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Differential effects of lovastatin on mitogen induced calcium influx in human cultured vascular smooth muscle cells

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1 In this study the effect of lovastatin, an inhibitor of cholesterol and isoprenoid synthesis, on the rises in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by platelet derived growth factor BB (PDGF-BB), angiotensin II (AII), low density lipoproteins (LDL) and foetal calf serum (FCS) was examined in human cultured vascular smooth muscle cells (VSMC) from saphenous vein. Changes in $[Ca^{2+}]_i$ were measured in cell suspensions by the Ca^{2+} sensitive probe, fura 2.

2 Incubation with lovastatin for 24–26 h markedly reduced the peak rise and sustained phase of $[Ca^{2+}]_i$ elevation in response to PDGF-BB but the responses to AII, LDL and FCS were unaffected. Further experiments showed that lovastatin pretreatment inhibited PDGF-BB induced Ca^{2+} influx but not intracellular Ca^{2+} release. This inhibition could be overcome by co-incubation with mevalonic acid.

3 Pretreatment of cells with the heterotrimeric G protein inhibitor pertussis toxin for up to 24 h completely abolished AII-induced $[Ca^{2+}]_i$ rises but the response to PDGF-BB was unaffected.

4 The tyrosine kinase inhibitor genistein largely abolished PDGF-BB-induced $[Ca^{2+}]_i$ elevation but had no significant effect on AII-induced responses.

5 Pre-incubation with lovastatin had no effect on the level of tyrosine phosphorylation of PDGF-β receptors (as measured by Western blot) in response to the PDGF-BB ligand.

6 PDGF-BB elicits Ca^{2+} influx via a tyrosine kinase-dependent mechanism distinct from the heterotrimeric G protein coupled pathway utilized by AII. Lovastatin most likely acts by inhibition of isoprenylation (via blockade of isoprenoid synthesis) of an intermediate molecule involved in PDGF-BB-induced Ca^{2+} influx.

Keywords: Lovastatin; platelet-derived growth factor; angiotensin II; vascular smooth muscle; calcium homeostasis; tyrosine phosphorylation

Introduction

Lovastatin is a plasma cholesterol lowering agent which competitively inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the enzyme which catalyses the first dedicated step in the mevalonate pathway (Endo, 1992). This has the effect of suppressing cholesterol synthesis but also inhibits the synthesis of the class of compounds known as isoprenoids, the non-sterol lipids which form an intermediate part of the mevalonate pathway (Goldstein & Brown, 1990). In particular, the isoprenoids farnesol and geranylgeraniol are able to interact with certain proteins in an enzyme catalysed posttranslational modification termed isoprenylation (Maltese, 1990). In this process, an isoprenoid becomes covalently bonded to a cysteine residue located near the C-terminal of the protein. Subsequent modifications, namely proteolytic cleavage, methyl esterification and (more variably) acylation finally culminate in the C-terminal being replaced by a hydrophobic lipid complex (Clarke, 1992; Casey, 1994). The net result is that the protein as a whole is more able to associate with membranes, thereby allowing a much increased interaction with membrane bound receptors, effector molecules etc (Glomset *et al*., 1990; Casey, 1995).

Proteins known to undergo the above modifications include the γ subunits of heterotrimeric G proteins and all members of the ras superfamily of small GTPases (Hancock *et al*., 1989; Clarke, 1992). These are molecules intimately involved in signal transduction, with the latter class in particular known to play a central role in the control of proliferation in numerous cell types (Bourne *et al*., 1990) including vascular smooth muscle cells (VSMC, Irani *et al*., 1994). Since the stimulation of growth-related processes by external agents such as growth factors involves the flow of information across the plasma membrane to the cytosol and on to the nuclear envelope, the correct division of molecules involved in this chain of events is critical. The current widespread view is that isoprenylation is an important mechanism by which this positioning may be achieved (Glomset *et al*., 1990; Casey, 1994).

We have previously investigated the role of the mevalonate pathway in the proliferation of human VSMC cultured from saphenous vein (Munro *et al*., 1994) and, like others (Corsini *et al*., 1993), have found that isoprenoids are required for DNA synthesis and subsequent proliferation in vascular smooth muscle in response to stimulation by foetal calf serum (FCS). The present experiments build on this earlier study and focus on one specific signalling process, namely the ability of many mitogenic agents to modulate $[Ca^{2+}]_i$ in smooth muscle (Bochov *et al*., 1992). This is thought to constitute an important signal for the induction of VSMC migration (Nakao *et al*., 1983; Nomoto *et al*., 1988) and more contentiously for proliferation (Nishimura *et al*, 1992; Mogani & Kojima, 1993; Kobayashi *et al*., 1994; Hughes *et al*., 1995) in addition to being a well established signal for contraction. We have investigated the role of the mevalonate pathway (with lovastatin) in the regulation of $[Ca^{2+}]_i$ in human cultured VSMC by platelet-derived growth factor-BB (PDGF-BB), angiotensin II (AII), low density lipoproteins (LDL) and FCS and have made a comparison of the mechanisms of $[Ca^{2+}]_i$ elevation by PDGF-BB and AII. Together with data from studies with pertussis toxin (PTX, an inhibitor of certain heterotrimeric G proteins) and genistein (a tyrosine kinase inhibitor) we demonstrate distinct differences between the mechanisms by which PDGF-BB and AII induce $[Ca^{2+}]_i$ elevation and provide evidence for the importance of isoprenylation growth factor-induced Ca^{2+} influx.

