinases (AG1296 and AG17) and EGF receptor kinases (AG1478) in airway smooth muscle [21], implicating a mechanism of growth factor receptor transactivation by thrombin. We were unable to observe any tyrosine phosphorylation of either PDGF or EGF receptors by immunoprecipitation (data not shown) but this may be limited by the sensitivity of this method. However, there is the potential for this mechanism of activation, downstream of a PAR, to link these G protein-coupled receptors to signalling intermediates such as PtdIns 3-kinase and Ras via growth factor activation to mediate cellular responses, such as growth and survival.

Other ASM spasmogens, such as bradykinin although able to activate phospholipase C and mobilise $Ca^{2+}$ have no mitogenic potential [9]. Our observations suggest that the ability of thrombin to act as a mitogen and activate phospholipase C occurs via either multiple receptors or

through a single receptor coupled to multiple signalling pathways. In this study, we have shown that the ability of thrombin to stimulate [$^3$H]InsP accumulation in BTSM cells is mimicked by the PAR1 (but not PAR2, PAR3 or PAR4) synthetic ligand suggesting that PAR1 mediates the $Ca^{2+}$ mobilising effects of thrombin in ASM. In addition, the PAR1-activating peptide was unable to induce PtdIns(3,4,5)$P_3$ generation or DNA synthesis indicating dissociation between phospholipase C activation and DNA synthesis following PAR1 ligation. Vouret-Craviari et al. [22] found that PAR-activating peptides were degraded over a few hours which explained their lack of effect to induce mitogenesis or induce MAPK activation in contrast to thrombin. Our previous report [8] showed that treatment of BTSM cells with thrombin for as short a time as 5 min was sufficient to induce a full mitogenic response and would anticipate that PAR-activating peptides would still be intact at these early time points. None of the peptides corresponding to any of the PARs were able to induce DNA synthesis nor did they induce PtdIns(3,4,5)$P_3$ accumulation however, the peptide corresponding to the activation sequence of PAR3 is known not to act as an agonist and this receptor may be a possible candidate to mediate this response.

Activation of PtdIns 3-kinase is a key signalling pathway in the induction of DNA synthesis in BTSM cells, its product PtdIns(3,4,5)$P_3$ is rapidly formed following exposure to a mitogen, such as thrombin and treatment of cells with wortmannin inhibits PtdIns 3-kinase activity and DNA synthesis. Furthermore, PPACK-treated thrombin does not cause PtdIns(3,4,5)$P_3$ generation confirming that retention of proteolytic activity is required for PtdIns 3-kinase activation, consistent with our observations for thrombin-induced DNA synthesis. It would therefore seem likely that thrombin acts on a PAR distinct from PAR1 to activate PtdIns 3-kinase and mediate its mitogenic effects.

All PARs so far identified have the classic heptahelical structure of GPCRs and are coupled to one, or in the case of PAR1, multiple G proteins [5,23,24]. Pertussis toxin treatment of BTSM cells, which causes ADP-ribosylation and inactivation of $G_{i/o}$, partially inhibited thrombin-induced DNA synthesis indicating the involvement of a pertussis toxin-sensitive G protein in mediating this response. This is in agreement with Panettieri et al. [9] who showed that human airway smooth muscle cells exhibited a similar sensitivity of thrombin-induced cell growth to pertussis toxin. They also demonstrated that inositol phosphate generation as a consequence of phospholipase C activation was insensitive to pertussis toxin suggesting dissociation between these two responses in human ASM.

The ability of wortmannin to inhibit thrombin-induced [$^3$H]thymidine incorporation demonstrated a key role for PtdIns 3-kinase in initiating cellular proliferation. Our experiments showed that thrombin-stimulated activation of PtdIns 3-kinase within p85 immunoprecipitates thus indicating the involvement of a class 1A PtdIns 3-kinase. Moreover, our previous study showed that both PDGF-BB and thrombin activate the same class 1A PtdIns 3-kinase to mediate DNA synthesis in BTSM cells [8] has been corroborated by Krymskaya et al. [25] using a transfection approach in human ASM. The sensitivity of thrombin-induced PtdIns 3-kinase activity to inhibition by pertussis toxin is similar to that of DNA synthesis and in conjunction with the ability of wortmannin to inhibit both responses suggests a mechanism of activation whereby a pertussis toxin-sensitive G protein is activated by thrombin presumably through a PAR and couples to PtdIns 3-kinase to initiate DNA synthesis.

Our results support the premise that the distinct physiological effects of thrombin in BTSM cells may be mediated by different PARs. In human platelets, thrombin acts on both PAR1 and PAR4 to cause activation, showing that thrombin can act on multiple PARs in a single cell type [6]. Moreover, it has been demonstrated that in mouse platelets PAR3 is unable to mediate transmembrane signalling directly but functions as a cofactor for the cleavage and activation of PAR4 by thrombin [26]. This ability of receptors to heterodimerise upon activation may also in part explain the ability of thrombin to cause transactivation of growth factor receptors as seen in BTSM cells. More recently, Bretschneider et al. [27] have shown that in isolated human vascular smooth muscle cells, thrombin and PAR3-AP were able to induce a transient mobilisation of $Ca^{2+}$, activation of ERK1/2 and DNA synthesis implicating a role for PAR3, at least in part, in mediating thrombin-induced responses. Our data suggest that thrombin-induced DNA synthesis in BTSM cells was mediated through a receptor which required to be acted on by catalytically-active thrombin and that this receptor was distinct from PAR1. Our results obtained with the selective PAR ligands and the observation that thrombin activates only PAR1, PAR3 and PAR4 [4] suggested that PAR3 may be a likely candidate. Support for this proposal was obtained using antisense oligonucleotides to PAR3 which were found to reduce expression of PAR3 protein, and to significantly inhibit thrombin-induced DNA synthesis while having no effect on PDGF-BB-induced responses. Furthermore, PtdIns(3,4,5)$P_3$ generation by thrombin was significantly inhibited by PAR3 antisense confirming the critical role of PtdIns 3-kinase in mediating DNA synthesis and that it appears to be regulated by stimulation of PAR3. Importantly, both PAR1-AP and thrombin-induced [$^3$H]InsP accumulation was unaffected by PAR3 antisense oligonucleotides confirming that this response, which leads to $Ca^{2+}$ mobilisation and smooth muscle contraction is mediated by a distinct receptor, namely PAR1, thereby indicating that thrombin can act on multiple PARs in BTSM cells to mediate distinct responses. These results must be carefully interpreted as the antisense oligonucleotides were designed against a region of consensus sequence between human and mouse PAR3, as no bovine receptor has thus far been fully sequenced. These results do, however, indicate a

mechanism involving PAR3 in mediating the mitogenic effects of thrombin in airway smooth muscle.

This study has provided evidence that thrombin acts on distinct receptors resulting in activation of disparate signalling pathways in BTSM cells: inducing phosphoinositide hydrolysis, leading to $Ca^{2+}$ mobilisation and contraction, through activation of PAR1 and induction of DNA synthesis through activation of a distinct PAR, which appears to be PAR3, and mediated by PtdIns 3-kinase. Thrombin-induced DNA synthesis involves PDGF/EGF receptor transactivation, which may occur through a heterodimerisation mechanism, and involves a predominantly $G_{i/o}$-dependent activation of a class 1A PtdIns 3-kinase. The precise mechanism and involvement of growth factor receptors in thrombin signalling awaits further investigation.

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