PDGF stimulates DNA synthesis in human vascular smooth muscle cells via a novel wortmannin-insensitive phosphatidylinositol 3-kinase

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Received 14 May 2003; revised 15 October 2003; accepted 15 October 2003

First published online 25 November 2003

Edited by Richard Marais

Abstract The class 1_A phosphatidylinositol 3-kinase enzymes consist of a number of heterodimeric complexes of regulatory and catalytic subunits and have been implicated in a number of cellular responses. While platelet-derived growth factor (PDGF)-induced chemotaxis of human vascular smooth muscle cells (HVSMC) is inhibited by both wortmannin and LY294002, DNA synthesis is only inhibited by LY294002. Serum-induced DNA synthesis however is inhibited by LY294002, wortmannin and rapamycin. Similarly PDGF-induced protein kinase B (PKB) activation is inhibited by LY294002 but not by wortmannin or rapamycin. In conclusion PDGF-induced DNA synthesis appears to occur through a phosphatidylinositol 3-kinase (PI3-K)-dependent, but wortmannin-insensitive, PKB/Akt pathway.

Key words: Platelet-derived growth factor; Vascular smooth muscle; DNA synthesis; Phosphatidylinositol 3-kinase

1. Introduction

Phosphatidylinositol 3-kinase (PI3-K) is a lipid kinase which specifically phosphorylates phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) or phosphatidylinositol 4,5-bisphosphate (PIP_2) on the D3 position of the inositol ring resulting in the production of phosphatidylinositol 3-phosphate [PI(3)P], phosphatidylinositol 3,4-bisphosphate [$PI(3,4)P_2$] and phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$]. Although PI(3)P has been shown to occur constitutively in cells $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are only detected in stimulated cells suggesting a role for these inositol phospholipids as intracellular mediators [1].

The PI3-K family of enzymes generally consist of a heterodimeric complex of regulatory (55–150 kDa) and catalytic (100–200 kDa) subunits and can be divided into classes with respect to structure, regulation and lipid specificity. The class 1_A PI3-K enzymes consist of an 85 kDa regulatory subunit (α, β and γ isoforms), a 110 kDa catalytic subunit (α, β and δ isoforms) and use PIP_2 as a lipid substrate [2]. The regulatory subunit contains several defined functional domains which allow it to bind to other cellular proteins resulting in the build-up of a signalling complex. The SH2 domains in this regulatory subunit bind to and are phosphorylated by a range of activated growth factor receptors including the platelet-derived growth factor (PDGF) receptor and the insulin receptor [3,4]. Although not essential for PI3-K activity, several studies have demonstrated that growth factor-stimulated tyrosine phosphorylation of the regulatory subunit correlates well with enzyme activation [3,5,6].

The major role of vascular smooth muscle cells (VSMC) in vivo is contraction and when fully differentiated these cells have a low proliferative index. Following injury or disease however these take on a synthetic phenotype which is capable of both chemotaxis and growth [7]. A similar dedifferentiation is seen in VSMC in culture suggesting that this condition may represent a model of injury. While the culture of animal-derived VSM cells is commonly used for experimental purposes work from this group has consistently demonstrated that data from animal cells do not always provide an accurate reflection of signalling mechanisms in human VSM cells [6,8–10].

Class 1_A PI3-K enzymes have recently been implicated in the chemotaxis [11], growth [12] and contractility [13] of several types of cell and have also been shown to act as a survival factor in a large number of cells [14,15].

Recent data from our laboratory have demonstrated that PDGF differentially induces chemotaxis and DNA synthesis in human VSMC (HVSMC) with the PDGFβ receptor acting as both a positive and a negative modulator of chemotaxis. Low concentrations of PDGF (1–10 ng/ml) induce chemotaxis but very little DNA synthesis whereas high concentrations (>30 ng/ml) promote DNA synthesis but inhibit chemotaxis. Differential patterns of tyrosine phosphorylation are also induced by these different concentrations of PDGF indicating that different signalling pathways are activated although increased tyrosine phosphorylation of an 85 kDa band is seen following stimulation with concentrations of PDGF that stimulate either chemotaxis or DNA synthesis suggesting the involvement of PI3-K [9].

Data presented here demonstrate that while PDGF-induced chemotaxis is inhibited by both of the commercially available PI3-K inhibitors, LY294002 and wortmannin, DNA synthesis appears to occur through a novel PI3-K-dependent but wortmannin-insensitive pathway.

