Differential regulation of extracellular signal-regulated protein kinases (ERKs) 1 and 2 by cAMP and dissociation of ERK inhibition from anti-mitogenic effects in rabbit vascular smooth muscle cells

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The inhibition of extracellular signal-regulated protein kinases (ERKs) is implicated in the negative regulation of vascular smooth muscle cell (VSMC) mitogenesis by cAMP-elevating agents and transforming growth factor β_1 (TGF- β_1). These factors inhibited rabbit aortic VSMC mitogenesis induced by platelet-derived growth factor (PDGF)-BB by preventing the entry of cells into S-phase. cAMP-elevating agents partly inhibited the late phase (1–4 h) of activation of ERKs 1 and 2 induced by PDGF-BB without inhibiting the early phase of activity (5–15 min) and had no effect on activity induced by basic fibroblast growth factor (bFGF). In contrast, cAMP elevation caused a marked inhibition of early ERK activation induced by angiotensin II and thrombin. TGF- β_1 had no inhibitory effect on ERK activation induced by PDGF-BB or bFGF. The inhibition of PDGF-BB-stimulated DNA synthesis by either forskolin/3-isobutyl-1-methylxanthine (IBMX) or TGF- β_1 was not decreased

when the agents were added up to 8 h after growth factor. In contrast, the selective ERK kinase inhibitor PD98059 was a weak inhibitor of DNA synthesis; a combination of PD98059 and forskolin/IBMX had an additive inhibitory effect on DNA synthesis. Forskolin/IBMX inhibited the growth factor-induced expression of *c-myc* mRNA and cyclin D₁ protein, and enhanced the protein expression of p27^{kip1}. TGF- β_1 had no effect on the expression of *c-myc* or p27^{kip1} and weakly attenuated the expression of cyclin D₁. These findings support the conclusion that the suppression of VSMC mitogenesis by cAMP and TGF- β_1 is independent of ERK inhibition. Anti-mitogenic effects of cAMP might be primarily mediated by events in late G₁.

Key words: angiotensin II, cyclin, platelet-derived growth factor (PDGF), protein kinase C (PKC).

INTRODUCTION

Vascular smooth muscle cell (VSMC) proliferation is important in the pathogenesis of atherosclerosis and its complications [1,2]. cAMP-elevating agents and transforming growth factor β_1 (TGF- β_1) are implicated in the negative regulation of VSMC proliferation [3–10]. Various cAMP-elevating agents and agonists inhibit mitogenesis of VSMCs in culture and VSMC hyperplasia *in vivo* [4–6]. TGF- β_1 inhibits the proliferation of VSMCs and is implicated in the suppression of VSMC proliferation in neointima formation *in vivo* [7–10] but has also been reported to potentiate VSMC proliferation, depending on species, growth state and TGF- β_1 receptor isotype expression [11–13]. However, the mitogenic effects of TGF- β_1 in VSMCs are thought to be indirect and to be mediated at least in part via the TGF- β_1 -induced autocrine expression of platelet-derived growth factor (PDGF)-AA [14,15].

The activation of extracellular signal-regulated protein kinases (ERKs) 1 and 2 is thought to be central in mitogenic signalling in many cell types. The major pathway for ERK activation by polypeptide ligands for receptor tyrosine kinases occurs via GRB-2/Ras-dependent stimulation of Raf-1 and mitogen-activated protein kinase kinase (MEK), the dual-specificity kinase that specifically activates ERKs 1 and 2 [16,17]. Protein kinase C (PKC) is thought to mediate the activation of ERKs by phorbol esters and by some agonists for seven-transmembrane-domain receptors linked to G-proteins that activate β isoforms of phospholipase C, leading to the generation of diacylglycerol [18–23]. cAMP-elevating agents have been reported to inhibit the

activation of ERK and Raf-1 in fibroblasts and VSMCs [24–27]. TGF- β has also been reported to inhibit ERKs [28]. However, it remains unclear whether the inhibition of ERKs is obligatory or important for the anti-mitogenic effects of cAMP-elevating agents or TGF- β in VSMCs. Furthermore, several reports show that cAMP-elevating agents do not inhibit the activation of ERKs in cells subject to cAMP-dependent inhibition of mitogenesis [29–32].

