

Ca²⁺-channel blockers modulate expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and low density lipoprotein receptor genes stimulated by platelet-derived growth factor

(gene activation/atherosclerosis)

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ABSTRACT The effects of Ca²⁺-channel blockers (amlodipine, nifedipine, nitrendipine, and verapamil) on expression of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.88) and low density lipoprotein receptor (LDL-R) genes stimulated by recombinant platelet-derived growth factor BB isomer (PDGF-BB) were evaluated in human skin fibroblasts. The drugs enhanced expression of the LDL-R protein on the plasma membrane of the cells; in contrast, they inhibited expression of the HMG-CoA reductase gene. In addition, PDGF-BB-dependent stimulation of transcription of *c-fos* mRNA was inhibited also by the Ca²⁺-channel blockers. We conclude that PDGF-BB-dependent activation of the two genes is inhibited effectively by the Ca²⁺-channel blockers, at therapeutic concentrations, although they are unable to lower systemic cholesterol levels at these concentrations; however, they do modify responses of the two genes that are involved crucially in regulation of cellular cholesterol homeostasis.

Evidence has accumulated suggesting that platelet-derived growth factor (PDGF), which is primarily known as a growth factor for various cell types, has an important role in development of atherosclerotic lesions (1). PDGF has numerous biological properties; among these are potent mitogenic, chemotactic, and vasoconstrictor effects on vascular smooth muscle cells (VSMC) (2). Ross *et al.* (3) have reported that various PDGF isoforms have unique expressions in different cell types that are involved in development of atherosclerotic lesions. Thus, *in situ* hybridization experiments with PDGF mRNAs in atherosclerotic lesions revealed that VSMC are the predominant site of PDGF AA isomer (PDGF-AA), whereas PDGF BB isomer (PDGF-BB) is found principally in macrophages. This implies distinct functions for the two PDGF isoforms in the atherosclerotic process.

In this context, we have shown recently that the PDGF isoforms have a distinct ability to stimulate expression of the two genes that are prominently involved in regulation of cholesterol biosynthesis: low density lipoprotein receptor (LDL-R) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.88) genes (4). Although all PDGF isoforms activate expression of the LDL-R gene, induction of the mRNA corresponding to HMG-CoA reductase was stimulated specifically by PDGF-BB.

Two Ca²⁺-channel blockers, verapamil and diltiazem, increased binding of LDL to fibroblasts, which led to an increase in the cells' endocytosomal capacity for binding LDL (5). This effect was apparently specific for the Ca²⁺-channel blockers and was interpreted as an increase in LDL-R mRNA. However, Ca²⁺-channel blockers, in gen-

eral, do not decrease the concentration of plasma cholesterol in humans or in other animals, although their antiatherosclerotic effect has been implicated in various experimental hypercholesterolemic conditions (6, 7).

We have shown that Ca²⁺-channel blockers have an inhibitory effect on PDGF-induced signal-transduction pathways, one of which is the pathway for phosphatidylinositol (PI) turnover (8, 9). Thus, the drugs blocked PDGF-induced elevation of intracellular free calcium concentration, [Ca²⁺]_i, as well as protein kinase C (PKC) activation (8). In view of our previous observations, it was desirable to study the potential effect of Ca²⁺-channel blockers on PDGF-induced expression of the genes for HMG-CoA reductase and LDL-R.

We report here that (i) Ca²⁺-channel blockers stimulate transcription of the mRNA for LDL-R and amplify PDGF-BB-induced expression of the LDL-R gene in human skin fibroblasts, (ii) Ca²⁺-channel blockers do not affect expression of the gene for HMG-CoA reductase, (iii) in contrast, PDGF-BB-induced transcription of the mRNA for HMG-CoA reductase is inhibited by Ca²⁺-channel blockers, and (iv) the potency of Ca²⁺-channel blockers in inhibiting the promotion of PDGF-BB-induced transcription of the HMG-CoA reductase gene is 2-3 orders of magnitude lower than that required for inhibiting DNA synthesis *de novo* in human fibroblasts.