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Methods

Cell culture

Human vascular smooth muscle was obtained from saphenous vein from patients undergoing cardiovascular surgery. Tissues were surplus to requirements and their use conformed to the guidelines of our local Ethics Committee. VSMCs were cultured in 80 cm^2 tissue culture flasks by use of an explant technique described previously (Munro *et al*., 1994) in a culture medium consisting of Dulbecco's modified Eagle's medium supplemented with FCS (15% v/v), 1-alanyl-1-glutamine (4 mM), penicillin (100 u ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), gentamicin (25 $\mu g\ ml^{-1}$) and buffered with HEPES (25 mM). Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. When confluence was reached the cells were subsequently subcultured under the same conditions described above, and were used for experiments when they reached confluence at passage 2. The identity of the cells as VSMCs was regularly confirmed by immunocytochemistry in which positive staining for α-actin (a marker for smooth muscle (Skalli *et al*., 1987)) was demonstrated. The effect of lovastatin or pertussis toxin (PTX) was examined by preincubating one of a matched pair of flasks from an individual with the relevant drug (24–26 h with lovastatin or up to 24 h with PTX) and the other with the relevant vehicle. The culture medium was changed in both flasks at the time of addition of the drug. Viability of the cells was confirmed as described previously (Munro *et al*., 1994).

Preparation of LDL

LDL was isolated from the plasma of a healthy male by density gradient centrifugation as previously described (Hughes *et al*., 1994).

Measurement of $[Ca^{2+}]_i$

Confluent monolayers were loaded with Fura-2 by means of incubation with Fura-2 AM (5 μM, added in 15 μl dimethyl sulphoxide) for 90 min. Monolayers were washed twice in phosphate buffered saline solution and followed by a brief trypsinization to yield a cell suspension. The cell suspension was then centrifuged (1000 r.p.m., 4 min) and resuspended in 16 ml of physiological saline solution (PSS) of the following composition (mM): NaCl 127, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 0.01, glucose 14 and HEPES 10.6, adjusted to pH 7.2 with NaOH. Before each experiment, 1 mM $CaCl_2$ or 0.5 mM EGTA was added to aliquots of 2.5 ml cell suspension.

Fluorescence was measured by a PTI Deltascan dual wavelength spectrofluorimeter with excitation wavelengths of 340 nm and 380 nm and emission at 510 nm. Data collection rate was 7 Hz and all experiments were carried out at 37°C.

Stimulants were added directly to the cell suspension. The concentrations used (PDGF-BB (25 ng ml^{-1}), AII (1 μM), LDL (20 $\mu g\ ml^{-1}$) and FCS (5% v/v)) were chosen on the basis of preliminary studies which established concentration-response relationships for these agents. In the cases of PDGF-BB, AII and LDL the doses correspond to maximal or near maximal and for FCS the dose corresponds approximately to the EC_{50}.

$[Ca^{2+}]_i$ was estimated according to the method of Grynkiewicz *et al*. (1985), with 0.1% Triton X-100 to lyse the cells. $[Ca^{2+}]_i$ rises were expressed as $\Delta[Ca^{2+}]_i$, the difference between peak $[Ca^{2+}]_i$ and resting $[Ca^{2+}]_i$ before the addition of the agonist. FCS and genistein caused a small degree of quenching of fluorescence but had no detectable effect on the calibration of $[Ca^{2+}]_i$ and therefore could be normalized. In contrast other selective inhibitors of tyrosine kinases, tyrphostin 23 and bistyrphostin caused marked quenching of fluorescence and consequently were inappropriate for use in these studies.

Measurement of tyrosine phosphorylation

Cells were plated onto Petri dishes and allowed to attach overnight (approximately 1×10^6 cell per dish) then incubated with or without lovastatin (10 μM, 24 h). All cells were then stimulated with PDGF-BB (25 ng ml^{-1}) for 1 min, washed twice with ice cold PBS and scraped into 1 ml of lysis buffer containing Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), EGTA (100 nM), NP-40 (1%) and Na deoxycholate (0.25%) containing PMSF, NaF, Na_3VO_4 (all 1 mM) and aprotinin, pepstatin and 1 $\mu g\ ml^{-1}$ leupeptin. The PDGF-β receptor was immunoprecipitated from cell protein (300 μg per sample) and captured on protein A-agarose in the manner described by Abedi *et al*. (1995). Immunoprecipitated samples (normalized by volume, 10 μl) were then run on 7.5% and 10% polyacrylamide gels, transferred to nitrocellulose and probed with an anti-phosphotyrosine antibody (PY20). Immunoreactive bands were visualized by chemiluminescence with horseradish peroxidase-conjugated anti-mouse IgG. Densitometry was carried out with a Bio Rad GS-690 Imaging Densitometer (Herts, U.K.).

