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Stimulation by endothelin-1 of mitogen-activated protein kinases and DNA synthesis in bovine tracheal smooth muscle cells

Kevin Malarkey, *Edwin R. Chilvers, *Mark F. Lawson & [1]Robin Plevin

Department of Physiology & Pharmacology, University of Strathclyde, Royal College, 204 George Street, Glasgow G1 1XW and *Respiratory Medicine Unit, Department of Medicine (RIE), Rayne Laboratory, University of Edinburgh, Medical School, Teviot Place, Edinburgh EH8 9AG

1 In cultures of bovine tracheal smooth muscle cells, platelet-derived growth factor-BB (PDGF), bradykinin (BK) and endothelin-1 (ET-1) stimulated the tyrosine phosphorylation and activation of both pp42 and pp44 kDa forms of mitogen-activated protein (MAP) kinase.

2 Both ET-1 and PDGF stimulated a sustained activation of MAP kinase whilst the response to BK was transient.

3 Activation of MAP kinase occurred in a concentration-dependent manner (EC_{50} values: ET-1, 2.3 ± 1.3 nM; BK, 8.7 ± 4.1 nM, PDGF, 9.7 ± 3.2 ng ml^{-1}).

4 Pretreatment with the protein kinase C (PKC) inhibitor Ro-318220, significantly reduced ET-1 activation of MAP kinase at 2 and 5 min but enhanced MAP kinase activation at 60 min.

5 Following chronic phorbol ester pretreatment, BK-stimulated activation of MAP kinase was abolished whilst the responses to PDGF and ET-1 were only partly reduced (80 and 45% inhibition respectively).

6 Pretreatment with pertussis toxin reduced ET-1 stimulated activation of MAP kinase particularly at later times (60 min), but left the responses to both PDGF and BK unaffected.

7 ET-1 also stimulated a 3 fold increase in [^{3}H]-thymidine incorporation which was abolished by pertussis toxin pretreatment. In contrast, PDGF stimulated a 131 fold increase in [^{3}H]-thymidine incorporation which was not affected by pertussis toxin.

8 These results suggest that a pertussis toxin-sensitive activation of MAP kinase may play an important role in ET-1-stimulated DNA synthesis but that activation of MAP kinase alone is not sufficient to induce the magnitude of DNA synthesis observed in response to PDGF.

Keywords: Endothelin-1; mitogen-activated protein kinase; tracheal smooth muscle; pertussis toxin; proliferation/ mitogenesis

Introduction

Endothelin-1 (ET-1), a 21 amino acid peptide (Yanagisawa *et al.*, 1988), elicits profound effects upon many cells of the respiratory system (Hay *et al.*, 1993). ET-1 is a potent bronchoconstrictor for human, guinea pig, rat and bovine airways smooth muscle (Henry *et al.*, 1990; 1992; Nally *et al.*, 1994) and has also been shown to stimulate cell growth and division in these cells (Glassberg *et al.*, 1994). The proliferative effects of ET-1 in airways smooth muscle may be of considerable pathological importance since it is now recognised that a major contribution to airflow limitation in chronic asthma is an increase in the number and size of airway smooth muscle cells (Knox, 1994). However, at present the intracellular signalling pathways which regulate cell growth and division in response to ET-1 and other bronchoconstrictor agents is unclear.

A major signalling pathway demonstrated in a number of other cell types to be involved in the regulation of cell growth and division involves the activation of intracellular proteins by phosphorylation upon tyrosine residues (Malarkey *et al.*, 1995b). One such enzyme family activated in this manner are the mitogen-activated protein (MAP) kinases (Davis, 1993). MAP kinases are believed to regulate a number of early events in mitogenesis including the activation of protein synthesis (Chen *et al.*, 1992), phosphorylation of oncogenes such as c-*Jun* (Pulverer *et al.*, 1991) and stimulation of glucose transport (Merrall *et al.*, 1993). It has recently been shown that expression of both the pp42 and pp44 kDa isoforms of MAP kinase is essential for agonist-induced cell cycle progression in fibroblasts (Pages *et al.*, 1993).

While the activation of the MAP kinase cascade in response to growth factors is now well characterized (Egan *et al.*, 1993; Malarkey *et al.*, 1995b), the regulation of this pathway in response to G-protein coupled receptor agonists is less well established. Protein kinase C (PKC)-dependent pathways have been implicated in the activation of MAP kinase in response to vasoconstrictors such as angiotensin II and vasopressin (Cribben *et al.*, 1993; Molloy *et al.*, 1993; Malarkey *et al.*, 1995a). However, recently a number of studies have shown that G-protein coupled agonists such as the mitogenic phospholipid lysophosphatidic acid (LPA) and thrombin may activate MAP kinase and stimulate growth via a pertussis toxin-sensitive mechanism (Kahan *et al.*, 1992; McLees *et al.*, 1995) involving the direct activation of nucleotide exchange upon the small molecular weight G-protein $p21^{ras}$ (van Corven *et al.*, 1993; Howe & Marshall, 1993; Hordjik *et al.*, 1994).