In the present study we investigated the contribution of ERK inhibition to the negative regulation of rabbit aortic VSMC mitogenesis by cAMP and TGF- β_1 . The results show that, although ERK activation in VSMCs is regulated by cAMP, this regulation is highly dependent on the activating ligand. Furthermore, TGF- β_1 had no effect on ERK activity and evidence is presented that the cAMP-dependent inhibition of ERKs can be dissociated from the anti-mitogenic action of either cAMP-elevating agents or TGF- β_1 . Instead, our findings suggest that events in later G₁ are more likely to mediate the inhibition of VSMC mitogenesis.

MATERIALS AND METHODS

Cell culture

VSMCs were cultured from explants of rabbit aortic tunica media in Dulbecco's modified Eagle's medium containing 20% (v/v) fetal calf serum and other supplements, as described previously [33]. For experimental purposes, confluent cultures were rendered quiescent by incubation with Dulbecco's modified Eagle's medium containing 0.5% (v/v) fetal calf serum for 40 h.

Abbreviations used: ERK, extracellular signal-regulated protein kinase; bFGF, basic fibroblast growth factor; IBMX, 3-isobutyl-1-methylxanthine; MBP, myelin basic protein; MEK, mitogen-activated protein kinase kinase; PDGF, platelet-derived growth factor; PGE₂, prostaglandin E₂; PKC, protein kinase C; TGF- β_1 , transforming growth factor β_1 ; VSMC, vascular smooth muscle cell.

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Figure 1 cAMP-elevating agents and TGF- β_1 inhibit the reinitiation of DNA synthesis in rabbit VSMCs

VSMCs were incubated with [3 H]thymidine for 30 h (0–30) or for a total of 3 h from 12–15, 15–18 or 18–21 h after the addition of PDGF-BB and other factors. At 0 h, cells were treated with no additions (control cells, open bars), or with 25 ng/ml PDGF-BB (hatched bars), 25 ng/ml PDGF-BB plus 6 μ M forskolin/100 μ M IBMX (**A**, filled bars), or 25 ng/ml PDGF-BB plus 10 ng/ml TGF- β_1 (**B**, filled bars). Values here and in Figure 7 are expressed as percentages of the maximum PDGF-induced incorporation and are means $\pm$ S.E.M. ($n = 3$). These correspond to PDGF-BB-induced [3 H]thymidine incorporation after labelling for 30 h or during the 12–15, 15–18 and 18–21 h periods of 49 864, 3082, 20 570 and 21 758 c.p.m. per 33 mm dish of VSMCs ($n = 3$) respectively. The incorporation of [3 H]thymidine under control conditions was 5–10% of the stimulated values; the stimulation of [3 H]thymidine incorporation by PDGF was 20–75% of the stimulation by serum.

All treatments with factors were performed in serum-free media. In experiments involving treatments with reagents dissolved in DMSO, all cell cultures were treated with an equal volume of this solvent.

Measurement of mitogenic activity

DNA synthesis was assessed by the determination of [3 H]-thymidine incorporation into trichloroacetic acid-precipitable material, as described [34]. Cell proliferation was determined by counting cells after incubation for 96 h in the presence of 0.5% (v/v) fetal calf serum and other additions as described [33].

cAMP measurements

Cells were washed twice with ice-cold PBS on ice; cAMP was extracted by the addition of 200 μ l of 0.1 M HCl. Samples were centrifuged at 15 000 g for 15 min at 4 $^{\circ}$ C and the supernatant was recovered and neutralized by the addition of 1 M K_3PO_4 as described [34]. cAMP was measured by enzyme-linked immunoassay, in accordance with the manufacturer's instructions (Amersham). Cellular protein in each sample was measured with a bicinchoninic acid protein assay kit (Pierce).

Northern analysis

RNA was extracted by using RNA-ZolBTM (Stratagene); *c-myc* expression was evaluated by Northern analysis by using standard procedures [35] with an 850 bp *Pst*I/*Hind*III fragment (nt 540–1390) of human *c-myc* cDNA (provided by Dr. H. Land, Imperial Cancer Research Fund, London, U.K.). Equal loading of RNA was verified by the hybridization of filters with a 28 S rRNA anti-sense oligonucleotide probe (Clontech).