Materials

Lovastatin was a gift from Merck, Sharpe and Dohme (Herts., U.K.). It was converted to the active form as previously described (Munro *et al*., 1994).

Recombinant PDGF-BB was obtained from Boehringer Mannheim (Lewes, U.K.). AII was from Ciba Geigy and foetal calf serum was obtained from JRH Biosciences (Les Ulis, France). All other cell culture materials were from Gibco Life Technologies (Paisley, U.K.). Fura-2 AM was purchased from Molecular Probes Inc (OR, U.S.A.). Anti-PDGF-β receptor antibody was from TCS Biologicals (Buckingham, U.K.). PY20 was obtained from Affiniti Research Products (Exeter, U.K.) and mouse IgG was from Dako (Bucks, U.K.). Protein A-agarose was from Cambridge Biosciences (Cambridge, U.K.). Chemiluminescence reagents were obtained from Amersham, U.K. All other drugs and chemicals were purchased from Sigma, U.K.

Statistics and data analysis

Data are presented as mean ± s.e.mean and results were compared by the Wilcoxon matched pairs test. A value of $P < 0.05$ was considered significant.

Results

The four agents used in this study raised $[Ca^{2+}]_i$ in cultured human VSMC. Figure 1 shows the contrasting profiles of $[Ca^{2+}]_i$ elevation induced by PDGF-BB, AII, FCS and LDL. In the presence of 1 mM extracellular Ca^{2+} resting $[Ca^{2+}]_i$ was 83 ± 12 nM, $n = 7$. The response to PDGF-BB (25 ng ml^{-1}) consisted of an initial transient rise followed by a sustained component which persisted for the duration of the experiment (3–6 min), although the level of this sustained component compared to the peak value was variable. However, AII (1 μM) produced a much smaller peak rise in $[Ca^{2+}]_i$ with little or no sustained component. A further difference concerned the latency between addition of the agonists to the chamber and the onset of the rise in $[Ca^{2+}]_i$. For PDGF-BB the delay was typically 4–12 s, whereas for AII it was 1–3 s.

In the absence of extracellular Ca^{2+} the resting $[Ca^{2+}]_i$ was reduced to 36 ± 10 nM, $n = 5$ PDGF-BB (25 ng ml^{-1}) elicited a smaller transient rise but with no accompanying sustained component. The latency was 30–60 s under these conditions, with the rate of rise (and therefore the time to peak $[Ca^{2+}]_i$) being variable.

Figure 2 illustrates the effect of pre-incubation with lovastatin on $[Ca^{2+}]_i$ transients induced by PDGF-BB. In 1 mM extracellular Ca^{2+} the peak response to PDGF-BB was attenuated by 70% ($\Delta[Ca^{2+}]_i = 118 \pm 10$ nM and 35 ± 8 nM in

control and lovastatin treated cells, respectively, $n=7$ matched pairs) and the second component was also attenuated and no longer sustained for the duration of the experiment. The response to AII, though, was not significantly affected (44 ± 13 nM and 30 ± 4 nM, $n=4$). Similarly, the responses to 20 μg ml^{-1} LDL (81 ± 30 nM and 97 ± 47 nM, $n=4$) and 5% FCS (260 ± 67 nM and 294 ± 64 nM, $n=5$) were also unaffected. When the experiment was carried out in the absence of extracellular Ca^{2+}, the response to PDGF-BB was not altered by lovastatin preincubation (41 ± 12 nM and 44 ± 15 nM, $n=5$). Lovastatin did not significantly alter resting $[Ca^{2+}]_i$ in the presence or absence of 1 mM extracellular Ca^{2+} (93 ± 19 nM, $n=7$ and 56 ± 23 nM, $n=5$, respectively). These results are summarized in Figure 3.