In this study, we have examined the characteristics of MAP kinase activation in bovine tracheal smooth muscle cells. We show that the activation of MAP kinase in response to ET-1 involves PKC-dependent and independent mechanisms and also involves the activation of a pertussis toxin-sensitive pathway. In addition we show that DNA synthesis in response to ET-1 is also dependent upon a pertussis toxin-sensitive event. However, the activation of MAP kinase alone does not appear sufficient to explain the efficacy of PDGF as a mitogen in bovine tracheal smooth muscle cells.

A preliminary account of some of these findings was presented to the XIIth IUPHAR meeting Montreal, Canada (Malarkey *et al.*, 1994).

[1] Author for correspondence.

Methods

Cell culture

Bovine tracheae were obtained from the local abbatoir. Small strips of the trachealis muscle, dissected free of epithelium and connective tissue, were washed in DMEM containing gentamicin 50 $\mu g\ ml^{-1}$ and amphotericin 2.5 $\mu g\ ml^{-1}$ and then incubated in a similar medium containing collagenase type IV 1 $mg\ ml^{-1}$ for 60 min at 37°C with intermittent shaking. Following addition of foetal calf serum (FCS), the solution was filtered through gauze and the tracheal smooth muscle cells collected by centrifugation at 250 *g* for 6 min. Cells were plated out and cultured in DMEM containing 10% FCS, L-glutamine 2 mM, gentamicin 50 $\mu g\ ml^{-1}$ and amphotericin 2.5 $\mu g\ ml^{-1}$. Cells from passages 3–10 of three separate cultures were used for all experiments. Cells were rendered quiescent by serum-deprivation for 48 h prior to experimentation. The identity of the tracheal smooth muscle cells was confirmed by immunocytochemical staining using a smooth muscle specific α-actin mouse monclonal antibody (DAKOM635) detected using rabbit anti-mouse HRP-linked antibody and 3-amino 9-ethyl-carbazole (Sigma) (results not shown).

Cell stimulation and immunoblotting

Cells were incubated in Hanks-buffered saline containing 10 mM glucose and 2% w/v BSA for 30 min then stimulated with agonist or vehicle for the time periods indicated. The reactions were terminated by rapid aspiration of the buffer followed by 3 washes of the cell monolayer in ice cold 20 mM HEPES buffer (pH 7.4) containing (mM): NaCl 150, NaF 50, Na_4PO_7 10, EDTA 4, EGTA 2, and Na_3VO_4 2. Following the final aspiration, 0.75 ml of SDS sample buffer (70°C) was added and the sample passed repeatedly through a 21G needle. The samples were boiled for 5 min and then stored at −80°C until analysis. Aliquots of each sample (50–75 μg protein) were run on 10% SDS-PAGE gels and then subjected to Western blotting. The nitrocellulose blots were blocked for non-specific binding for 3 h in 50 mM Tris buffer containing 150 mM NaCl, 0.2% v/v Tween pH 7.4 (NaTT) and 2% BSA, then incubated in the same buffer containing 0.2% BSA and 200 $ng\ ml^{-1}$ of a rabbit antiphosphotyrosine antibody overnight at room temperature. The blots were washed in NaTT buffer for 90 min (6 changes of NaTT buffer) and then incubated for 60 min in buffer containing 0.2% BSA and 1/10,000 dilution of donkey anti-rabbit Ig linked to HRP. The blots were then washed for 120 min (8 changes of NaTT buffer) and bands visualized using ECL detection (Amersham). The blotting procedure was assessed for specificity for phosphotyrosine, appropriate antibody dilution, and linearity with protein concentration (Saville *et al.*, 1994). For MAP kinase immunoblotting, conditions were employed as outlined above except that 10 $ng\ ml^{-1}$ of a mouse monclonal MAP kinase antibody, recognising both the 42 and 44 kDa isoforms of MAP kinase (MK12), was used as the primary antibody and the second antibody was a anti-mouse Ig linked to HRP.

MAP kinase activity

Bovine tracheal smooth muscle cells grown in 75 cm^2 flasks and quiesced as outlined above, were incubated with agonists for the times given. The reactions were terminated by the replacement of the buffer with ice-cold 150 mM NaCl. The cells were scraped into 10 ml tubes and centrifuged for 5 min at 1000 *g*. The pellet was transferred to Eppendorf tubes and dispersed into 10 mM Tris buffer (pH 7.4) containing: NaCl 150 mM, EGTA 2 mM, DTT 2 mM, Na_3VO_4 1 mM, PMSF 1 mM, leupeptin 10 $\mu g\ ml^{-1}$ and aprotinin 10 $\mu g\ ml^{-1}$, by repeated dispersion through a G 26 needle. The samples were centrifuged for 15 min at 25 000 *g* and the supernatant assayed for MAP kinase activity with an assay kit (BIOTRAK, Amersham) which measures the incorporation of [γ-^{32}P]-ATP into a specific MAP kinase substrate peptide (KRELVEPLT^{669}PAGEAPNALLR). The reactions were carried out in 75 mM HEPES buffer (pH 7.4) containing 1.2 mM $MgCl_2$, 2 mM substrate peptide and 1.2 mM ATP/1μCi [γ-^{32}P]-ATP in a volume of 30 μl at 37°C for 30 min. The phosphorylated peptide was separated by ion exchange chromatography on P81 ion exchange paper using 75 mM H_3PO_4 and quantified by liquid scintillation counting.

