Efficacy of agonist-stimulated MEK activation determines the susceptibility of mitogen-activated protein (MAP) kinase to inhibition in rat aortic smooth muscle cells

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In rat aortic smooth muscle cells, platelet-derived growth factor (PDGF) stimulated a sustained activation of mitogen-activated protein kinase (MAP kinase) while the response to angiotensin II (AII) was transient. This was due to a relatively greater initial activation of MAP kinase kinase (MEK) and a correspondingly greater residual MEK activity at later time points. Pretreatment of cells with the novel MEK inhibitor PD 098059 reduced MEK activation at 5 min in response to each agonist by a similar proportion (70%); however, at this time point MAP kinase activation in response to PDGF was only marginally affected while the response to AII was substantially reduced. PD 098059 did, however, reduce PDGF-stimulated MEK activity after 30 min and this correlated with a loss in MAP kinase activity and DNA synthesis. Pretreatment with forskolin also caused a similar pattern of inhibition of agonist-stimulated MEK and MAP

kinase activity. Only following protein kinase C down-regulation were both AII- and PDGF-stimulated MAP kinase activation substantially reduced and this correlated with the virtual loss of both MEK and c-Raf-1 activity in response to both agents. The differential inhibition of MAP kinase activation by forskolin was not due to specific activation of A-Raf by PDGF; both PDGF and AII stimulated A-Raf kinase and this activity was strongly inhibited by forskolin. These results suggest that the efficacy of MEK activation determines the duration of MAP kinase activation and the susceptibility of MAP kinase activation to inhibition by different agents. The results also argue against the selective activation of A-Raf by PDGF as a mechanism to explain the differences in the kinetics of MAP kinase activity stimulated by AII and PDGF.

INTRODUCTION

A major cell signalling pathway involved in the regulation of cell growth and division in response to both growth factors and G-protein-coupled receptor agonists is the mitogen-activated protein kinase (MAP kinase) cascade [1]. The MAP kinases are believed to play a key role in initiating early events in mitogenesis [2–4]. Previously, we and others have shown that a number of growth factors and constrictor peptides such as angiotensin II (AII) and bradykinin (BK) stimulate the activation of MAP kinase in smooth muscle cells of the vasculature and the airways [5–9].

A number of previous studies have used different approaches to inhibit MAP kinase activation with a view to defining the mechanisms that may be involved in the activation of the pathway. These include protocols designed to down-regulate protein kinase C (PKC) isoforms [8], raising intracellular cyclic AMP to stimulate protein kinase A (PKA)-mediated inactivation of c-Raf-1 [10,11], the expression of c-Raf-1 antisense mRNA [12] and assay of MAP kinase kinase (MEK) activities under different conditions [13,14]. These approaches have led to the implication of novel MEK and Raf isoforms in the regulation of MAP kinase activity which can be specifically activated by some mitogens but not others. Recently a number of compounds have been developed which may be useful in further identification and function

of novel MAP kinase cascades. These include SB 203580 which is an inhibitor of Reactivating Kinase (also known as p38 MAP kinase) [15,16] and the recently characterized MEK inhibitor PD 098059 [17,18].

In this study we show that, in vascular smooth muscle cells, PD 098059 substantially inhibits AII-stimulated MAP kinase activation, but has a small effect upon stimulation by platelet-derived growth factor (PDGF). This relative insensitivity is due to a greater MEK activation in response to PDGF, since PD 098059 reduced MEK activation in response to both classes of agonist by the same proportion. In addition, we find that the relative sensitivity of PDGF and AII to inhibition by agents such as forskolin and PKC down-regulation can be explained primarily by the relative magnitude of MEK activation by different agents.