Western blotting

Whole cell extracts were prepared and assayed for ERK activation by Western blotting with specific antibodies against the activated forms of ERKs, as described [36]. Cyclin D₁ immunoblotting was performed on whole cell extracts. Immunoreactive bands were detected by enhanced chemiluminescence with horseradish peroxidase-conjugated anti-rabbit IgG and ECLTM reagent.

Autoradiograms were semi-quantified by scanning densitometry with an LKB Ultrosan XL densitometer. The values shown in the Figures have been calculated from the peak areas corresponding to the 42 kDa ERK protein bands.

Myelin basic protein (MBP) kinase assay

The MBP assay for ERK activity was performed as described previously [37]. Immunoprecipitates were prepared from cell lysates with the use of a polyclonal anti-ERK2 antibody and resuspended in 40 μ l kinase buffer [20 mM Hepes (pH 7.4)/10 mM $MgCl_2$ /1 mM dithiothreitol/10 mM *p*-nitrophenyl phosphate] containing 10 μ g of MBP and 50 μ M ATP with 3 μ Ci of [γ - 32 P]ATP, then incubated for 10 min at 30 $^{\circ}$ C. Reactions were terminated by the addition of 40 μ l of 2 $\times$ SDS/PAGE sample buffer. Samples were heated at 95–100 $^{\circ}$ C for 5 min and SDS/PAGE was performed with 12% polyacrylamide gels. Gels were fixed, dried and exposed to X-ray film.

Statistical analysis

Statistical analysis of the difference between means obtained from multiple experiments was performed by using one-way analysis of variance. $P < 0.05$ was considered to be significant.

Materials

PDGF-BB and TGF- β_1 were from R&D Systems. PMA, angiotensin II, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and prostaglandin E₂ (PGE₂) were from Sigma-Aldrich Ltd. Prostaglandin analogues were provided by Schering AG. PD 98059 and Ro-31-8220 were from Calbiochem. Antibodies against activated forms of ERKs and against total ERKs (ERKs 1 and 2) were from Promega and New England Biolabs. Polyclonal anti-ERK2 and antibody against p27^{kip1} were from Santa Cruz. Rabbit polyclonal cyclin D1 antibody was the gift of Dr. Gordon Peters (ICRF, London, U.K.); 28 S rRNA anti-sense oligonucleotide probe (Clontech) was from Clontech UK Ltd. [3 H]Thymidine (84 Ci/mmol), cAMP assay kits, [γ - 32 P]ATP (approx. 5000 Ci/mmol) and ECLTM reagents were from Amersham. All other materials and reagents used were of the purest grade available.

RESULTS

Inhibition of VSMC mitogenesis by cAMP and TGF- β

TGF- β_1 and forskolin/IBMX were effective inhibitors of rabbit aortic VSMC DNA synthesis and proliferation induced by PDGF-BB, basic fibroblast growth factor (bFGF) or serum (results not shown). To determine whether the inhibition of mitogenesis by cAMP-elevating agents and TGF- β_1 was due to the prevention of entry of cells into S-phase, cells were treated with PDGF-BB in the presence or absence of either TGF- β or

Figure 2 cAMP partly inhibits the late phase of PDGF-BB-induced ERK activation

VSMCs were preincubated for either 30 min (A, top panel, and B–D) or for 3 h (A, bottom panel) in the absence (control, con, –) or the presence (FK + IBMX, +) of 6 μ M forskolin/100 μ M IBMX; either 25 ng/ml PDGF-BB (A–C) or 10 ng/ml bFGF (D) was then added for the indicated durations in minutes. ERK activation was determined by Western blotting (A, B and D) or by MBP kinase assay (C) as described in the Materials and methods section. Semi-quantification of four experiments similar to that in the top panel of (A) is presented as fold increases (means $\pm$ S.E.M.) in p42 ERK activity above the basal level (B). * $P < 0.03$ for PDGF-BB + FK/IBMX compared with PDGF-BB alone at 60 min. Other results shown are representative of three to six experiments.

Figure 3 Partial inhibition of late-phase PDGF-BB-induced ERK activation by PGE₂ and prostacyclin agonists

VSMCs were preincubated for 30 min in the absence (control) or the presence of 6 μ M forskolin/100 μ M IBMX (FK + IBMX) (A), 10 μ M PGE₂/100 μ M IBMX (PGE₂ + IBMX) (B), 100 nM iloprost/100 μ M IBMX (ILO + IBMX) (C) or 100 nM cicaprost/100 μ M IBMX (CICA + IBMX) (D); 25 ng/ml PDGF-BB was then added for the durations indicated in minutes. ERK activation was determined by Western blotting. PGE₂/IBMX, iloprost/IBMX and cicaprost/IBMX decreased ERK activity to mean values ($n = 3$) of 45%, 36% and 35% of PDGF-BB-induced ERK activity at 60 min respectively.