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01350-4

2. Materials and methods

2.1. Materials

Cell culture plastics, media, supplements and PDGF were obtained from Life Technologies (Paisley, UK). Foetal calf serum (FCS) was obtained from M.B. Meldrum Ltd (Bourne End, UK). Bovine serum albumin fraction V was purchased from Boehringer Mannheim (Lewes, UK). BCA protein assay kits were obtained from Pierce Warriner (Chester, UK). Hybond C nitrocellulose, enhanced chemiluminescence reagents and streptavidin complex were obtained from Amersham (Buckinghamshire, UK). All antibodies were obtained from TCS (Buckingham, UK). All other reagents were obtained from Sigma (Poole, UK).

2.2. VSMC culture

Human vascular smooth muscle was obtained from saphenous vein of patients undergoing cardiovascular surgery. Tissues were surplus to requirements, and their use conformed to the guidelines of our local ethics committee. VSMC were cultured using an explant technique as previously described [16] and were routinely used at third passage. VSMC were cultured in Dulbecco's modified Eagle's medium (DMEM) buffered with 25 mmol/l HEPES and supplemented with 15% (vol/vol) FCS, 4 mmol/l 1-alanyl-1-glutamine (glutamax-I), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (25 μg/ml). Cell cultures were maintained in a humidified atmosphere of 5% CO_2 (vol/vol) in air at 37°C. Identity of the cells as VSMC was routinely checked by immunocytochemical studies for SM α-actin staining.

2.3. Chemotaxis

Chemotaxis assays were performed in modified Boyden chambers as previously described [8,9]. Briefly the cell suspension (2.25×10^5 cells/ml) was preincubated in the presence or absence of the PI3-K inhibitors LY294002 and wortmannin for 30 min prior to the migration assay. The cell suspension was separated from the chemoattractant (2 ng/ml PDGF) by gelatin-coated polycarbonate filters and the chambers were incubated for 5 h at 37°C prior to fixing in absolute ethanol and staining with 1% toluidine blue.

2.4. DNA synthesis

DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine into acid-insoluble material as previously described [8]. Cells were maintained in serum-free medium for a period of 5 days prior to the assay. Quiescent cells were preincubated in the presence or absence of inhibitors (10 μM LY294002, 100 nM wortmannin, 100 nM rapamycin, 30 μM PD98059) for 30 min prior to stimulation with either PDGF (30 ng/ml) or 15% FCS. [^{3}H]thymidine was added to the cells after 24 h and the stimulation terminated after a further 6 h.

2.5. Cell stimulation

This was performed as previously described [6,10]. Briefly HVSMC were subcultured and plated onto Petri dishes at a known density (1 000 000 cells/dish) and allowed to attach for 24 h in DMEM supplemented with 15% (vol/vol) FCS. Cells were then washed twice with phosphate buffered saline (PBS) and maintained in serum-free medium for a period of 5 days. Quiescent cells were then stimulated with PDGF (30 ng/ml) for a period of 30 min at 37°C. Following stimulation cells were washed twice with ice-cold PBS and scraped into 1 ml of ice-cold lysis buffer (50 mmol/l Tris (pH 7.4); 150 mmol/l NaCl; 1 mmol/l ethyleneglycol-bis-(β-aminoetylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA); 1% (vol/vol) NP-40; 0.25% sodium deoxycholate; 1 mmol/l sodium fluoride, sodium orthovanadate, phenymethylsulphonyl fluoride; 1 μg/ml aprotinin, pepstatin, leupeptin). The lysate was allowed to stand on ice for 10 min prior to centrifugation (14 000 rpm, 15 min, 4°C). The resulting supernatants were used for protein measurement.

2.6. Immunoprecipitation

A known concentration of cellular protein (approximately 100 μg) was removed from all samples and precleared for 1 h with albumin-agarose prior to incubation with the anti-phosphotyrosine antibody PY-20 (1 μg antibody/100 μg protein) for 3 h at 4°C. Immunoprecipitates were captured on protein A-agarose in the manner previously described [6].

2.7. Immunoblotting

Immunoblotting was performed in the manner previously described [6,10]. Briefly proteins were separated on 10% sodium dodecyl sulphate–polyacrylamide gels prior to transfer to nitrocellulose using a Bio-Rad wet gel transfer system. Following successful transfer nitrocellulose blots were blocked in 5% bovine serum albumin (BSA) for 1 h and washed three times in Tris buffered saline containing 0.05% Tween 20 (TTBS) prior to probing with primary antibody for 1 h. Blots were washed several times in TTBS before being probed with the appropriate secondary antibody and developed using enhanced chemiluminescence.