MATERIALS AND METHODS

Materials

All materials were of the highest commercial grades available. Enhanced chemiluminescence (ECL) detection reagents and the BIOTRAK[®] MAP kinase assay kit were obtained from Amersham International (Amersham, Bucks., U.K.), anti-MEK and

Abbreviations used: AII, angiotensin II; BK, bradykinin; EGF, epidermal growth factor; LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase or ERK kinase; PKA, protein kinase A; PDGF, platelet-derived growth factor $\beta\beta$ isoform; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RASM, rat aortic vascular smooth muscle cell; BTSMC, bovine tracheal smooth muscle cell.

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anti-(MAP kinase) antibodies were from Affiniti Research Products Ltd (Exeter, U.K.). Cell culture materials were from Gibco (Paisley, Scotland, U.K.). Anti-C-Raf-1 (c-20) and anti-A-Raf (c-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The pRSETB plasmids containing MEK and MAP kinase were a kind gift from Dr. G. Johnson (University of Denver, CO, U.S.A.). PD 098059 was a kind gift from Dr. A. R. Saltiel (Parke Davis Research Division, Warner Lambert Company, Ann Arbor, MI, U.S.A.).

Expression and purification of histidine-tagged MAP kinase

Wild-type and inactive *Xenopus* MAP kinase and MEK were synthesized as a histidine-tagged fusion protein in the *Escherichia coli* strain BL21(DE3)pLysS following transfection with the pRSETB plasmid containing cDNA inserts as previously defined [19]. The protein was purified from the bacterial cell lysates using a Ni-nitrotri-acetic acid-Sepharose matrix.

Cell culture

Primary cultures of rat aortic smooth muscle cells (RASMCs) and bovine tracheal smooth muscle cells (BTSMCs) were established by enzymic dispersion and cultured as outlined previously [8,9]. All experiments were performed on cells quiesced in serum-free Dulbecco's modified Eagle's medium for 48 h.

MAP kinase immunoblotting and activity

Aliquots of each sample (30–50 µg of protein) were subjected to Western blotting and analysed for MAP kinase content as outlined previously [20]. MAP kinase activity was assayed in detergent lysates using the BIOTRAK® MAP kinase assay kit utilizing the epidermal growth factor (EGF) receptor peptide (EGFR^{661–680}) as substrate [8,9,20].

MEK activity

MEK activity was assayed *in vitro* using partially purified histidine-tagged substrates following immunoprecipitation of the relevant kinase as outlined previously [19]. Briefly, cell extracts were solubilized in 20 mM Tris/HCl (pH 7.5) containing 137 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM Na₂P₂O₇, 1 mM Na₃VO₄, 10 µg/ml aprotinin, 0.1 mM PMSF, 1% Triton X-100 and 10% (v/v) glycerol for 90 min on ice. The samples were then centrifuged at 13000 rev./min at 4 °C for 5 min to collect any insoluble material. Equal amounts (150–200 µg of protein) of precleared lysates were incubated with 1 µg/ml rabbit anti-MEK polyclonal antibody for 90 min at 4 °C with mixing and then for a further 90 min with Pansorbin (Calbiochem). The immunoprecipitates were recovered by centrifugation at 13000 rev./min on a microfuge for 60 s, washed twice in solubilization buffer and twice in the same buffer which did not contain detergents.

For MEK activity assay, immunoprecipitates were further washed twice in 25 mM Hepes buffer, pH 7.4, containing 25 mM β-glycerophosphate, 15 mM MgCl₂ and 1 mM dithiothreitol and then incubated in the same buffer containing 50 µM ATP/5 µCi of [γ -³²P]ATP in the presence of 1 µg of wild-type MAP kinase and 200 µM EGF receptor peptide substrate in a final volume of 30 µl at 30 °C for 15 min. The reaction was terminated on ice and a proportion of the sample assayed for peptide phosphorylation by ion-exchange chromatography while another portion was subjected to SDS/10%-PAGE, the gels dried and then exposed to autoradiographic film.