Inositol phosphate accumulation

Cells were grown to confluence on 6 well culture plates and labelled for 48 h in the absence of serum with 1–2.5 $\mu Ci\ ml^{-1}$ of [^{3}H]-myo-inositol. Cells were washed (×3) in phosphate buffered saline (PBS) and incubated with PBS containing 10 mM LiCl for 10 min prior to stimulation. Reactions (2 ml) were terminated by the addition of 400 μl ice cold 3M trichloroacetic acid (TCA) and samples left to extract on ice for a further 20 min. After cell scraping, the content of each dish was transferred to 5 ml insert vials and neutralized extracts prepared from the supernatants using 1,2,-trichlorotrifluoroethane : tri-n-octylamine as detailed previously (Chilvers *et al.*, 1990). An [^{3}H]-IP_{1-4} fraction was separated from [^{3}H]-inositol and [^{3}H]-IP_{5-6} using AG1-×8 Dowex colums eluted with ammonium formate/formic acid buffers and quantified by liquid scintillation (Chilvers *et al.*, 1990).

[^{3}H]-thymidine incorporation

Confluent and quiescent cells were washed twice in serum-free DMEM, agonists added, and the cells incubated for a further 24 h. [^{3}H]-thymidine (0.1 $\mu Ci\ ml^{-1}$) was added for the final 6 h of the incubation. Cells were washed twice in PBS, three times in 5% TCA and twice with ethanol. Cells were solubilised in 0.3M NaOH and [^{3}H]-thymidine incorporation assayed by liquid scintillation counting.

Materials

All antibodies were purchased from Affiniti Research Products Ltd. (Nottingham). Second antibodies, ECL detection reagents and [γ-^{32}P]-ATP (Sp. Act, 3000 $Ci\ mmol^{-1}$) were purchased from Amersham International (Amersham, Bucks). The BIOTRAK MAP kinase assay kit was from Amersham International (Amersham, Bucks). *Myo*-[2-^{3}H]-inositol (17–20 $Ci\ mmol^{-1}$) and [^{3}H]-thymidine (38 $Ci\ mmol^{-1}$) were from New England Nuclear. Other reagents were purchased from Sigma, BDH or were of the highest commercial purity available.

Statistics

Statistical analysis of the data was performed using an unpaired *t* test. Concentration-response curve analysis was performed with an iterative curve fitting procedure (DeLean *et al.*, 1980).

Results

In bovine tracheal smooth muscle cells, PDGF (30 $ng\ ml^{-1}$) stimulated the tyrosine phosphorylation of a number of proteins (Figure 1a). These included two proteins of approximately 40–45 kDa molecular weight which corresponded to the 42 and 44 kDa isoforms of MAP kinase. Phosphorylation was maximal between 5 and 10 min of stimulation and was sustained for up to 60 min. Of the other proteins phosphorylated in response to PDGF, the 120 kDa protein was identified by immunoprecipitation as focal adhesion kinase pp125^{FAK} (Saville *et al.*, 1994). In addition, the tyrosine phosphorylation of a 180 kDa band associated with the autophosphorylation of the PDGF receptor was also observed. The tyrosine phos-

phorylation of pp42 and pp44 MAP kinase was associated with a reduction in the electrophoretic mobility of the phosphorylated form of MAP kinase upon SDS-PAGE (Figure 1b).

BK (100 nM) and ET-1 (100 nM) also stimulated the tyrosine phosphorylation of pp42 and pp44 MAP kinase (Figure 2) and a shift in the mobility of MAP kinase on SDS-PAGE (results not shown). For BK this response was transient peaking between 2 and 5 min before returning to basal values by 15 min (Figure 2a). The maximum response to ET-1 was also obtained between 2 and 5 min; however, the signal was sustained for at least 60 min (Figure 2b).