2.8. Statistical analysis

With the exception of cell signalling studies where data were expressed as % basal phosphorylation stimulated data were defined as 100% and all experimental data expressed as mean ± S.E.M. in relation to this. Statistical differences between means in terms of dose-dependent effects were compared using a Friedman non-parametric analysis of variance (ANOVA) for multiple repeated measures. A value of $P \leq 0.05$ was considered significant.

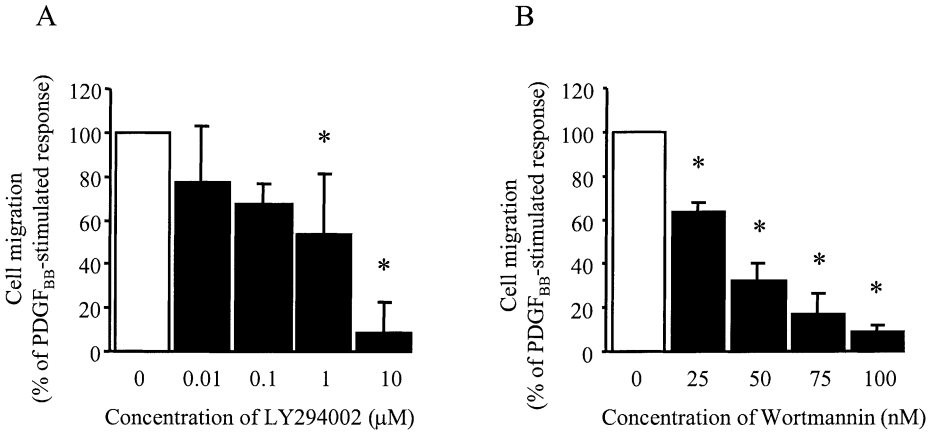

Fig. 1. Effect of PI3-K inhibitors on PDGF-induced cell chemotaxis. A: Histogram shows the effect of increasing concentrations of LY294002 on PDGF-induced cell chemotaxis. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using four separate cell strains. * represents a P value <0.05. B: Histogram shows the effect of increasing concentrations of wortmannin on PDGF-induced cell chemotaxis. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using four separate cell strains. * represents a P value <0.05.

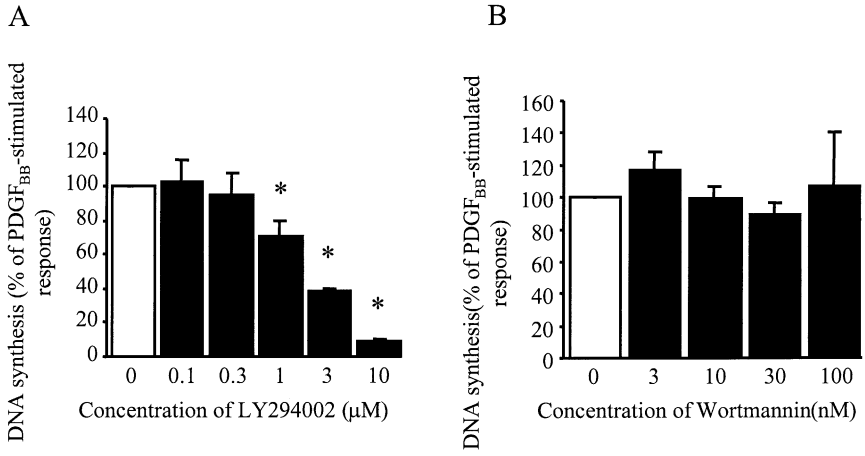

Fig. 2. Effect of PI3-K inhibitors on PDGF-induced DNA synthesis. A: Histogram shows the effect of increasing concentrations of LY294002 on PDGF-induced [^{3}H]thymidine incorporation in HVSMC. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using four separate cell strains. * represents a P value <0.05. B: Histogram shows the effect of increasing concentrations of wortmannin on PDGF-induced [^{3}H]thymidine incorporation in HVSMC. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using four to 16 separate cell strains. * represents a P value <0.05.