Raf kinase activity

Confluent and quiescent RASMCs were stimulated as described previously and c-Raf-1 or A-raf was immunoprecipitated from precleared lysates as outlined above except that 1 µg/ml of either anti-c-Raf-1 or anti-A-Raf polyclonal antibody was used in the initial immunoprecipitation step. The immunoprecipitates were recovered and washed in 10 mM Pipes, pH 7.0, containing 100 mM NaCl, 15 mM MnCl₂ and 10 µg/ml aprotinin. The immunoprecipitates were then incubated in the same buffer containing 50 µM ATP/10 µCi of [γ -³²P]ATP and 1 µg of catalytically inactive MEK (MEKB) in a final volume of 30 µl for 15 min at 30 °C. An aliquot of the sample was subjected to SDS/PAGE and analysed by autoradiography.

Initial immunoblotting experiments confirmed full recovery of each kinase in the immunoprecipitates and no difference in recovery following either agonist stimulation or preincubation with inhibitors. Both MEK and Raf assays were initially characterized for linearity with time over a 20 min incubation period (results not shown).

DNA synthesis

Cells were incubated with agents for 18 h and then incubated with 0.1 µCi/ml [³H]thymidine for a further 8 h. Cells were washed once with ice-cold PBS, three times in 5% trichloroacetic acid and then twice in ethanol. The cells were solubilized in 0.5 ml of 0.3 M NaOH for 30 min at room temperature and the radioactivity associated with each sample measured by liquid scintillation counting [9].

Statistical analysis

Statistical analysis employed a paired *t*-test.

RESULTS

Figure 1 shows the time course of MEK and MAP kinase activation in AII- and PDGF-stimulated vascular smooth muscle cells. For both agonists the activation of MEK was transient with the initial peak of activation at 5 min before returning towards basal values by 15–30 min (Figures 1A and 1B). However, it was found that the maximal activation of MEK was some 2–3-fold greater for PDGF than for AII and this gave rise to a higher absolute activation of MEK at later time points relative to the AII signal. The kinetics of MAP kinase activation did not appear to follow the kinetics of MEK activation; MAP kinase activity was only sustained for PDGF and was transient for AII (Figure 1C). Similar results have been obtained previously using phosphotyrosine immunoblotting and retention of MAP kinase on SDS/PAGE [8].

Figure 2 shows the effect of the MEK inhibitor PD 098059 upon AII- and PDGF-stimulated activation of MAP kinase in RASMCs. Preincubation of the cells with PD 098059 substantially inhibited the retardation of MAP kinase on SDS/PAGE in response to a 5 min stimulation of the cells with AII. However, PDGF-stimulated retardation of MAP kinase was inhibited to a lesser extent (Figure 2A) and this was reflected at the level of *in vitro* kinase activity (see Table 1) where AII-stimulated MAP kinase activity was reduced by 65% but PDGF-evoked activity was only decreased by 12% following inhibitor pretreatment. Preincubation with PD 098059, however, reduced maximum MEK activity in response to both AII and PDGF by

Figure 1 The kinetics of MEK and MAP kinase activation in AII- and PDGF-stimulated RASMCs

Cells were incubated with either AII (100 nM; ●) or PDGF (50 ng/ml; □) for the times indicated and then assayed for either MEK (A and B) or MAP kinase activity (C) as outlined in the Materials and methods section. Each value is representative of a single experiment from at least three others.

approximately 70% (71% versus 73% for AII and PDGF respectively; Figure 2B and Table 1).

Preincubation with PD 098059 also substantially reduced the activation of MEK following a 30 min stimulation with PDGF (Figure 2B and Table 1). The degree of inhibition was approximately 90%, reducing the fold stimulation from 6.06- to 1.61-fold. This resulted in an inhibition of MAP kinase activation as assessed by mobility shifts (Figure 2A) and by *in vitro* kinase assay (2.66- to 1.31-fold, see Table 1). In contrast, PD 098059 did not affect the initial activation of c-Raf-1 kinase activity by either agent (results not shown).