The tyrosine phosphorylation of MAP kinase and shift in migration on SDS-PAGE was also associated with an increase in the activity of MAP kinase as determined by the phosphorylation of a specific MAP kinase substrate namely the EGF receptor peptide (Table 1 and Figure 3). The fold activation of MAP kinase was greatest in response to ET-1 and PDGF with BK giving a response approximately 40% of that observed with the other agonists (Table 1). This corresponded well with the relative fold increase in the stimulated accumulation of [^{3}H]-IP observed in response to each agonist (Table 1). Histamine (1 mM) had no significant effect upon MAP kinase activity and only a very small effect upon [^{3}H]-IP accumulation (Table 1). Kinetic analysis showed that BK stimulated a transient increase in MAP kinase activity whilst the response to ET-1 was sustained for at least 60 min (Figure 3). Single time point determinations of MAP kinase activity in response to PDGF at 60 min gave an increase in MAP kinase activity of approximately 50% compared to that obtained at 10 min.

Both BK and ET-1 activated MAP kinase in a concentration-dependent manner (Figure 4). For BK and ET-1, activation was observed in the low nanomolar range (EC_{50} values: ET-1, 2.31 ± 1.3nM; BK, 8.71 ± 4.1nM; $n = 3$). The protein kinase C activator, tetradecanoyl phorbol acetate (TPA, 100 nM) also stimulated pp42 and pp44 MAP kinase in bovine tracheal smooth muscle cells as determined by a shift in the mobility of the proteins on SDS-PAGE (Figure 5a). This activation was abolished by the protein kinase C inhibitor, Ro-318220, which was maximally effective at 10 μM (Figure 5b). TPA-stimulated

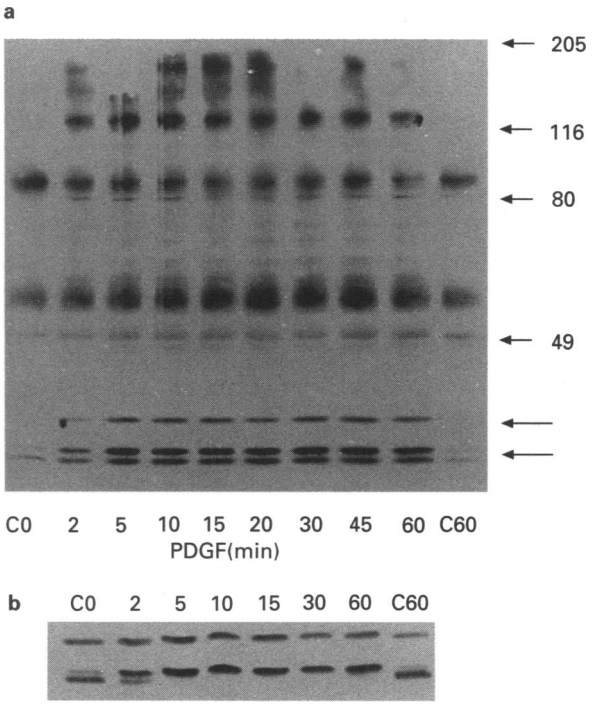

Figure 1 PDGF-stimulated tyrosine phophorylation of pp42 and pp44 MAP kinases in bovine tracheal smooth muscle cells. Cells were incubated with 30 ng ml^{-1} PDGF for the times indicated and then assayed for phosphotyrosine (a) or MAP kinase (b) content as outlined in the Methods section. Each blot is representative of at least three others. Annotations indicate time in min, C = control, P = PDGF. Arrows indicate position of the 42 and 44 kDa proteins.

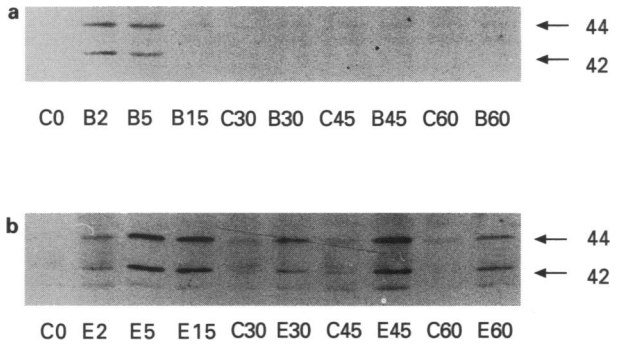

Figure 2 BK and ET-1-stimulated tyrosine phosphorylation of pp42 and pp44 MAP kinase in bovine tracheal smooth muscle cells. Cells were incubated with 100 nM BK (a) or 100 nM ET-1 (b) for the times indicated and then assayed for phosphotyrosine content as outlined in the Methods section. Each blot is representative of at least three others.

Table 1 Agonist stimulation of MAP kinase activity and [^{3}H]-inositol phosphate accumulation in bovine tracheal smooth muscle cells

	Fold stimulation over control values	
	MAP kinase	*[^{3}H]-IP accumulation*
BK	5.4 ± 2.4	5.65 ± 0.94
PDGF	13.7 ± 5.0	6.54 ± 0.58
ET-1	14.8 ± 5.3	9.01 ± 0.97
Histamine	1.3 ± 0.5*	1.2 ± 0.07

For analysis of MAP kinase activity cells were incubated with 100 nM BK for 2 min, 100 nM ET-1 for 5 min, 30 ng ml^{-1} PDGF for 10 min or 1 mM histamine for 5 min, and MAP kinase activity assayed as outlined in the Methods section. For [^{3}H]-IP accumulation cells labelled with [^{3}H]-inositol were incubated with agonist at the concentrations indicated for 30 min then assayed for [^{3}H]-IP accumulation as outlined in the Methods. Each value is the mean ± s.e.mean of at least three experiments performed in duplicate. * Performed twice.