3. Results

3.1. Effect of PI3-K inhibitors on HVSMC chemotaxis

Treatment of HVSMC with both the PI3-K inhibitors, LY294002 and wortmannin, prior to their exposure to a directed gradient of PDGF (2 ng/ml) resulted in a concentration-dependent decrease in the ability of these cells to migrate in response to PDGF (Fig. 1).

3.2. Effect of PI3-K inhibitors on HVSMC DNA synthesis

Treatment with LY294002 resulted in a concentration-dependent reduction in the ability of HVSMC to synthesise DNA in response to PDGF but treatment with wortmannin had no significant effect on DNA synthesis induced by PDGF in HVSMC (Fig. 2). Neither replacing the media with fresh wortmannin-containing media nor topping up the wortmannin concentration 6 hourly resulted in any additional inhibition of PDGF-induced DNA synthesis (data not shown). In light of these results the effects of PI3-K inhibitors on serum (15%)-induced DNA synthesis of HVSMC were investigated. Treatment with 10 μM LY294002 resulted in complete inhibition of serum-induced DNA synthesis while treatment with 100 mM wortmannin also resulted in a moderate but significant inhibition of DNA synthesis (Fig. 3).

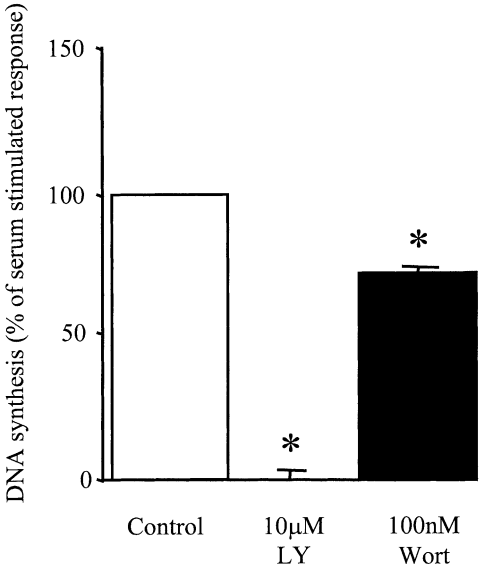

Fig. 3. Effect of PI3-K inhibitors on serum-induced DNA synthesis. Histogram shows the effect of 10 μM LY294002 and 100 nM wortmannin on PDGF-induced [^{3}H]thymidine incorporation in HVSMC. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using four separate cell strains. * represents a P value <0.05.

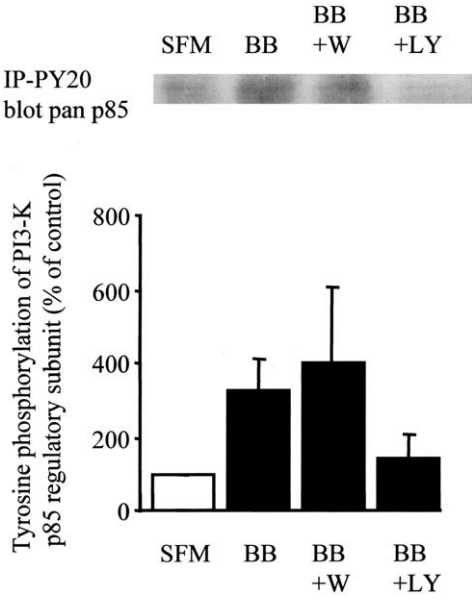

Fig. 4. Effect of PI3-K inhibitors on PDGF-stimulated p85 phosphorylation. Histogram shows the effect of 100 nM wortmannin and 10 μM LY294002 on tyrosine phosphorylation of the p85 regulatory subunit of PI3-K. Phosphorylation of control, unstimulated cells was taken to be 100% and all other data were normalised against this. Data represent the mean ± S.E.M. of two separate cell strains. Representative Western blot is shown above the histogram.

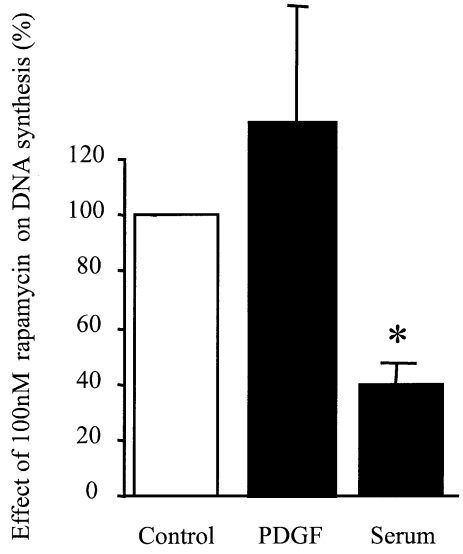

Fig. 5. Effect of $p70^{S6kinase}$ inhibitor on PDGF- and serum-induced DNA synthesis. Histogram shows the effect of 100 nM rapamycin on PDGF- and serum-induced [^{3}H]thymidine incorporation in HVSMC. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using five to six separate cell strains. * represents a P value < 0.05.