In order to determine if the effect of PD 098059 was cell-type-specific, the compound was tested in airways smooth muscle cells and in Rat-1 fibroblasts (Figure 3). In BTSMCs, PD 098059 abolished BK-stimulated activation of MAP kinase but did not affect the response to PDGF (Figure 3A). However, a similar degree of inhibition was observed in Rat-1 fibroblasts stimulated with either EGF or lysophosphatidic acid (LPA) (Figure 3B).

Figure 4 and Table 2 show the effect of raising intracellular cAMP on the activation of MAP kinase and MEK in both vascular and airways smooth muscle cells. In vascular smooth

Figure 2 The effect of PD 098059 on AII- and PDGF-stimulated activation of MAP kinase and MEK in RASMCs

Cells were incubated with 50 μ M PD 098059 for 30 min (PD) and then stimulated with either 100 nM AII (A) or 50 ng/ml PDGF (P) for a further 5 or 30 min. Samples were assayed for MAP kinase levels (A) or MEK activity (B) as outlined in the Materials and methods section. Each experiment is a representative example of at least two others.

Table 1 Effect of PD 098059 on MEK and MAP kinase activation in RASMCs

Cells were incubated with 50 μ M PD 098059 for 30 min and then stimulated with either 100 nM AII or 50 ng/ml PDGF for 5 min. A proportion of the extracts were assayed for MEK and MAP kinase activity as outlined in the Materials and methods section. Results represent the means $\pm$ S.E.M. from four separate experiments. * P < 0.05 compared with stimulation in the absence of PD 098059.

	MEK (fold stimulation)	MAP kinase (fold stimulation)
AII (5 min)	12.62 $\pm$ 1.0	5.1 $\pm$ 0.13
AII + PD 098059	3.42 $\pm$ 1.3*	1.8 $\pm$ 0.4*
PDGF (5 min)	29.2 $\pm$ 6	6.0 $\pm$ 0.24
PDGF + PD 098059	7.9 $\pm$ 2.8*	5.3 $\pm$ 0.5
PDGF (30 min)	6.06 $\pm$ 1.79	2.66 $\pm$ 0.15
PDGF + PD 098059	1.61 $\pm$ 0.2*	1.31 $\pm$ 0.03*

muscle cells, pretreatment with forskolin abolished AII-stimulated MAP kinase activation; however, the maximal response to PDGF was only reduced by 5% (Figure 4A and Table 2). In cells stimulated with PDGF for 30 min the effect of forskolin was much greater, with MAP kinase activity being reduced from 4-fold to 1.2-fold (Table 2).

In contrast, forskolin pretreatment reduced maximal AII- and PDGF-stimulated MEK activation activity by approximately 80% (88% and 83.5% AII versus PDGF respectively; Figure 4B and Table 2). However, because of the differences in the maximal activation of MEK for AII and PDGF, the residual MEK activity was 2–3-fold greater for PDGF than for AII (4.6- versus 2.0-fold, Table 2). Forskolin pretreatment abolished the PDGF-stimulated MEK activity at 30 min (4-fold stimulation to 1-fold; Figure 4B and Table 2), consistent with a loss in MAP kinase activity at this time point.

Activation of c-Raf-1 was affected in the same manner with a comparable loss in kinase activity relative to different levels of maximal stimulation by AII and PDGF (Figure 4C). Forskolin

Figure 3 The effect of PD 098059 upon agonist activation of MAP kinase in BTSMCs and Rat-1 fibroblasts

BTSMCs (A) or Rat-1 fibroblasts (B) were incubated with 50 μ M PD 098059 (PD) for 30 min before stimulation with BK (1 μ M; 2 min), PDGF (50 ng/ml; 5 min) (P), EGF (100 nM) (E), or LPA (10 μ M; 2 min) (L). Samples were assayed for MAP kinase as outlined in the Materials and methods section. Each blot is representative of two others.