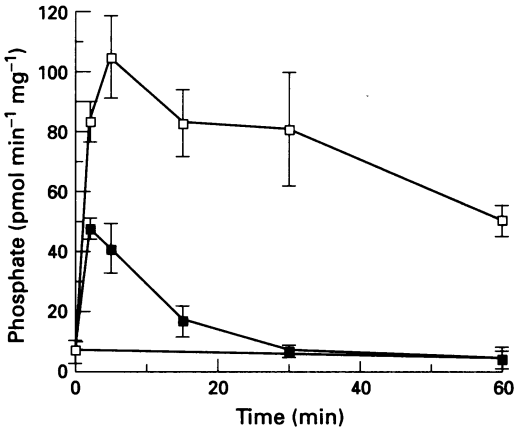

Figure 3 Time course of MAP kinase activity stimulated by ET-1 and BK in bovine tracheal smooth muscle cells. Cells were incubated with vehicle (●), 100 nM BK or 100 nM ET-1 (□) for the times indicated. Cell extracts were assayed for *in vitro* MAP kinase activity as outlined in the Methods section. Each point represents the mean ± s.e.mean of duplicate determinations from a single experiment which is representstive of 2 others.

in vitro MAP kinase activity was also abolished by 10 μM Ro-318220 (pmol phosphate min^{-1} mg^{-1} : control, 17.5±4; TPA, 99.0±6; TPA+Ro-318220, 21.0±7; $n=2$).

Pretreatment of the cells with Ro-318220 also reduced activation of MAP kinase in response to ET-1 (Figure 6a and b); however, this inhibition was dependent upon the duration of agonist stimulation. At 2 min the response to ET-1 was reduced, whilst at later times (60 min) the response to the agonist was increased in the presence of Ro-318220.

Chronic pretreatment of the cells with 300 nM TPA for 24 h also reduced the activation of MAP kinase in response to ET-1 (Table 2). Following 5 min of ET-1 stimulation, the degree of inhibition was approximately 50% (51±7%, $n=3$); however, after 60 min stimulation the inhibition was less (22±7% $n=3$). The response to PDGF at 10 min was reduced by 76±9% ($n=3$) at 10 min whilst the response to BK at 2 min was abolished (Table 3).

Pretreatment of the cells with 50 ng ml^{-1} pertussis toxin for 18 h reduced ET-1 activation of MAP kinase (Figure 7 and Table 3). However, the inhibition was again time-dependent. Approximately 30% of the signal was inhibited after 5 min of stimulation (34±6%, $n=4$); however, the degree of inhibition

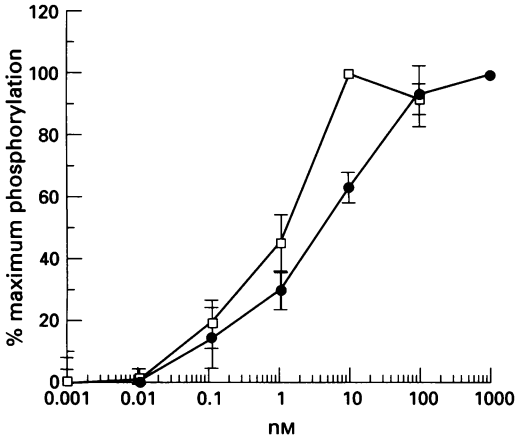

Figure 4 Concentration response curves for ET-1 and BK-stimulated tryosine phosphorylation of pp42 MAP kinase in bovine tracheal smooth muscle cells. Cells were stimulated with increasing concentrations of BK (●) or ET-1 (□) for 5 min and then assayed for phosphotyrosine content. Phosphotyrosine blots were quantified by scanning densitometry. Each point represents the mean±s.e.mean of four individual experiments.