METHODS

PDGF-BB Isomer. The PDGF-BB isomer was obtained from GIBCO/BRL. Its activity with respect to proliferation of human skin fibroblasts was dose dependent between 0.5 and 50 ng/ml; a suboptimal dose of 5 ng/ml was added to cell cultures during challenge.

Measurement of Mitogenicity. The mitogenic effect was measured by the amount of [³H]thymidine incorporated into the DNA of the fibroblasts according to the procedure of Chesterman *et al.* (10).

Fibroblasts. Three different primary fibroblast cell lines derived from human skin by sterile punch biopsies were cultivated in minimal essential medium (MEM) with Earle's salts (GIBCO) that was supplemented with 10% fetal calf serum (Seromed, Munich), 1% vitamins (GIBCO), and 8 mM L-glutamine (GIBCO) in 250-ml Falcon tissue culture flasks until the cells became confluent (11). The growth medium was changed every second day; no antibiotic or antimycotic

Abbreviations: LDL-R, low density lipoprotein receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PDGF, platelet-derived growth factor; rPDGF-BB, recombinant PDGF BB isomer; PKC, protein kinase C; PI, phosphatidylinositol; VSMC, vascular smooth muscle cells.

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Table 1. Inhibitory effect of various Ca²⁺-channel blockers on [³H]thymidine incorporation in human skin fibroblasts induced by recombinant PDGF-BB (10 ng/ml)

Ca ²⁺ -channel blocker	IC ₅₀ value, M
Amlodipine	8 × 10 ⁻⁸
Verapamil	3 × 10 ⁻⁸
Nifedipine	2 × 10 ⁻⁷
Nitrendipine	9 × 10 ⁻⁶

Average values of experiments done in triplicate are presented.

agents were employed; cells were used between the fourth and sixth passage. Two days before cells were challenged with PDGF-BB, the medium was replaced by S-medium (MEM/1% vitamins/8 mM L-glutamine).

Extraction of Total RNA. Total RNA was extracted according to the protocol of Chomczynski and Sacchi (12). The RNA was quantitated by spectrophotometry (260 nm/280 nm).

Northern and Dot Blot Analyses. Equal amounts of RNA were denatured at 95°C for 5 min in sample buffer (50% formamide/10% glycine/1% bromophenol blue) and then size-fractionated by electrophoresis through a 1% agarose gel containing 0.02 M morpholinisulfonate, 2% formaldehyde, and 0.2 μg of ethidium bromide per ml (13).

Dot blots were performed as described by Constanzi and Gillespie (14). Membranes (for Northern blot and/or dot blot) were dried after blotting and were UV-irradiated to fix the RNA. The prehybridization procedure was performed at 45°C for 2 hr using a solution of 50% formamide/8% dextran sulfate/100 mg of sonicated salmon sperm DNA per ml/0.5 M NaCl/1% SDS. Twenty-five nanograms of the labeled cDNA probe was added for hybridization and kept at 65°C for at least 12 hr. The hybridized membranes were washed twice with 2× standard saline citrate [(SSC) supplemented with 0.1% SDS] at 65°C, followed by two additional washes in 1×

SSC (0.1% SDS). The nylon membranes were dried, wrapped in plastic foil, and exposed to x-ray film for 24–48 hr.

Quantitation of the synthesis *de novo* of mRNAs was assessed using autoradiography.

Expression of LDL-R. Expression of the LDL-R on the cell membrane was determined according to the protocol of Gherardi *et al.* (15) using a murine anti-human LDL-R monoclonal antibody (Amersham). Briefly, vinyl microtiter plates were coated with 5 μg of anti-LDL-R antibody per well in 0.05 ml of 0.05 M Na₂CO₃/0.001 M NaN₃, pH 9.6, and incubated overnight at room temperature. Plates were washed with Tris-buffered saline [(TBS) 0.025 M Tris-HCl/0.15 M NaCl, pH 7.4] and blocked with 50 μl of bovine serum albumin (25 mg/ml) in 0.05 M Na₂CO₃ for 60 min at 37°C.