Figure 4 Effect of forskolin pretreatment on AII- and PDGF-stimulated MAP kinase, MEK and c-Raf-1 kinase activation in RASMCs

Cells were incubated with 10 μ M forskolin/100 μ M isobutylmethylxanthine (F) for 20 min before stimulation with 50 ng/ml PDGF (P) or 100 nM AII for 5 min or 30 min (A) and then assayed for MAP kinase content (A), MEK activity (B) or c-Raf-1 kinase activity (C). Each experiment is representative of at least three others.

Table 2 Effect of forskolin pretreatment on MEK and MAP kinase activation in vascular and airways smooth muscle cells

Cells (RASMCs or BTSMCs) were incubated with 10 μ M forskolin/100 μ M isobutylmethylxanthine (F) for 20 min before stimulation with 50 ng/ml PDGF (P) or 100 nM AII (A) or 1 μ M BK and then assayed for MAP kinase or MEK activity. Each value is the mean $\pm$ S.E.M. of at least four experiments. $\dagger P < 0.05$ in comparison with non-treated values; ns, not significantly different from controls. Abbreviation: nd, not determined.

		MEK activation (fold stimulation)	MAP kinase activation (fold stimulation)
RASMC	AII (5 min)	16.5 $\pm$ 0.8	5.1 $\pm$ 0.4
	AII + F	2.0 $\pm$ 0.5 $\dagger$	1.2 $\pm$ 0.2 $\dagger$
	PDGF (5 min)	27.8 $\pm$ 3.2	5.88 $\pm$ 0.5
	PDGF + F	4.6 $\pm$ 1.6 $\dagger$	5.2 $\pm$ 0.5 ^{ns}
	PDGF (30 min)	4**	4.04 $\pm$ 1.5*
	PDGF + F	1**	1.21 $\pm$ 1.5*
BTSMC	BK (2 min)	2.58**	2.47 $\pm$ 0.02*
	BK + F	1.2**	1.48 $\pm$ 0.1*
	PDGF	14.47**	5.45 $\pm$ 0.6*
	PDGF + F	3.29**	4.66 $\pm$ 0.6*
	PDGF (30 min)	nd	3.05 $\pm$ 0.15*
	PDGF + F	nd	1.05 $\pm$ 0.08*

* Performed three times
** Performed twice.

Figure 5 Effect of chronic PMA pretreatment on AII- and PDGF-stimulated MEK and c-Raf-1 activity in RASMCs

Cells were incubated with 100 nM PMA or vehicle for 24 h then stimulated with 100 nM AII (A) or 50 ng/ml PDGF (P) for 5 min. Samples were assayed for MEK activity (A) or c-Raf-1 kinase (C). In (B) and (D) MEK and Raf kinase activity was quantified by either EGF peptide phosphorylation (MEK assay, B) or by densitometry (Raf assay, D). Each value represents the mean $\pm$ S.E.M. from at least three experiments. * indicates significantly different from non-treated cells ($P < 0.05$).

Figure 6 Effect of forskolin on AII- and PDGF-stimulated A-Raf kinase activity in RASMCs

Cells were incubated with 10 μ M forskolin/100 μ M isobutylmethylxanthine (F) for 20 min before stimulation with 50 ng/ml PDGF (P) or 100 nM AII for 5 min (A) and then assayed for A-Raf kinase activity. (A) A representative autoradiograph of a single experiment repeated three times. (B) Autoradiographs were quantified by densitometry. Each value is the mean $\pm$ S.E.M. of four experiments. *Indicates significantly different from non-treated cells ($P < 0.05$).

reduced AII-stimulated c-Raf-1 kinase activity by $96 \pm 5\%$ and PDGF by $78 \pm 10\%$ ($n = 4$), resulting in a greater residual c-Raf-1 kinase activity remaining after PDGF stimulation. These differences seemed to be a function of the initial magnitudes of c-Raf-1 activation by both agents because when 25 ng/ml PDGF was used to stimulate Raf-1 kinase activity to a level similar in magnitude to AII, kinase activity was completely abolished (results not shown). Forskolin pretreatment prior to 30 min stimulation with PDGF resulted in a complete abolition of c-Raf-1 kinase activity (Figure 4).