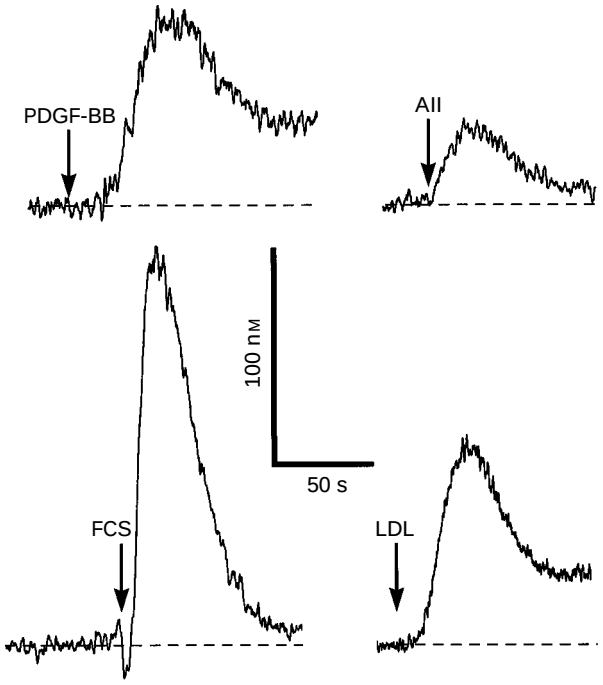

Figure 1 Rises in intracellular calcium concentration ($[Ca^{2+}]_i$) elicited by platelet derived growth factor-BB (PDGF-BB), angiotensin II (AII), foetal calf serum (FCS) and low density lipoproteins (LDL) in human cultured vascular smooth muscle cells in 1 mM extracellular Ca^{2+}. Estimation of $[Ca^{2+}]_i$ was as described in Methods. Times of addition of agonists are indicated by arrows. The traces indicate the contrasting latencies, peak responses and sustained components associated with the four agents used in this study and are representative of 4–7 separate experiments.

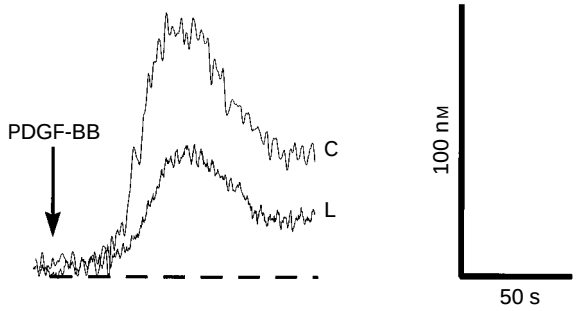

Figure 2 Effect of preincubation with lovastatin (10 μM for 24–26 h) on platelet derived growth factor-BB (PDGF-BB) induced $[Ca^{2+}]_i$ transients in the presence of 1 mM extracellular Ca^{2+}. C and L denote control and lovastatin-treated cells, respectively. Arrow denotes time of addition of PDGF-BB. The trace is representative of 7 separate experiments.

To determine whether the inhibitory effect of lovastatin on Ca^{2+} influx originated due to the effect on HMG CoA reductase or was due to some other mechanism, cells treated with the drug were co-incubated with mevalonic acid (100 μM, 24–30 h). Specifically, HMG CoA reductase catalyses the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonate. The primary action of lovastatin is therefore to deplete intracellular mevalonate levels. The addition of mevalonate overcomes this depletion and provides a direct means of distinguishing between an inhibitory effect on HMG CoA re-

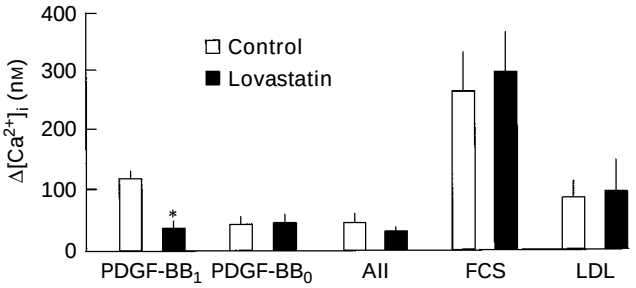

Figure 3 Effect of pre-incubation with lovastatin (10 μM for 24–26 h) on $\Delta[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$ – resting $[Ca^{2+}]_i$) induced by platelet derived growth factor-BB ($PDGF\text{-}BB_1$), angiotensin II (AII), foetal calf serum (FCS) and low density lipoproteins (LDL) in 1 mM extracellular Ca^{2+}, and for platelet derived growth factor-BB in extracellular Ca^{2+} free conditions with 0.5 mM extracellular EGTA ($PDGF\text{-}BB_0$). Columns represent mean$\pm$s.e.mean of 4–7 separate experiments. *Denotes $P<0.05$ by the Wilcoxon matched pairs test.