6 μ M forskolin/100 μ M IBMX and then pulsed for 3 h with [³H]thymidine at different times after the addition of factors. The degrees of inhibition by either forskolin/IBMX (Figure 1A) or TGF- β_1 (Figure 1B) were very similar after incubating cells with [³H]thymidine from 12 to 15 h, from 15 to 18 h, from 18 to 21 h or from 0 to 30 h. These results indicate that TGF- β and forskolin/IBMX act in G₁ to inhibit DNA synthesis by decreasing the number of cells entering S-phase. Half-maximal inhibitory effects of forskolin/IBMX and TGF- β_1 were given by 0.5 μ M forskolin and 5–7.5 ng/ml TGF- β_1 ; both factors also inhibited cell proliferation as determined by cell counting (results not shown). PGE₂ and the specific prostacyclin agonist cicaprost, which increase intracellular cAMP concentration via receptor-mediated activation of adenylate cyclase, were also effective inhibitors of PDGF-BB-induced DNA synthesis and proliferation in the presence of IBMX (results not shown). Forskolin/IBMX, cicaprost/IBMX and PGE₂/IBMX induced increases in intracellular cAMP above control levels (6.6 pmol/mg of protein; $\pm$ S.E.M., $n = 10$) of 9.8 ± 1.9 -fold, 7.8 ± 0.6 -fold and 4.5 ± 0.3 -fold, respectively ($\pm$ S.E.M., $n = 4$ –8).

Effects of cAMP and TGF- β on ERK activation

PDGF-BB induced a time-dependent increase in the level of activated ERKs 1 and 2 in VSMCs with a rapid and transient increase in activity (maximum from 5 to 15 min) followed by a period of decreased activity sustained for 1–2 h (Figure 2).

Figure 4 Inhibition of angiotensin II- and thrombin-induced ERK activation by cAMP-elevating agents

VSMCs were preincubated for 30 min in the absence (control) or the presence of either 6 μ M forskolin/100 μ M IBMX (FK + IBMX) (**A–C**) or 100 nM cicaprost/100 μ M IBMX (CICA + IBMX) (**A** and **B**); 10 ng/ml angiotensin II (**A,B**) or 1 i.u./ml thrombin (**C**) was then added for the indicated durations in minutes. ERK activation was determined by Western blotting. (**B**) Semi-quantified results presented as fold increases (means $\pm$ S.E.M.; $n = 3$) in p42 ERK activation.

Western blotting of parallel samples with antibody against active and inactive ERKs showed that cell extracts contained equal amounts of total ERK immunoreactivity (results not shown). Forskolin/IBMX had no detectable effect on the early phase of maximum ERK activation induced by PDGF-BB but consistently caused a partial decrease in the late phase of ERK activation to 35 % and 32 % ($n = 6$) of the stimulated value after 60 and 120 min, respectively (Figures 2A and 2B). Pretreatment with forskolin/IBMX for up to 3 h caused a similar decrease in ERK activation after 60 min of treatment with PDGF-BB without decreasing the acute phase of activation (Figure 2A, lower panel). Determination of ERK activation with the MBP kinase assay showed that pretreatment with forskolin/IBMX had no effect on MBP phosphorylation stimulated by a 15 min treatment with PDGF-BB, but decreased MBP phosphorylation after 60 min to near the basal level (Figure 2C). In the presence of IBMX, PGE₂ and the prostacyclin agonists iloprost and cicaprost partly decreased PDGF-BB-stimulated ERK activation at 60 min to 45 %, 36 % and 35 % of the stimulated level ($n = 3$) respectively, without decreasing the early phase (Figures 3A–3D). Similarly to results with PDGF-BB, forskolin/IBMX had no effect on ERK activation induced by bFGF after 15 min. In some experiments forskolin/IBMX caused a modest decrease in