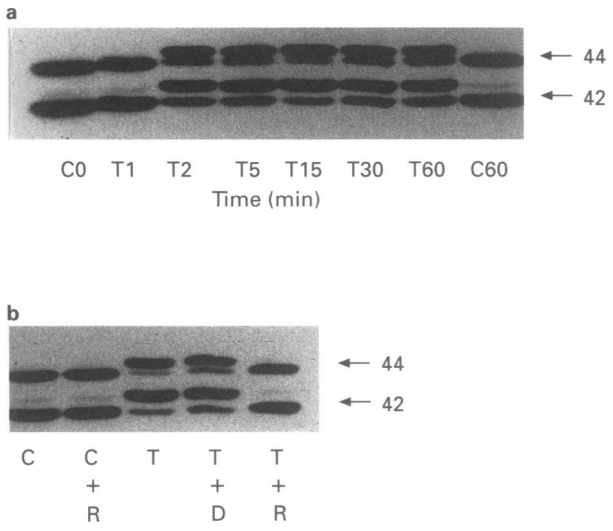

Figure 5 The effect of Ro-319220 on TPA-stimulated activation of pp42 and pp44 MAP in bovine tracheal smooth muscle cells. In (a) cells were incubated with 100 nM TPA for the times indicated. Cell extracts were assayed for MAP kinase content as outlined in the Methods section. In (b) cells were incubated with (0.05%) DMSO (D) or 10μM Ro-318220 (R) for 30 min before stimulation with 100 nM TPA for 10 min. Each blot is a representative example of at least three other experiments.

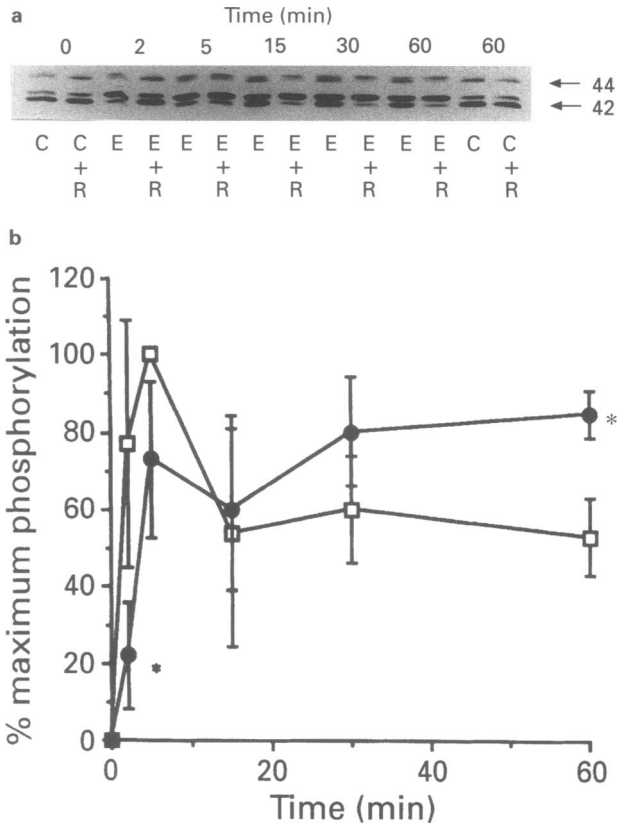

Figure 6 The effect of Ro-318220 on ET-1-stimulated tyrosine phosphorylation of pp42 kinase in bovine tracheal smooth muscle cells in culture. Cells were preincubated with vehicle (□) or 10 μM Ro-318220 (●) for 30 min before stimulation with 100 nM ET-1 for the times indicated. In (a) MAP kinase content was determined was outlined in the Methods section. Each blot is representative of at least three others. In (b) phosphotyrosine blots were quantified by scanning densitometry. Each point represents the mean±s.e.mean of four experiments. (* $P<0.05$ compared to vehicle values).

Table 2 The effect of 24 h TPA pretreatment on agonist-stimulated MAP kinase activity in bovine tracheal smooth muscle cells

	MAP kinase activity (pmol phosphate min^{-1} mg^{-1})	
	Control	*TPA*
Basal	10.5±3.0	8.0±0.5
BK 2 min	62.5±7.0	8.0±1.0*
ET-1 5 min	108.6±9.0	62.0±13.0*
ET-1 60 min	79.5±12.0	50.0±2.0*
PDGF 10 min	168±15.5	39.0±1.3*

Cells were incubated with 300 nM 4α-phorbol (control) or TPA for 24 h before agonist stimulation. Cell extracts were assayed for MAP kinase activity as outlined in the Methods section. Each value (pmol phosphate min^{-1} mg^{-1}) represents the mean±s.e.mean of at least three experiments performed in duplicate (*$P<0.05$ compared to control values).

was approximately 70–80 after 60 min (81±9%, $n=4$). Pertussis toxin pretreatment also reduced ET-1-stimulated accumulation of [^{3}H]-IP over 30 min by approximately 50% (46.8±4.4%, $n=3$). In contrast, pertussis toxin was without effect upon either PDGF- or BK-stimulated MAP kinase activation or [^{3}H]-IP accumulation.

In bovine tracheal smooth muscle cells, ET-1 (100 nM) also stimulated a 3 fold increase in [^{3}H]-thymidine incorporation whilst the response to TPA was approximately 2 fold (Table 4). However, BK (100 nM-1 μM) failed to stimulate a significant response over a number of experiments. Pretreatment with pertussis toxin abolished [^{3}H]-thymidine incorporation in response to ET-1 (Table 4).