Similar results were obtained in airways smooth muscle cells for BK and PDGF at the level of both MAP kinase and MEK (Table 2). A 2.5-fold activation of MEK by BK was substantially inhibited by forskolin ($> 95\%$) and this resulted in an approximate 60% inhibition of MAP kinase activity (2.47-fold to 1.48-fold). PDGF-stimulated MEK activity was also substantially reduced from 14.47-fold to 3.29-fold, but this resulted in only a 14.5% decrease in MAP kinase activity decreasing from 5.45- to 4.66-fold.

Differential inhibition of MAP kinase was further investigated following down-regulation of PKC isoforms using chronic phorbol 12-myristate 13-acetate (PMA) pretreatment. Previously, we have shown that down-regulation of PKC α and ϵ isoforms following 24 h chronic PMA pretreatment resulted in the abolition of AII-stimulated retardation in MAP kinase on SDS/PAGE [8]. A similar down-regulation protocol reduced both MEK and c-Raf-1 activation in response to both AII and PDGF by more than 95% (Figures 5A–5C). This resulted in complete abolition of MAP kinase activation in response to AII and a substantial inhibition in response to PDGF of approximately 90% (c.p.m.: control = 4180 ± 173 ; control + PMA = 5455 ± 123 ; AII = 12766 ± 2467 ; AII + PMA = 3396 ± 226 ; PDGF = 18122 ± 1277 ; PDGF + PMA = 6559 ± 294 , $n = 3$).

Table 3 Effect of PD 098059 and forskolin on PDGF-stimulated DNA synthesis in RASMCs

Cells were incubated with 50 μ M PD 098059 (PD) for 30 min or 10 μ M forskolin (F) for 20 min before stimulation with 50 ng/ml PDGF for 26 h. After 18 h 0.1 μ Ci/ml of [3 H]thymidine was added and [3 H]thymidine incorporation measured as outlined in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of three experiments performed in triplicate. Abbreviation: Cont., untreated control cells.

	Thymidine incorporation (d.p.m.)
Cont.	967 $\pm$ 231
Cont. + PD	1415 $\pm$ 447
PDGF	12895 $\pm$ 1200
PDGF + PD	1053 $\pm$ 695
Cont. + F	605 $\pm$ 89
PDGF + F	1654 $\pm$ 759

In order to exclude the potential for another, PDGF-specific, mechanism of MEK or MAP kinase activation which did not involve c-Raf-1 we further examined the characteristics of A-Raf activation, a Raf isoform known to be expressed in cells of mesodermal origin. Figure 6 shows that both AII and PDGF stimulated A-Raf kinase activity in RASMCs. The magnitude of activation over a number of experiments was approximately similar to that observed for c-Raf-1 (PDGF was 1.6-fold greater than AII). Forskolin substantially reduced A-Raf kinase activity in response to either agonist (percentage inhibition: AII = 78 ± 6 ; PDGF = 64 ± 7).

Finally, we assayed PDGF-mediated stimulation of [3 H]thymidine incorporation into RASMCs to determine whether inhibition of MAP kinase at late time points by PD 098059 or forskolin was sufficient to prevent DNA synthesis in response to PDGF. Table 3 shows that PD 098059 substantially reduced [3 H]thymidine incorporation stimulated by PDGF ($> 90\%$ inhibition) while forskolin abolished the response. Forskolin also inhibited basal [3 H]thymidine incorporation by approximately 30%.

DISCUSSION

In this study we have examined the effects of inhibitors of intermediates of the MAP kinase cascade on both vasoconstrictor and growth factor activation of MAP kinase. We have done this since a number of recent reports have shown differences in the sensitivity of growth factor and G-protein-coupled receptor activation of MAP kinase to inhibition, particularly in airways smooth muscle cells [10–12]. From these findings, it has been proposed that specific growth factor-mediated activation of novel Raf and MEK isoforms may underlie this difference in sensitivity and may also explain the well-recognized differences in the kinetics of MAP kinase activation by these two classes of agonist (see [1] for a review). However, our evidence suggests that this could be mainly accounted for by differences in the relative magnitude of Raf and MEK activation by growth factors relative to G-protein-coupled receptor agonists, with little evidence for additional, growth factor-specific, mechanisms of activation.