Figure 5 Effects of PKC inhibitors on ERK activation

VSMCs were preincubated for 30 min in the absence (control, con) or the presence of 3 μ M Ro-31-8220 (Ro), 3 μ M GF-109203X (GF) or 6 μ M forskolin/100 μ M IBMX (FK + IBMX, FK/I). After pretreatments, 25 ng/ml PDGF-BB (**A, B**), 100 ng/ml PMA (**C**, left panel), 1 i.u./ml thrombin (**C**, right panel) or 10 ng/ml angiotensin II (**D**) was added for the indicated durations in minutes. ERK activation was determined by Western blotting. (**B**) Semi-quantified results presented as fold increases (means $\pm$ S.E.M.; $n = 3$) in p42 ERK activation. Other results are representative of three (**C**) or five (**D**) experiments.

bFGF-induced activation at later times from 1 to 4 h, but this was not observed consistently and was not significant (Figure 2D). Forskolin/IBMX also had no effect on the early phase of ERK activation induced by serum but partly decreased the later phase of serum-stimulated ERK activation (results not shown).

We examined whether cAMP-elevating agents could inhibit ERK activation induced by two factors that act through G-protein-coupled receptors, namely angiotensin II and thrombin [29,30]. As shown in Figure 4, both forskolin/IBMX and cicaprost/IBMX caused a striking decrease in ERK activation induced by treatment with either angiotensin II or thrombin for 15 and 60 min.

The selective PKC inhibitors Ro-31-8220 [38,39] and GF109203X [40] were used to test the possibility that early and late PDGF-BB-induced ERK activation might be mediated via PKC-independent and PKC-dependent pathways respectively that are differentially sensitive to inhibition by cAMP. Neither inhibitor decreased the early or later phases of PDGF-BB-induced ERK activity, but Ro-31-8220 consistently enhanced the late phase of PDGF-BB-stimulated ERK activity (Figures 5A and 5B). Ro-31-8220 inhibited the activation of ERKs by PMA

Figure 6 TGF- β_1 does not inhibit PDGF-BB-induced ERK activation in rabbit VSMCs

VSMCs were preincubated for either 30 min (top panel of **A**) or 3 h (bottom panel of **A**) in the absence (PDGF-BB, —) or presence of 10 ng/ml TGF- β_1 (PDGF-BB + TGF- β_1 , +, TGF- β_1); either 25 ng/ml PDGF-BB (**A**) or 10 ng/ml bFGF (**B**) was then added for the indicated durations in minutes unless indicated otherwise. ERK activation was determined by Western blotting. The results are representative of six (top panel of **A**), and three (bottom panel of **A**, **B**) experiments.

and thrombin (Figure 5C). Neither inhibitor decreased the activation of ERK by angiotensin II but Ro-31-8220 alone markedly increased angiotensin II-induced ERK activity after 1 h to 5.6 ± 1.6 -fold ($n = 5$) above the basal level, compared with a 1.8 ± 0.2 -fold ($n = 5$) increase after 1 h without pretreatment with Ro-31-8220 (Figure 5D).

Pretreatment of VSMCs with TGF- β_1 for 30 min caused no decrease in either the early (15 min) or later (60 and 120 min) phases of ERK activation induced by PDGF-BB (Figure 6A) or bFGF (Figure 6B). TGF- β_1 similarly caused no inhibition when cells were pretreated with TGF- β_1 for 3 h before the addition of growth factors instead of 30 min (Figure 6A).

Role of inhibition of ERK in anti-mitogenic effects of cAMP

To investigate the dependence of anti-mitogenic effects of cAMP-elevating agents and TGF- β_1 on ERK activation during early G₁, we tested the effects of the addition of these factors at different times after mitogens. As shown in Figure 7(A), PDGF-BB-stimulated DNA synthesis determined after continuous incubation with [³H]thymidine for 30 h was markedly inhibited when forskolin/IBMX was added either simultaneously with growth factors or at 4, 8 or 12 h after the addition of growth factor. The inhibition of DNA synthesis was greatly decreased when forskolin/IBMX was added 24 h after PDGF-BB. Delaying the addition of TGF- β_1 for different times after that of growth factor produced very similar results to those obtained for forskolin/IBMX (Figure 7B). There was no evidence for ERK activation by PDGF-BB after 6, 10 or 20 h (Figure 7A, inset); the addition of forskolin/IBMX for a further 4 h after a 6 h treatment with PDGF-BB caused no further decrease in basal ERK activity (results not shown).