3.3. *Effect of PI3-K inhibitors on PDGF-stimulated p85 phosphorylation*

Western blotting for the p85 regulatory subunit of PI3-K following immunoprecipitation of tyrosine-phosphorylated proteins demonstrated that high-dose PDGF stimulated a 3-fold increase in the phosphorylation state of this subunit. This PDGF-stimulated phosphorylation was not affected by treatment with wortmannin but was inhibited by treatment with LY294002 (Fig. 4).

3.4. *Role of $p70^{S6kinase}$ in HVSMC DNA synthesis*

In order to determine whether the wortmannin-insensitive pathway required activation of $p70^{S6kinase}$ the effect of rapamycin (1–100 nM) on PDGF-induced DNA synthesis in HVSMC was investigated. Rapamycin did not affect the level of PDGF-induced DNA synthesis at any concentration. Serum-induced DNA synthesis however, was significantly inhibited by 1 nM rapamycin (48 ± 8% of the stimulated response). The level inhibition of serum-induced DNA synthesis was not affected by increasing concentrations of the inhibitor up to 100 nM (Fig. 5).

3.5. *Role of mitogen-activated protein kinase (MAPK) in HVSMC DNA synthesis*

In order to determine whether the wortmannin-insensitive pathway activated by PDGF involves the activation of MAPK the effect of the MEK inhibitor PD98059 on PDGF-induced DNA synthesis was investigated. PD98059 resulted in a concentration-dependent inhibition of both PDGF- and serum-induced DNA synthesis albeit to differing extents. PDGF-induced DNA synthesis was almost completely inhibited by 30 μM PD98059 while serum-induced DNA synthesis was only partially inhibited (30 μM PD98059 – 56 ± 4% of stimulated response) (Fig. 6).

3.6. *Effect of inhibitors on the activation of downstream targets*

Protein kinase B (PKB) or Akt1 is a 60 kDa serine/threonine protein kinase which has been identified as being a downstream target of PI3-K. PDGF stimulation of HVSMC resulted in a 2.5-fold increase in the level of activation of PKB, as determined by the increase in the level of phosphorylation of serine 473. This activation of PKB/Akt was completely inhibited by treatment with 10 μM LY294002 but unaffected by 100 nM wortmannin, 100 nM rapamycin or 30 μM PD98059 (Fig. 7A).

$P70^{S6kinase}$ may also be a possible downstream target of PI3-K. PDGF stimulation of HVSMC did not however result in any significant increase in the activity of $p70^{S6kinase}$ (as measured by phosphorylation of threonine 412) above control levels, although treatment with rapamycin signifi-