LDL-R proteins were solubilized in 50 mM Tris-HCl/50 mM NaCl/2 mM CaCl₂/1 mM phenylmethylsulfonyl fluoride/10 mg of Triton X-100 per ml and equal amounts of protein were added to the plates and incubated for 1 hr at 37°C, followed by a 3-hr incubation at 4°C. Plates were washed three times with TBS, an ¹²⁵I-labeled anti-LDL-R antibody was added, and plates were incubated for 1 hr at room temperature followed by an additional 3-hr incubation at 4°C. Plates were washed as described above and air-dried; radioactivity in single wells was assessed with a scintillation counter.

Ca²⁺-Channel Blockers. Amlodipine was obtained from Pfizer (Sandwich, England); verapamil was from Knoll (Liestal, Switzerland); and nifedipine and nitrendipine were from Bayer (Leverkusen, F.R.G.). Incubations were performed either with Ca²⁺-channel blockers alone or after a 4-hr preincubation of the cells, after which PDGF-BB was added.

RESULTS

Effects of Ca²⁺-Channel Blockers on Recombinant PDGF-BB (rPDGF-BB)-Induced DNA Synthesis *de Novo* in

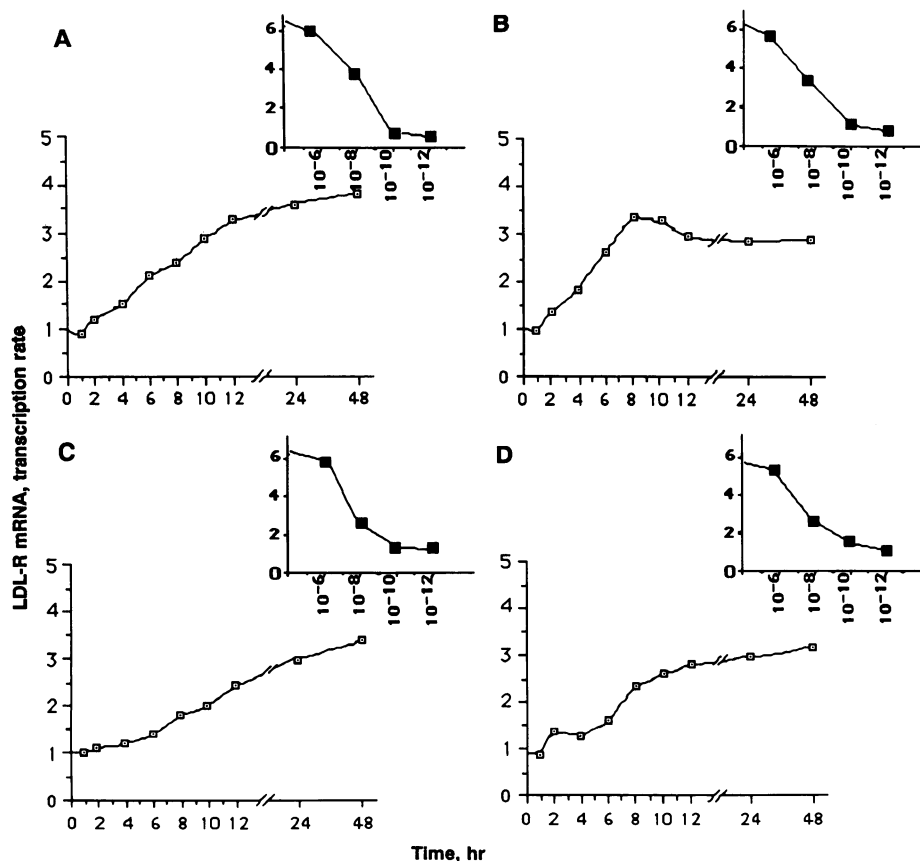


FIG. 1. Induction of LDL-R mRNA by four Ca²⁺-channel blockers (1 × 10⁻⁸ M) in human skin fibroblasts as a function of time and concentration: amlodipine (A), verapamil (B), nifedipine (C), nitrendipine (D). (Insets) Effects of various concentrations of Ca²⁺-channel blockers (in molar concentrations) after 24 hr of incubation. Data points are average values of experiments done in triplicate with three different cell lines.