The selective MEK inhibitor PD98059 [36,41,42] completely inhibited PDGF-BB-stimulated ERK activation in the range 30–50 μ M (Figure 7C, inset). PD98059 at 40 μ M caused a small but statistically significant 20% decrease ($P < 0.001$) in PDGF-

BB-induced DNA synthesis but the addition of PD98059 4–24 h after PDGF-BB had no significant inhibitory effect. A comparison of the effects of forskolin/IBMX and PD98059 in parallel cultures showed that, whereas PD98059 and forskolin/IBMX alone decreased PDGF-BB-stimulated DNA synthesis to 80% and 22% of the maximum respectively, a combination of PD98059 and forskolin/IBMX caused a further additive decrease to 6% of the maximum (Figure 7D).

Regulation of expression of *c-myc*, cyclin D₁ and p27^{kip1} by cAMP

We next examined the effects of forskolin/IBMX on the expression of three components thought to be important for regulating progression through G₁: the *c-myc* proto-oncogene [43,44], cyclin D₁ [45] and the cyclin-dependent kinase inhibitor p27^{kip1} [46,47]. As shown in Figure 8(A), forskolin/IBMX markedly decreased the stimulation of *c-myc* mRNA expression induced in response to an 8 h incubation with either 10% serum or 25 ng/ml PDGF-BB. TGF- β_1 caused no decrease in the growth-factor-stimulated expression of *c-myc* mRNA (results not shown). The effects of forskolin/IBMX on PDGF-BB and serum-induced *c-myc* mRNA expression were concentration-dependent, with a detectable decrease at 0.1 μ M forskolin and a maximum inhibition at 1 μ M (Figure 8A). The induction of cyclin D₁ protein expression by PDGF-BB and serum was evident after 6–10 h, and increased for up to 20 h after treatment with growth factor (Figure 8B). Forskolin/IBMX decreased the growth-factor-induced increase in cyclin D₁ expression after 6, 10 or 20 h (Figure 8B). The cAMP-elevating agents decreased the cyclin D₁ level to 44% and 52% of that induced by PDGF-BB and serum after 10 h respectively ($n = 2$). TGF- β_1 modestly attenuated the growth-factor-induced expression of cyclin D₁ over a similar time course but its effect was noticeably less marked than that of forskolin/IBMX (Figure 8B). Treatment with forskolin/IBMX for 12–24 h promoted an increase in the protein expression of p27^{kip1} as determined by Western blotting (Figure 8C). Semi-quantification showed that, in three independent experiments, forskolin/IBMX increased p27^{kip1} expression above the control level by approx. 2-fold, 2.5-fold and 3-fold after 12, 16 and 24 h respectively. TGF- β did not significantly increase the expression of p27^{kip1} (Figure 8C).

DISCUSSION

A salient feature of the findings presented here is that various cAMP-elevating agents did not inhibit the early phase (up to 15 min) of ERK activation (1–3 h) induced by PDGF-BB and bFGF and only partly inhibited the late phase of PDGF-BB-induced activation; they had no significant effect on the late response to bFGF. These results contrast with the report of Graves et al. [27] that forskolin partly inhibited the maximum early PDGF-BB-induced ERK activation in human VSMCs by approx. 30%. This might reflect species-specific differences in the regulation of PDGF-stimulated ERK activation. Human VSMCs express α - and β -receptors for PDGF, whereas rabbit VSMCs express only β -receptors [33]; this could plausibly underlie differential regulation by cAMP. Additionally, because the earlier study employed a different assay of ERK activation, inter-assay variability might have contributed to the contrasting findings. Overall, our results indicate that cAMP-elevating agents are weak inhibitors of late ERK activation induced by PDGF-BB and bFGF in rabbit aortic VSMCs.