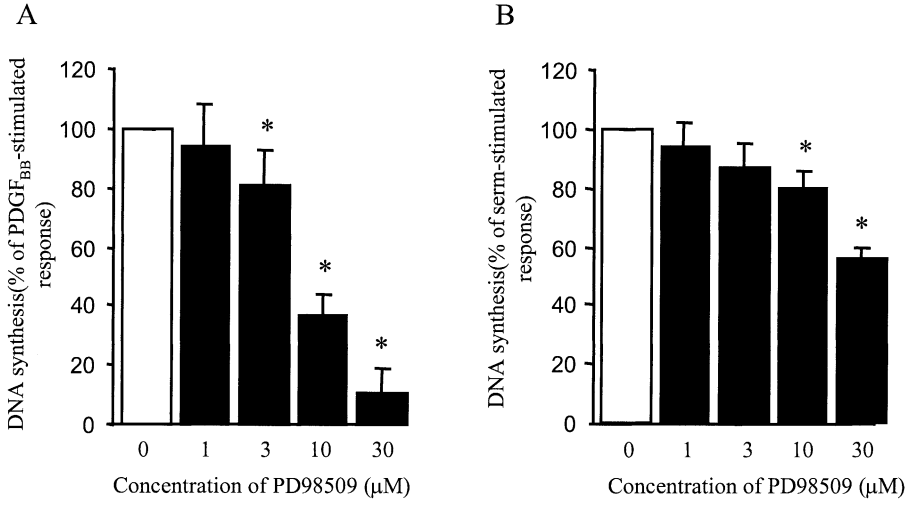

Fig. 6. Effect of MEK inhibitor on PDGF- and serum-induced DNA synthesis. A: Histogram shows the effect of increasing concentrations of PD98059 on PDGF-induced [^{3}H]thymidine incorporation in HVSMC. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using five separate cell strains. * represents a P value < 0.05. B: Histogram shows the effect of increasing concentrations of PD98059 on serum-induced [^{3}H]thymidine incorporation in HVSMC. Data are expressed as a percentage of the stimulated response which was taken to be 100% and represent the mean ± S.E.M. of experiments using six separate cell strains. * represents a P value < 0.05.

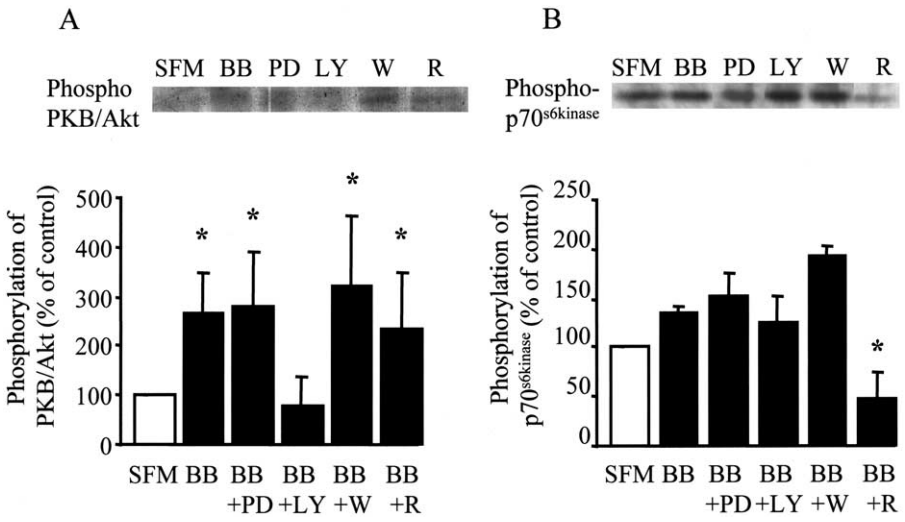

Fig. 7. Effect of inhibitors on PDGF-induced activation of PKB. A: Histogram shows the activation of PKB/Akt (mean ± S.E.M.) as determined by densitometric analysis of the anti-phospho Akt antibody. Phosphorylation of control, unstimulated cells was taken to be 100% and all other data were normalised against this. Data represent the mean ± S.E.M. of three separate strains. * represents a P value < 0.05. Representative Western blot is shown above the histogram. 100 nM wortmannin (W), 10 μM LY294002 (LY), 30 μM PD98059 (PD) and 100 nM rapamycin (R). B: Histogram shows activation of $p70^{S6kinase}$ as determined by densitometric analysis of the anti-phospho-$p70^{S6kinase}$ antibody. Phosphorylation of control, unstimulated cells was taken to be 100% and all other data were normalised against this. Data represent the mean ± S.E.M. of three separate cell strains. Representative Western blot is shown above the histogram.

cantly reduced $p70^{S6kinase}$ activity to below basal levels (Fig. 7B).

4. Discussion

Data presented here suggest that PDGF stimulates DNA synthesis in HVSMC via a novel PI3-K-dependent but wortmannin-insensitive pathway which is distinct from that stimulated by serum. Similarly the PI3-K isoforms involved in this signalling pathway can be differentiated from those involved in PDGF-induced chemotaxis by their sensitivity to the PI3-K inhibitors, LY294002 and wortmannin.

Our initial concern with respect to the PDGF-induced DNA synthesis data was that wortmannin activity was being lost over the course of the assay although a number of groups had previously demonstrated inhibition of growth factor-induced DNA synthesis by wortmannin using similar assay conditions [5,17–19]. However, since regular addition of fresh wortmannin did not result in any additional inhibition of PDGF-induced DNA synthesis and serum-induced DNA synthesis was significantly inhibited by wortmannin, it is unlikely that loss of wortmannin activity accounts for the results. The incomplete inhibition of serum-induced DNA synthesis by wortmannin correlates well with data obtained from vascular endothelial cells [20] and probably results from activation of the pathway stimulated by PDGF that is present in the serum.