Initially we found transient activation of MEK in response to both AII and PDGF. Transient activation of MEK has been previously observed in a number of cell types in response to several different classes of growth factor and also to certain G-protein-coupled agonists [21–23]. This is irrespective of the time course of the MAP kinase activation observed for each agonist, suggesting that prolongation of MEK activity is not necessary to sustain the MAP kinase signal. However, a significant difference

was observed between AII and PDGF in the initial magnitude of MEK activation which resulted in a greater absolute amount of MEK activation in response to the growth factor, particularly at later times. Our studies suggest that this residual MEK activation may exceed a threshold activity which can sustain MAP kinase activity in several cell types, including smooth muscle cells.

The relative difference in the magnitude of MEK activation in response to PDGF and AII was significant in determining how much inhibition of MAP kinase occurred in response to different agents and at different stimulation times. PD 098059 reduced by a similar proportion (approximately 70%) MEK activation by AII and PDGF. However, AII-stimulated MAP kinase activation was reduced by a much greater proportion. Similar findings were presented by Alessi et al., in Swiss 3T3 fibroblasts [24], which confirm a large signalling reserve at the level of MEK, suggesting that a limiting feature of the cascade is the amount of MAP kinase available for activation. However, we have shown here that the inhibition of the MEK signal by PD 098059 after 30 min of PDGF stimulation results in a corresponding decrease in MAP kinase activity, suggesting that at this time point the inhibition of MEK activation has an important bearing on whether the activation of MAP kinase is sustained.

These results were not restricted to vascular smooth muscle. BTSMCs stimulated with BK and PDGF showed a similar difference in sensitivity to PD 098059 and a corresponding difference in the magnitude of MEK activation (results not shown). However, in some cell types we found little difference in the sensitivity of MAP kinase activation to PD 098059 such as in EGF- and LPA-stimulated Rat-1 fibroblasts. This is consistent with recent results which indicate that there is vast cellular variation in the level of MEK activation in relation to MAP kinase activation across different cell types and in response to different agonists [24].

A similar phenomenon was also observed when cells were pretreated with forskolin. Forskolin reduced MEK and c-Raf-1 activation by approximately 80% and again this allowed differential sensitivity to be manifest at the level of MAP kinase. These results adequately explain the observations by a number of other laboratories which have shown differential effects of forskolin upon the activation of MAP kinase and upstream kinases in response to growth factors in both vascular and airways smooth muscle [11,25]. Furthermore, forskolin, like PD 098059, was more effective at later times, indicating that the inhibition of low levels of MEK activity limits MAP kinase activation generated by a growth factor.

Down-regulation of PKC also resulted in almost complete abolition of both c-Raf-1 and MEK activation. This was reflected in a relatively small difference in sensitivity at the level of MAP kinase where a substantial proportion (> 90%) of the PDGF-stimulated signal was lost. This once again may explain the observations from a number of laboratories which show marked differences in the relative sensitivity of MAP kinase activation by different growth factors and G-protein-coupled agonists to PKC down-regulation [8,9,26,27]. It has been widely assumed from these findings that G-protein-coupled receptor agonists and growth factors activate c-Raf-1 by different mechanisms [1]. In this regard it is of particular interest that PKC down-regulation abolishes c-Raf-1 activation irrespective of the nature of the stimulus. Previously we have shown that PKC ϵ is most likely to be involved in the regulation of MAP kinase in vascular smooth muscle cells [8]. Since we also find that down-regulation of PKC α by bryostatin-1 does not affect the activation of c-Raf-1 by either PDGF or AII (results not shown), it is possible that PKC ϵ , rather than PKC α as initially proposed [28], may activate c-Raf-1 in addition to other factors such as Src or lipids [29,30].