In contrast with these findings, cAMP-elevating agents strikingly decreased the early phase of ERK activity induced by angiotensin II and thrombin, factors that act via G-protein-

Figure 7 Anti-mitogenic effects of cAMP-elevating agents, TGF- β_1 and PD98059 at different times during G₁

(A–C) VSMCs were incubated for 30 h with [³H]thymidine, either with no further additions (C) or with 25 ng/ml PDGF-BB (P). To some PDGF-treated cells, 6 μ M forskolin/100 μ M IBMX (A), 10 ng/ml TGF- β_1 (B) or 40 μ M PD98059 (C) were added at the times indicated after the addition of PDGF-BB and [³H]thymidine from 0 to 24 h. The values shown in (A–C) are means $\pm$ S.E.M. ($n = 3$). The inset to (A) shows the effect of 25 ng/ml PDGF-BB on ERK activity after 0, 6, 10 and 20 h of treatment; the inset to (C) shows the effect of preincubating VSMCs for 30 min with different concentrations of PD98059 followed by the addition of 25 ng/ml PDGF-BB for 10 min. ERK activation was determined by Western blotting. (D) VSMCs were incubated for 40 h with [³H]thymidine with either no additions (C) or the addition of 25 ng/ml PDGF-BB (P), 25 ng/ml PDGF-BB plus 40 μ M PD98059 (P + PD), 25 ng/ml PDGF-BB plus 6 μ M forskolin/100 μ M IBMX (P + FK/I) or 25 ng/ml PDGF-BB plus 6 μ M forskolin/100 μ M IBMX/40 μ M PD98059 (P + PD + FK/I). The values shown are means $\pm$ S.E.M. ($n = 5$).

coupled receptors to stimulate phospholipase C- β isoforms, leading to the formation of diacylglycerol and the subsequent activation of PKC. The lack of effect of two PKC inhibitors on PDGF-BB-induced ERK activity indicates that PKC does not have a major role in the activation of ERKs by PDGF-BB and suggests that a selective effect of cAMP on PKC-dependent ERK activation is unlikely to account for the partial inhibition of late activity. The finding that angiotensin II-induced ERK activation was not inhibited by PKC inhibitors indicates that, like PDGF-BB, angiotensin II activates ERKs via a PKC-independent pathway. Some findings have suggested an involvement of PKC in angiotensin II-induced ERK activation [48]; others show that angiotensin II activates the G-protein-mediated activation of Ras in cardiac myocytes [49] and causes Ras-mediated and PKC-independent ERK activation in rat VSMCs [50]. The striking enhancement of the late phase of ERK activation induced by Ro-31-8220 is unlikely to be due to the inhibition of PKC because GF109203X had no effect; instead it might be mediated through Ro-31-8220's reported ability to inhibit the induction of the expression of ERK phosphatase 1 ('MKP-1') [51]. Ro-31-8220 and GF109203X are also potent inhibitors of p70 S6 kinase and mitogen-activated-protein-kinase-activated protein ('MAPKAP') kinase 1 β [52]. Because PKC inhibition blocked thrombin-induced ERK activation, cAMP-mediated inhibition

of PKC-dependent ERK activation could plausibly explain the inhibition of the response to thrombin. The mechanisms underlying differential cAMP-dependent inhibition of ERK activation through different receptors in VSMCs warrant further investigation.

Several lines of evidence indicated that the anti-mitogenic effects of cAMP-elevating agents and TGF- β_1 can be dissociated from inhibition of ERKs. Addition of cAMP-elevating agents and TGF- β_1 several hours after treatment with growth factors had a strongly anti-mitogenic effect even though growth-factor-induced ERK activity had returned to basal levels. Secondly, the MEK inhibitor PD98059 inhibited the stimulation of DNA synthesis by growth factors more weakly and was not inhibitory when added 2–8 h after PDGF-BB. PD98059 also acted together with forskolin/IBMX to produce an additive inhibitory effect on mitogenesis, consistent with the contention that these agents act through distinct pathways. The finding that PD98059 was a weak inhibitor of DNA synthesis was unexpected. However, it has been shown that this compound can stimulate as well as inhibit mitogenesis in human VSMCs by blocking the production of growth-inhibitory PGE₂ via ERK-dependent activation of cytosolic phospholipase A₂ [42]. PDGF-BB and bFGF induced a modest elevation of intracellular cAMP concentration in rabbit VSMCs, which was inhibited by PD98059 (R. Cospedal and I.