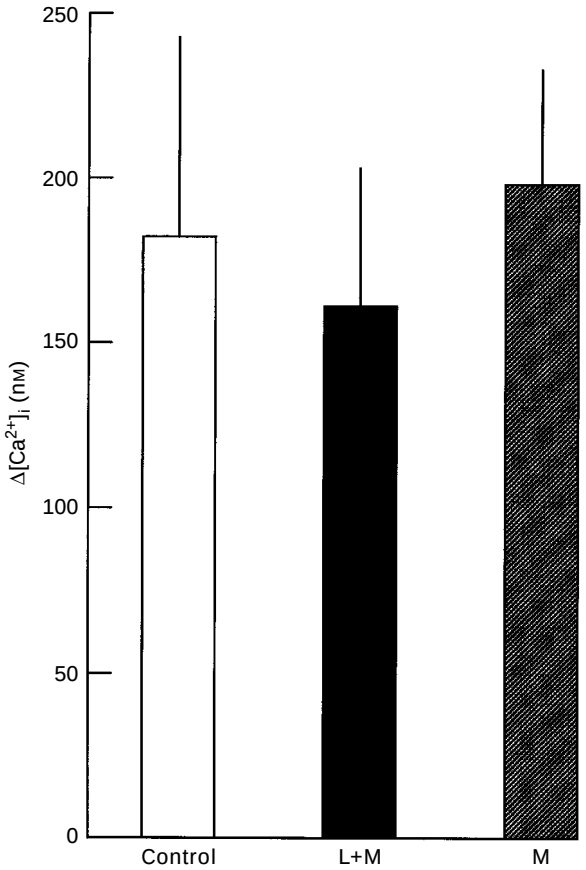

Figure 4 Effect of mevalonate (M; 100 μM, 24–26 h) alone or in combination with lovastatin (L; 10 μM, 24–26 h) on $\Delta[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$ – resting $[Ca^{2+}]_i$) induced by platelet derived growth factor-BB. Columns represent mean$\pm$s.e.mean of 4 separate experiments.

ductase or other unspecified actions. Figure 4 shows that replenishment of mevalonate in lovastatin-treated cells recovered the ability of PDGF-BB to induce a rise in $[Ca^{2+}]_i$ but addition of mevalonate alone did not promote an increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i = 182 \pm 61$ nM, 161 ± 42 nM and 198 ± 35 nM in control, lovastatin + mevalonate and mevalonate only treated cells, respectively, $n = 4$ matched pairs). This indicates that this effect lovastatin arose from depletion of mevalonate levels, i.e. from inhibition of HMG CoA reductase activity.

In many cell types Ca^{2+} influx and release from intracellular stores is mediated by heterotrimeric G proteins (Berridge, 1993; Fasolato *et al*., 1994). So it was decided to examine the effect of pertussis toxin (PTX, which inhibits the action of certain heterotrimeric G proteins) on the Ca^{2+} mobilizing ability of PDGF-BB and AII. Incubation with 0.5 μg ml^{-1} PTX for 4 h was sufficient to abolish completely the AII-induced increase in $[Ca^{2+}]_i$, whereas incubation with 1 μg ml^{-1} PTX for up to 24 h had no effect on the PDGF-BB induced $\Delta[Ca^{2+}]_i$ or sustained component ($\Delta[Ca^{2+}]_i = 104 \pm 28$ nM and 82 ± 20 nM for control and PTX treated cells, respectively (Figure 5).

One important way of classifying receptor coupled events is to divide them according to whether or not they act via tyrosine phosphorylation. AII has been shown to signal through heterotrimeric G protein and tyrosine kinase dependent pathways (Paxton *et al*., 1994; Hollenberg, 1994). So it was relevant to examine whether tyrosine kinase activity was necessary for the AII-induced $[Ca^{2+}]_i$ signal. Genistein (50 μM), a tyrosine kinase inhibitor, was applied to the cells 5 min before addition to AII. Genistein had no discernible effect on resting $[Ca^{2+}]_i$ or the response to AII ($\Delta[Ca^{2+}]_i = 47 \pm 20$ nM and 55 ± 25 nM for control and genistein treated cells, respectively, $n = 4$), as depicted in Figure 6. The same treatment used when PDGF-BB was the agonist nearly completely blocked the ability of this agent to induce a rise in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i = 91 \pm 12$ nM and 8 ± 8 nM, $n = 4$).

Since the incubation period with lovastatin was long (24–26 h) it was possible that the effect of lovastatin on PDGF-BB-induced signalling was being mediated by a reduction in expression and/or autophosphorylation of PDGF receptors. PDGF-BB binds to and activates both PDGF-α and PDGF-β receptors. We have previously found that the PDGF-β receptor subtype is predominantly responsible for $[Ca^{2+}]_i$ elevation in these cells (Hughes *et al*., 1995), because PDGF-AA (which only activates α receptors) elicited no rise in $[Ca^{2+}]_i$. PDGF-β receptors of cells stimulated with PDGF-BB (25 ng ml^{-1} for one minute, sufficient to induce the $[Ca^{2+}]_i$ signal; see Figure 1) were therefore first immunoprecipitated with a polyclonal antibody, then probed with an anti-phosphotyrosine antibody to determine the level of receptor tyrosine autophosphorylation. Figure 7 shows that under the experimental conditions shown to inhibit Ca^{2+} influx, there was no detectable reduction in PDGF-β receptor tyrosine phosphorylation, indicating that under these conditions lovastatin had no overall effect on PDGF-Rβ expression/tyrosine kinase activity. Several other bands were seen (at approximately 50 kDa and 70 kDa) but these were also unaffected by lovastatin treatment.