It is possible, however, that the results obtained in this study may be explained by differential regulation, by AII in comparison with PDGF, of phosphatases such as the MAP kinase phosphatases and PP2A. However, in agreement with recent reports (for example [21]), we do not find that MKP-1 plays a significant role in the regulation of MAP kinase activation or termination in RASMCs [8]. In further preliminary experiments, we have found that the recently described, cytosolically located MAP kinase phosphatase, MKP-2 [31], is not expressed in this cell type (A. Graham and R. Plevin, unpublished work). It is also possible that phosphatases such as PP2A, acting at the level of MEK or Raf, may be involved [21]; however, the finding that the kinetics of AII- and PDGF-stimulated MEK activity are very similar suggests that agonist specific differences in the regulation of phosphatases upstream of MAP kinase are unlikely to completely account for the differences in the kinetics of MAP kinase activation. However, without a complete *in vitro* and *in vivo* characterization of the individual phosphatases involved at each level of the cascade it is impossible to determine the relative influences of kinase or phosphatase activation upon the system.

Although activation of a single c-Raf-1/MEK cascade was deemed sufficient to explain the differential effects of inhibitors upon AII- and PDGF-stimulated MAP kinase activity, we have also examined the activation of another Raf isoform expressed in vascular smooth muscle cells, A-Raf. It has been suggested that in some cell types other Raf isoforms such as B-Raf and A-Raf may show reduced sensitivity to PKA-mediated phosphorylation under different conditions [31,32] and this may explain the relative insensitivity of MAP kinase activation to inhibition. We observed nothing about the activation of A-Raf which could provide an alternative explanation for the differences in sensitivity of AII- and PDGF-stimulated MAP kinase activity to inhibition except that based on the efficacy of the initial signal. Both PDGF and AII activated A-Raf with similar relative magnitude to c-Raf-1, and kinase activity in both cases was sensitive to forskolin pretreatment. Again there was a residual PDGF-stimulated A-Raf activity following forskolin pretreatment and while this will contribute to the reduced sensitivity of MEK and MAP kinase to forskolin as indicated in another study [32], in itself it does not constitute evidence for a novel A-Raf/MEK cascade activated solely by PDGF.

While the efficacy of the MEK signal may make growth factor activation of MAP kinase relatively insensitive to PD 098059 and forskolin, nevertheless, these agents will be important in preventing cell growth in response to PDGF and other growth factors because, as shown here, they are able to terminate MAP kinase activation more rapidly. Sustained activation of MAP kinase is believed to be important in the activation of vascular smooth muscle cell growth [32] and division, and the finding that PDGF stimulates DNA synthesis and AII does not, supports this hypothesis [33]. Indeed, we have found that both PD 098059 and forskolin substantially reduce PDGF-stimulated DNA synthesis (Table 3). Future studies examining the effects of PD 098059 and other inhibitors of the MAP kinase cascade may reveal these as useful therapies for hypertension and asthma where smooth muscle cell hyperplasia is a feature, allowing activation of cellular events which are regulated by acute activation of MAP kinase but inhibiting events where sustained activation of MAP kinase is required.

We would like to thank Dr. I.M. Varndell from Affiniti Research Products, (Exeter, U.K.) for the initial gift of specific anti-MEK antibodies, Drs. D.A. Cohen and L. Cowell (Amersham International, Bucks., U.K.) for the kind gift of the BIOTRAK™ MAP kinase assay kit, Dr. G.L. Johnson (Denver, CO, U.S.A.) for the gift of the MAP kinase and MEK plasmids and Dr. Alan Saltiel (Park Davis, Warner Lambert, Ann Arbor, U.S.A.) for the kind gift of PD 098059. The work carried out for this paper was funded

by grants to R.P. and G.W.G. from the Medical Research Council and Wellcome Trust. G.W.G. is a 1992 Lister Institute of Preventative Medicine Research Fellow.

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