In contrast to ET-1, there was approximately a 130 fold increase in [^{3}H]-thymidine incorporation in response to 30 ng ml^{-1} PDGF. The EC_{50} value for this response was 7.30±4.4 ng ml^{-1}, $n=3$ and this compared favourably with that obtained for MAP kinase activity, 9.70±3.2 ng ml^{-1}, $n=3$ (Figure 8).

Discussion

In this study we have examined the characteristics of agonist-stimulated MAP kinase activation in cultures of bovine tracheal smooth muscle cells and sought to correlate these findings with the potential mitogenic capacity of each agonist. We found that the bronchoconstrictor agonists, BK and ET-1, both stimulated the tyrosine phosphorylation and activation of pp42 and pp44 MAP kinase but displayed markedly different kinetics. Unlike a number of other G-protein coupled receptor agonists, ET-1 caused a sustained activation of MAP kinase (Molloy *et al.*, 1993). This compares favourably with growth factors such as PDGF which has been shown to sustain the activation of MAP kinase in other smooth muscle cell systems including vascular smooth muscle cells (Malarkey *et al.*, 1995a). As the kinetics of MAP kinase activation is believed to be important in determining the ability of G-protein coupled receptor agonists such as thrombin to stimulate growth (Kahan., 1992; Pages *et al.*, 1993), this may be an important feature of the effects of ET-1 in this cell type. This conclusion is supported by our observation that BK stimulates only a transient activation of MAP kinase and fails to induce a mitogenic response.

We further investigated the regulatory mechanisms which contributed to ET-1 stimulation of MAP kinase activation. Using the PKC inhibitor, Ro-318220, or chronic TPA pretreatment to downregulate PKC, we found a substantial reduction in ET-1 -stimulated MAP kinase activation, suggesting that a component of the MAP kinase signal involves PKC-mediated activation of an intermediate kinase. This is likely to be either Raf-1 which has been shown to be phosphorylated by PKCα *in vitro* (Kolch *et al.*, 1993) or possibly MEK kinase, a recently discovered MAP kinase kinase kinase which is also believed to be activated in a PKC-dependent manner (Lange-Carter *et al.*, 1993). These results are consistent with studies

Table 3 The effects of pertussis toxin pretreatment on agonist-stimulated MAP kinase activity in bovine tracheal smooth muscle cells

	MAP kinase activity (pmol phosphate min^{-1} mg^{-1})	
	Control	*Pertussis toxin*
Basal	12.0±5.0	14.5±3.0
BK 2 min	66.5±8.0	60.0±9.0$^{n.s.}$
ET-1 5 min	99.0±10.0	69±6.0*
ET-1 60 min	71.5±5.5	25.5±8.5*
PDGF 10 min	108.0±11.0	121.0±13$^{n.s.}$

Cells were incubated with 50 ng ml^{-1} pertussis toxin for 18 h before agonist stimulation. Cell extracts were assayed for MAP kinase activity as outlined in the Methods section. Each value (pmol phosphate min^{-1} mg^{-1}) represents the mean±s.e.mean of at least three experiments performed in duplicate. (*$P<0.05$ versus control stimulation; n.s. not significant).

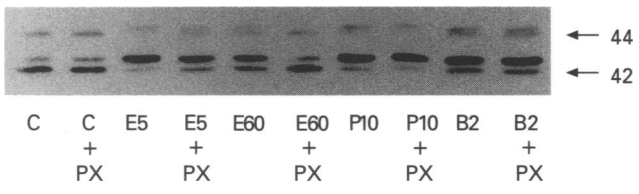

Figure 7 The effect of pertussis toxin pretreatment on ET-1-stimulated activation of MAP kinase mobility shift in bovine airway smooth muscle cells. Cells were incubated with 50 ng ml^{-1} pertussis toxin for 18 h before incubation for the times indicated. Cell extracts were assayed for MAP kinase content as outlined in the Methods section. Each blot is representative of at least 3 others.

Table 4 The effect of pertussis toxin pretreatment on agonist-stimulated [^{3}H]-thymidine incorporation in bovine tracheal smooth muscle cells

	[^{3}H]-thymidine incorporation (d.p.m.)	
	Control	*Pertussis toxin*
Basal	508±21	485±72
Bradykinin (1 μM)	463±58	ND
PMA (100 nM)	927±89	ND
ET-1 (100 nM)	1544±110	664±75*
PDGF (30 ng ml^{-1})	66883±6018	77836±7391^{NS}

Serum-deprived cells were incubated with vehicle or 50 ng ml^{-1} pertussis toxin for 18 h, stimulated with agonists and then assayed for [^{3}H]-thymidine incorporation 24 h later as outlined in the Methods section. Each value is the mean±s.e.mean from four experiments performed in triplicate. (*$P<0.05$ compared to control values; NS, not significant). ND, not determined.