Discussion

This study presents evidence that inhibition of HMG CoA reductase activity leads to attenuation of the $[Ca^{2+}]_i$ signal elicited by a potent tyrosine kinase-linked VSMC growth factor, PDGF-BB. In contrast, the $[Ca^{2+}]_i$ signal elicited by the G protein linked agents AII and LDL was unaffected. Recovery of inhibition by addition of mevalonate provides direct evidence that lovastatin primarily acted via inhibition of HMG CoA reductase activity, thereby implicating indirect suppres-

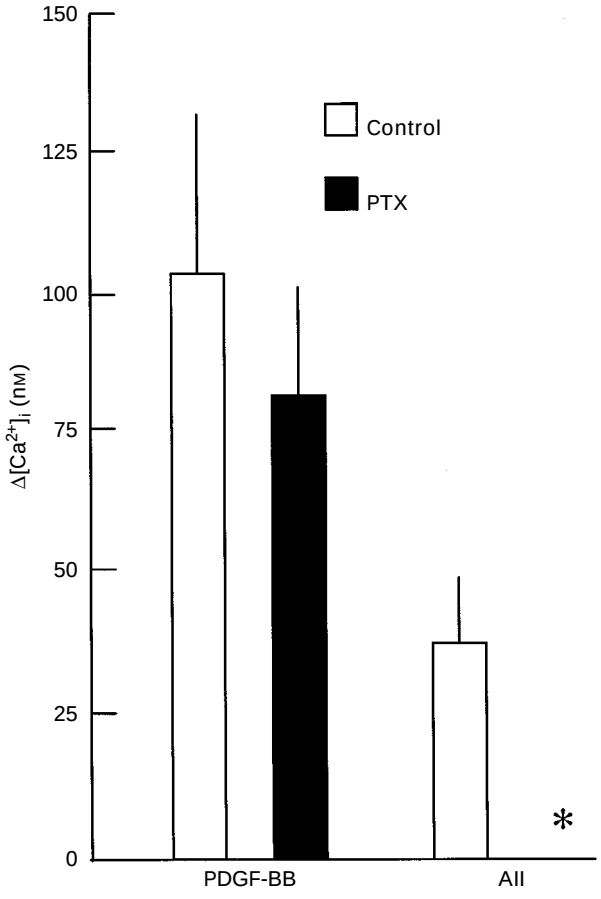

Figure 5 Effect of preincubation with pertussis toxin (PTX; up to 1 μg ml^{-1} for 24 h) on $\Delta[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$ − resting $[Ca^{2+}]_i$) induced by platelet derived growth factor-BB (PDGF-BB) and angiotensin II (AII) in 1 mM extracellular Ca^{2+}. Columns represent mean ± s.e.mean of 4 separate experiments. *Denotes $P < 0.05$ by the Wilcoxon matched pairs test.

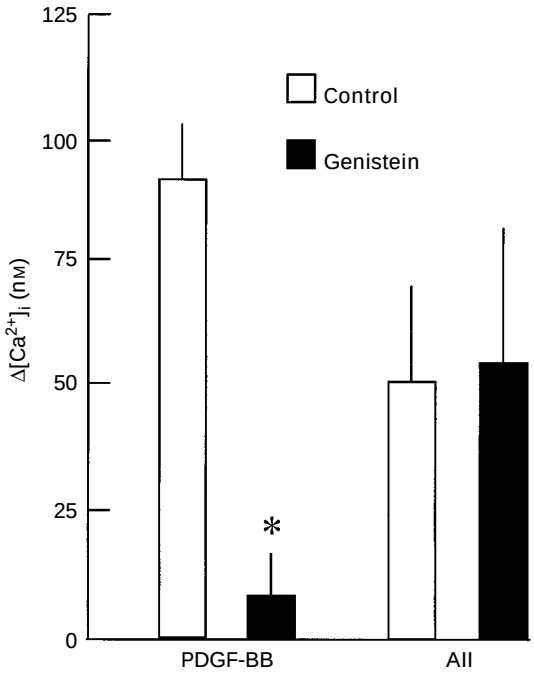

Figure 6 Effect of genistein (50 μM for 5 min) on $\Delta[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$) − resting $[Ca^{2+}]_i$) induced by platelet derived growth factor-BB (PDGF-BB) and angiotensin II (AII) in 1 mM extracellular Ca^{2+}. Columns represent mean ± s.e.mean of 4 separate experiments. *Denotes $P < 0.05$ by the Wilcoxon matched pairs test.

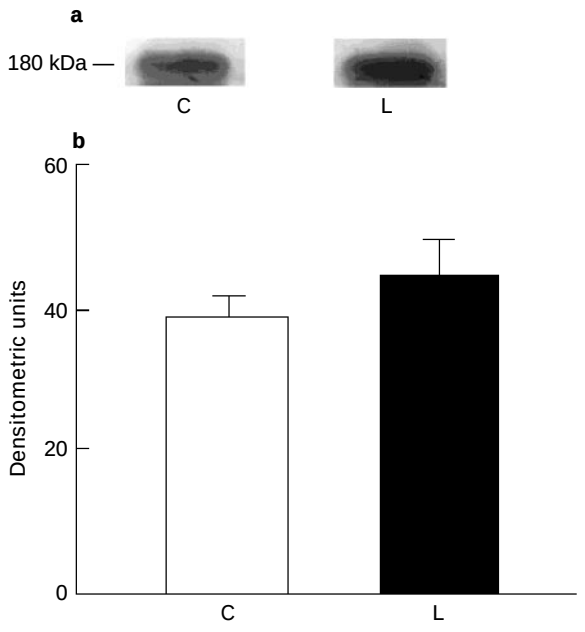

Figure 7 Effect of lovastatin (L; 10 μM for 24 h) on tyrosine phosphorylation of platelet-derived growth factor-β receptors as described in Methods. (a) Representative Western blot of immunoprecipitated PDGF-β receptors probed with anti-phosphotyrosine antibody. (b) Densitometry measurements of 3 separate experiments. Columns represent mean $\pm$ s.e.mean.