Further to this we investigated whether the differential effects of wortmannin and LY294002 on PDGF-stimulated DNA synthesis were specifically related to activation of PI3-K isoforms. To this end we determined the effect of these inhibitors on the PDGF-stimulated tyrosine phosphorylation of the p85 regulatory subunit of PI3-K which we have previously shown to be an accurate reflection of growth factor-induced enzyme activation in these cells [6]. High-dose PDGF resulted in an approximately 3-fold increase in the level of p85 tyrosine phosphorylation, which was not affected by wortmannin but was inhibited by LY294002, suggesting that high-dose PDGF does selectively activate a wortmannin-insensitive isoform of PI3-K in HVSMC.

A number of signalling proteins have been reported to lie downstream of PI3-K activation with the most well established being the serine threonine kinases $p70^{S6kinase}$ and PKB/Akt [21,22]. In an effort to further clarify the mechanisms involved in our observation we investigated the possible roles of these signalling proteins in the wortmannin-insensitive signalling pathway. The $p70^{S6kinase}$ inhibitor rapamycin did not result in any inhibition of PDGF-induced DNA synthesis, although it did result in a significant inhibition of serum-induced DNA synthesis. These data suggest that the wortmannin-insensitive cell signalling pathway is independent of the activation of $p70^{S6kinase}$. Indeed cell stimulation with PDGF did not result in activation of $p70^{S6kinase}$ as measured by phosphorylation-specific antibodies. The involvement of rapamycin with serum-stimulated DNA synthesis correlates well with data from a variety of vascular endothelial cell lines [20], while the lack of involvement of $p70^{S6kinase}$ in PDGF-stimulated DNA synthesis has previously been demonstrated in both rat and human aortic cells [23].

PDGF stimulation did, however, result in a 2.5-fold activation/phosphorylation of PKB/Akt, which was inhibited by LY294002 but not by wortmannin, indicating that PDGF-induced DNA synthesis in HVSMC occurs via a PI3-K- and PKB-dependent pathway.

PDGF-induced DNA synthesis was inhibited by the MEK inhibitor PD98059 suggesting that activation of ERK1/ERK2 is necessary for this wortmannin-insensitive PDGF-induced signalling pathway. The lack of effect of PD98059 on PKB/Akt phosphorylation, coupled with the complete inhibition of DNA synthesis by LY294002, suggests that activation of ERK1/ERK2 may occur downstream of PKB in this PI3-K-dependent signalling pathway. Similar observations have been reported in both Chinese hamster ovary (CHO) and Cos7 cells [24,25] and a physical association between PKB and Raf has been demonstrated in A7r5 cells [26].

This report clearly demonstrates PDGF-induced stimulation of DNA synthesis through an LY294002-sensitive but wortmannin-insensitive pathway. While both inhibitors lose specificity at high concentrations a recent report has suggested that LY294002 may inhibit casein kinase 2 (CK2) at concentrations similar to those required for PI3-K inhibition [27]. There are, however, no data to suggest that CK2 can activate PKB/Akt or that it is involved in growth factor-induced DNA synthesis [28] indicating that the results seen here are likely to occur through a PI3-K/PKB-dependent pathway. While LY294002 and wortmannin have previously been thought to show relatively little selectivity for PI3-K isoforms a novel wortmannin-insensitive class 2 PI3-K has been reported [29]. Similarly Coulonvol et al. demonstrated differential sensitivities of PI3-K-dependent $p70^{S6kinase}$ activity to wortmannin and LY294002 [18].

In summary, these data indicate that PDGF induces DNA synthesis of HVSMC through a novel PI3-K-dependent but wortmannin-insensitive pathway. The multiplicity of PI3-K regulatory and catalytic subunit isoforms gives rise to the possibility of a variety of distinct p85p110 heterodimeric complexes which have been suggested to be involved in specific cellular responses [30–32]. It is likely that it is this variety of heterodimers that gives rise to the differential ability of PI3-K inhibitors to inhibit specific cellular responses. The development of novel selective inhibitors could represent a major pharmacological advance in the treatment of human disease.

Acknowledgements: This work was supported by the British Heart Foundation (PG/98141 awarded to J.S.L. and A.D.H.).

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