Figure 8 Regulation of the expression of *c-myc*, cyclin D₁ and p27^{kip1} by cAMP

(A) Quiescent VSMCs were treated for 8 h with (+) or without (−) either 25 ng/ml PDGF-BB (top) or 10% (v/v) serum (bottom) in the presence of 100 μ M IBMX plus the indicated concentrations of forskolin. RNA was extracted and *c-myc* expression was examined by Northern blot analysis. Equal loading was verified by the intensity of the 28 S ribosomal RNA band. (B) VSMCs were preincubated for 30 min without (control) or with either 6 μ M forskolin/100 μ M IBMX (FK + IBMX) or 10 ng/ml TGF- β_1 ; either 25 ng/ml PDGF-BB (P) or 10% serum (S) was then added for the indicated durations in hours. Cyclin D₁ expression was determined by Western blotting. (C) VSMCs were incubated for the indicated durations in hours with 25 ng/ml PDGF-BB, 6 μ M forskolin/100 μ M IBMX (FK + IBMX) or 10 ng/ml TGF- β_1 . p27^{kip1} expression was determined by Western blotting. All results are representative of three to five experiments.

Zachary, unpublished work). These results suggest that the inhibition of ERKs might have a complex effect on cell growth by blocking endogenous inhibitory mechanisms. Our findings showed further that TGF- β_1 had no effect on ERK activation. Although it has been reported that long-duration, but not short-duration, pretreatment with TGF- β_1 inhibits bFGF-induced ERK activity in pig aortic VSMCs [28], we found no inhibition of PDGF-BB-induced ERK activity by using longer-duration pretreatment with TGF- β_1 .

Taken together, our findings strongly suggest that cAMP-elevating agents and TGF- β_1 inhibit mitogenesis in rabbit aortic VSMCs via mechanism(s) independent of ERK activation. This conclusion is supported by reports that cAMP-elevating agents block fibroblast mitogenesis induced by thrombin, bFGF and serum without inhibiting ERK activation [29]. Our findings, taken together with other findings, support the more general conclusion that cAMP-elevating agents can inhibit mitogenesis in VSMCs and other mesenchymal cells independently of ERK

inhibition. Because our results showed that cAMP was an effective inhibitor of activation of ERKs 1 and 2 by angiotensin II and thrombin, we cannot exclude the possibility that the inhibition of ERKs might be important in mediating the inhibition of VSMC proliferation involving other mitogens or inhibitors.

The finding that cAMP and TGF- β_1 were effective inhibitors of DNA synthesis when added at up to 8–12 h after the addition of PDGF-BB suggests that anti-mitogenic effects of cAMP-elevating agents and TGF- β_1 are temporally independent of other early signalling pathways and are more likely to block VSMC mitogenesis by inhibiting events later in G₁. This conclusion agrees with a previous observation that the PGE₁-mediated inhibition of PDGF-induced DNA synthesis in human VSMCs increased when PGE₁ was added at up to 12 h after the growth factor but markedly decreased when added at later times after the re-initiation of DNA synthesis [53]. Consistent with cAMP acting in mid-to-late G₁, cAMP analogues have been reported to prevent *c-myc* proto-oncogene mRNA expression in mid-G₁ in macrophages without blocking the initial induction of *c-myc* [54]; cAMP-elevating agents decreased the serum-induced protein expression of cyclin D₁ between 6 and 20 h after the addition of serum [45]. The results presented here show that the addition of forskolin/IBMX during mid to late G₁ inhibited the growth-factor-stimulated expression of both *c-myc* mRNA and cyclin D₁ protein and enhanced the protein expression of the cyclin-dependent kinase inhibitor p27^{kip1}. A growing body of evidence indicates that cyclin D₁ has a crucial role in governing cell cycle progression in G₁ [45] and is therefore an attractive candidate target for anti-mitogenic effects of cAMP. Because cAMP-elevating agents partly inhibited the mitogen-induced expression of cyclin D₁, and TGF- β_1 only modestly attenuated expression, these factors might also exert their effects through other components of the cell cycle control mechanism. TGF- β_1 had no effect on the expression of *c-myc* mRNA and p27^{kip1}, further indicating a difference in the regulation of later events in G₁ by TGF- β_1 and cAMP. Although these findings are consistent with the hypothesis that anti-proliferative actions of cAMP in VSMCs are mediated through cellular events in late G₁, the underlying cellular mechanisms involved remain unclear. It is expected that a further analysis of cAMP and TGF- β_1 on events late in the G₁ phase of the cell cycle will yield new insights into the mechanisms of negative regulation of VSMC mitogenesis.

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