sion of synthesis of one or more components of the mevalonate pathway as a potential mechanism of attenuation of $[Ca^{2+}]_i$ elevation. We have previously measured the cellular cholesterol:phospholipid (C:P) ratio in these cells (Munro *et al*., 1994) under the same experimental conditions and found that there was no substantial change in this parameter over 24 h, most likely due to a compensatory increased uptake of LDL present in the serum. This provides evidence that lovastatin acts via a mechanism other than alteration of C:P ratio to inhibit PDGF-BB induced $[Ca^{2+}]_i$ elevation. The most feasible alternative interpretation is that suppression of isoprenoid synthesis prevents membrane association of a normally isoprenylated protein essential for the mediation of this $[Ca^{2+}]_i$ signal.

The isoprenoid requirement appears specifically confined to the Ca^{2+} influx component, since lovastatin had no effect on $[Ca^{2+}]_i$ signalling in experiments carried out in the absence of extracellular Ca^{2+}. This observation, combined with the lack of effect on PDGF-β receptor autophosphorylation, suggests that any disruption of ligand-receptor coupling is not due to a direct effect on the receptors themselves. It further implies that the mediator affected is involved in the opening of Ca^{2+} permeable channels in the plasma membrane, but is not essentially required in release of Ca^{2+} from intracellular stores (although these results cannot be used to determine whether or not the mediator is involved with subsequent store refilling). PDGF has been shown to induce Ca^{2+} influx through a variety of pathways, including L-type and T-type voltage operated calcium channels (Estacion & Morden, 1993; Hughes, 1995a; Wijetunge & Hughes, 1995) and non-voltage operated channels (Szollosi *et al*., 1991) depending on cell type and the PDGF isoform used. Under the culture and experimental conditions described here Ca^{2+} influx cannot be elicited in human VSMC by KCl-induced depolarization and $[Ca^{2+}]_i$ transients induced by agonists are not affected by dihydropyridine calcium channel antagonists (data not shown). This implies that the functional channels are not voltage-dependent calcium channels. They most likely belong to the class of receptor-operated channels or second messenger operated channels, permeable to cations and identified in many cell types including VSMC (reviewed in Hughes, 1995b). Similar channels have been shown to be activated by PDGF in fibroblasts (Frace & Gargus, 1989).

Application of genistein, a selective inhibitor of tyrosine kinases, had the effect of nearly completely abolishing both the PGDF-BB induced influx and release components. Presumably, this effect occurs by preventing tyrosine phosphorylation of proteins in the signalling pathway, most likely the PDGF receptors themselves. This is thought to be a necessary step before activation of most downstream signals linked to this growth factor (Claesson-Welsh, 1994). The lack of effect of genistein on AII-induced responses can be taken to mean that tyrosine kinase activity is not essential for the generation of the $[Ca^{2+}]_i$ signal in response to this agent in these cells, despite the demonstration that AII can elicit tyrosine phosphorylation in VSMC (Hollenberg, 1994; Marrero *et al*., 1994) and has been shown by our group to be mitogenic for the human cultured VSMC used in this study (Patel *et al*., 1996).

The intracellular release of Ca^{2+} by the growth factor can be attributed to a mechanism by which tyrosine phosphorylated PDGF receptors directly activate phospholipase Cγ (Meisenhelder *et al*., 1989), leading in turn to the liberation of 1,4,5-inositol trisphosphate (IP_3) and subsequent release of Ca^{2+} from the sarcoplasmic reticulum. Such a mechanism would need tyrosine kinase activity but would not require an isoprenylated intermediate or a heterotrimeric G protein, thereby making this pathway susceptible to inhibition by genistein, but not by lovastatin or PTX.

The mechanism of PDGF-BB-induced Ca^{2+} influx is unknown. In addition to phospholipase Cγ, a large number of signalling molecules are recruited and activated in a complex formed with tyrosine phosphorylated PDGF-β receptors. Amongst these is the monomeric G protein ras, which couples to the receptors via adapter proteins. Ras is isoprenylated and also represents an early signal, which is a critical requirement in this system, since the rise in $[Ca^{2+}]_i$ was initiated only a few seconds after PDGF-BB addition. Another possibility is rho, which has recently been demonstrated to bind directly to activated PDGF-β receptors (Zubiaur *et al*., 1995). Two recent studies have indicated roles for these proteins in mobilization of $[Ca^{2+}]_i$ levels in response to agonists (Chong *et al*., 1994; Ma *et al*., 1996). However, PDGF acts via different pathways in different cell types (Satoh *et al*., 1993), so the situation in human VSMC cannot be predicted by simple extrapolation. However, the idea of a small G protein (Ras or Ras related) as a mediator of PDGF-BB-induced Ca^{2+} influx is consistent with a requirement for tyrosine kinase activity coupled with a requirement for an isoprenylated intermediate. Such a mechanism would also predict the observed insensitivity to PTX.