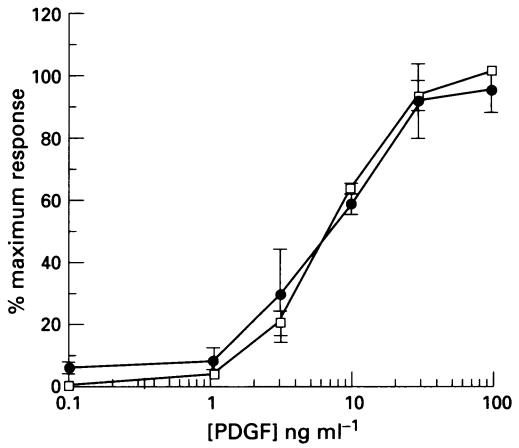

Figure 8 Concentration response curves for PDGF-stimulated *in vitro* MAP kinase activity and [^{3}H]-thymidine incorporation in bovine airway smooth muscle cells. Cells were incubated with increasing concentrations of PDGF and then assayed for *in vitro* MAP kinase (10 min ●) or [^{3}H]-thymidine incorporation (24 h □) as outlined in the Methods section. Each value represents the mean ±s.e.mean of a single experiment performed in triplicate and representative of two others.

which examine MAP kinase activation in response to other G-protein coupled agonists such as angiotensin II and vasopressin (Molloy *et al.*, 1993; Malarkey *et al.*, 1995a). Furthermore, we found that ET-1 also stimulated a substantial increase in the accumulation of [^{3}H]-IP further supporting the potential activation of PKC isoforms by ET-1.

We also observed a substantial PKC dependency in the activation of MAP kinase in response to PDGF. This finding is consistent with observations in vascular smooth muscle cells (Malarkey *et al.*, 1995b). In keeping with these observations we found that PDGF stimulated a substantial increase in [^{3}H]-IP accumulation suggesting that the activation of PLCγ may be a signalling event employed by this agonist in airway as well as in vascular smooth muscle cells (Homma *et al.*, 1993).

In addition, we found that the reduction of the MAP kinase activity in response to ET-1 following inhibition or down regulation of PKC was not as marked at later times and in some instances an enhanced response was observed (e.g. following pretreatment with Ro-318220). A possible explanation for such data may relate to the recent identification of a MAP kinase phosphatase in vascular smooth muscle which is induced by PKC-activating agonists (Duff *et al.*, 1993). It is possible therefore that the inhibition of a PKC-dependent activation of a MAP kinase phosphatase may prolong the duration of the MAP kinase signal.

In contrast to BK, ET-1-stimulated activation of MAP kinase was only partially reduced by PKC inhibition suggesting an additional PKC-independent component of the MAP kinase response. We found that ET-1-stimulated activation of MAP kinase was also sensitive to pertussis toxin. Recently, it has been shown that a number of G-protein coupled agonists such as LPA and thrombin may activate MAP kinase via a pertussis toxin-sensitive, PKC-independent stimulation of-p21^{ras} (van Corven *et al.*, 1993; McLees *et al.*, 1995) and this may well explain part of the ET-1-induced MAP kinase response shown here. However, our experiments have also shown that pertussis toxin pretreatment reduces ET-1-induced accumulation of [^{3}H]-IP in these cells suggesting that the receptor-PLC coupling may be in part transduced by a pertussis toxin-sensitive G-protein. However, in other studies it has been shown that ET-1 stimulates Ca^{2+} influx and that this is also pertussis toxin-sensitive (Simonson & Herman, 1993; Kasuya *et al.*, 1992). Since in both airways and vascular smooth muscle cells (Block *et al.*, 1989; Chilvers *et al.*, 1994) Ca^{2+} influx has been shown to contribute to the activation of polyphosphoinositide hydrolysis, it is thus possible that the effect of pertussis on [^{3}H]-IP accumulation may be indirect.

ET-1 stimulated a 3 fold increase in [^{3}H]-thymidine incorporation and this response was abolished by pertussis toxin pretreatment. This suggests that the activation of a pertussis toxin-sensitive pathway, possibly MAP kinase, may play an important role in the initiation of DNA synthesis. However, although ET-1 stimulated a substantial and sustained activation of MAP kinase which was comparable to that obtained with PDGF, PDGF was approximately 50 times more efficacious in stimulating DNA synthesis. This suggests that a component of the growth factor signalling cascade other than MAP kinase may be involved in stimulating DNA synthesis to the magnitude observed in response to PDGF. Nevertheless the sustained activation of MAP kinase may be essential for the movement of cells from G_0 into the cell cycle following mitogenic stimulation (Pages *et al.*, 1993) and consistent with this proposal is the finding that PDGF-stimulated MAP kinase activity and DNA synthesis exhibit comparable EC_{50} values (Figure 8).

In summary, we have shown that ET-1 stimulates the sustained activation of MAP kinase in bovine tracheal smooth muscle cells and that this is regulated by protein kinase C-dependent and pertussis toxin-sensitive signalling events. Activation of MAP kinase may contribute to the effects of ET-1 as a mitogen in this cell system.

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