In contrast to the case of PDGF-BB, inhibition of HMG CoA reductase by lovastatin had no effect on $[Ca^{2+}]_i$ transients induced by AII, LDL or FCS. This is consistent with the view that PDGF-BB elicits Ca^{2+} influx via a different mechanism from AII and LDL. FCS contains a wide range of vasoactive agents and is therefore capable of elevating $[Ca^{2+}]_i$ through multiple pathways, some of which may be insensitive to lovastatin. This result, coupled to our previous findings (Munro *et al*., 1994) suggests that receptor-mediated elevation of $[Ca^{2+}]_i$ in these cells is not sufficient to induce DNA synthesis.

The complete blockade of the AII-induced $[Ca^{2+}]_i$ rise by PTX indicates that AII raises $[Ca^{2+}]_i$ in human VSMCs via a PTX-sensitive heterotrimeric G protein. Previous studies have concluded that G proteins are involved in $[Ca^{2+}]_i$ elevation in response to this agent in smooth muscle, but have yielded no consensus regarding PTX sensitivity (Bruns & Marme, 1987; Dostal *et al*., 1990; Ohya & Sperelakis, 1991). The failure of lovastatin to affect responses to AII and LDL is surprising. It is established that all the currently known γ subunits of heterotrimeric G proteins are isoprenylated and that this modification is essential in order for membrane association of $\beta\gamma$ dimers (Casey, 1994). Thus it might be expected that lovastatin would inhibit AII and LDL-induced $[Ca^{2+}]_i$ rises by inhibition of G protein function. However, this was not observed. There are several possible reasons why lovastatin may not have an

effect. Inhibition of isoprenylation will only inhibit posttranslational modification of newly synthesized proteins leaving mature proteins unaffected. This makes the effect of lovastatin over the period of incubation (24–26 h in this case) critically dependent on the steady state number and the half-life of $\beta\gamma$ dimers, which may not become depleted to an extent which affects their ability to mediate the $[Ca^{2+}]_i$ signal.

Such a proposal would be consistent both with the relatively long half-life of heterotrimeric G proteins (Hadcock 1990) and the excess of $\beta\gamma$ to α subunits (Watkins *et al*., 1987). Indeed, in a recent study exposure to 30 μM lovastatin for 48 h reduced $G\alpha_i$ levels by only 15–30% (Chiloeches *et al*., 1995). In contrast the turnover of key monomeric G proteins is more rapid; for example $\sim$2 h for rhoB (Lebowitz *et al*., 1995) and 24 h for ras (Ulsh & Shih, 1984). These differences could account for our observations and may indicate a role for small G proteins in this system.

Another study performed on smooth muscle (A10 cells, Ng *et al*., 1994) has shown that Ca^{2+} influx in response to vasopressin was reduced by simvastatin (another HMG CoA reductase inhibitor). Vasopressin receptors are linked to heterotrimeric G proteins in VSMCs (Xuan *et al*., 1987; Zeng *et al*., 1989) and inhibition of $\beta\gamma$ subunit isoprenylation was put forward as a possible reason for the effect. These findings contrast with our observations with AII and LDL. The reason for this discrepancy is unclear and could simply reflect differences in methodology or cell type. An alternative is that vasopressin, despite being linked to G proteins, activates Ca^{2+} influx by mechanisms similar to PDGF-BB, thereby making it susceptible to inhibition by lovastatin. Such a proposal would explain the surprising finding that lovastatin did not affect vasopressin-induced intracellular Ca^{2+} release in the same study, despite this also being a heterotrimeric G protein-dependent process (Zeng *et al*., 1989).

There is increasing evidence that the actions of HMG CoA reductase inhibitors in the vasculature are attributable to more than their cholesterol lowering function (see Hughes, 1996, for example). Here we showed that lovastatin inhibits Ca^{2+} influx in response to PDGF, the most potent VSMC chemoattractant *in vivo*. Elevation of $[Ca^{2+}]_i$ is thought to constitute a critical signal in this process. Under these conditions the Ca^{2+} route of entry is not through L-type channels and is thus insensitive to the current range of clinically available calcium channel antagonists. Work in our laboratory has recently shown that lovastatin (at the same or lower concentrations than used here) can inhibit chemotaxis in these cells in response to PDGF (unpublished observations). Thus a novel action underlying the inhibition of VSMC migration by HMG CoA reductase inhibitors could be, at least in part, inhibition of Ca^{2+} influx by tyrosine kinase-linked growth factor receptors. The functional studies presented to date are now being supplemented by experiments in which the effect of lovastatin on the level of isoprenylation of established signalling molecules is measured.

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