Fibroblasts. Addition of the rPDGF-BB to fibroblasts resulted in a dose-dependent increase in incorporation of [³H]thymidine into DNA; it is in the range of its dissociation constant for binding to specific cell surface receptors (16). In contrast, addition of Ca²⁺-channel blockers to the fibroblasts negated the effect of PDGF-BB in stimulating DNA synthesis *de novo*. The IC₅₀ values of the various Ca²⁺-channel blockers are summarized in Table 1. Note that amlodipine has a 2-fold higher order of magnitude compared to nitrendipine in its ability to inhibit PDGF-BB-stimulated DNA synthesis *de novo*.

Effects of Ca²⁺-Channel Blockers on Expression of the LDL-R and the HMG-CoA Reductase Genes. It was reported earlier that the benzothiazepine diltiazem and the phenylalkylamine verapamil stimulate synthesis of LDL-R in skin fibroblasts (17). In the current study, we determined the relative potency of the other Ca²⁺-channel blockers to influence transcription of mRNA corresponding to LDL-R and HMG-CoA reductase. Fibroblasts were treated with various concentrations of the different Ca²⁺-channel blockers, after which the amounts of mRNA corresponding to the genes for LDL-R and HMG-CoA reductase were determined. The LDL-R transcription of mRNA began to increase within 2 hr following addition of the Ca²⁺-channel blockers; verapamil reached its maximal effect after 8 hr; amlodipine, nifedipine, and nitrendipine reached a plateau between 12 and 48 hr (Fig. 1). However, HMG-CoA reductase mRNA transcription was not affected by the presence of the various Ca²⁺-channel blockers when tested at various concentrations and at different time points (Fig. 2). The stimulatory effect of Ca²⁺-channel blockers on LDL-R transcription was abolished by treating the cells with actinomycin D (5 μg/ml) (data not shown). This also indicated that the effect of Ca²⁺-channel blockers occurs at the mRNA level.

Effects of Ca²⁺-Channel Blockers on PDGF-BB-Stimulated Expression of the LDL-R Gene. To substantiate whether the LDL-R gene expression by PDGF-BB can be enhanced by Ca²⁺-channel blockers, fibroblasts were treated with various Ca²⁺-channel blockers at different concentrations and LDL-R gene transcription was evaluated. An electrophoretic analysis of the mRNA formed revealed that PDGF-BB induced an increased transcription rate after 2 hr; however, this effect was heightened apparently by the presence of Ca²⁺-channel blockers (Fig. 3). HLA-β was consistently transcribed constitutively in all experiments. Note that the LDL-R mRNA transcription, in response to PDGF-BB

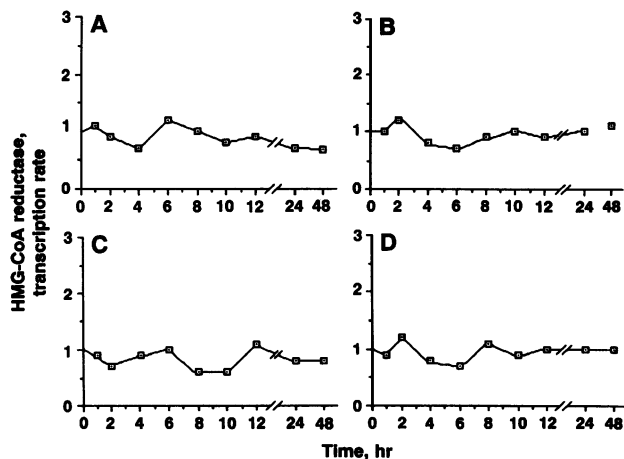


FIG. 2. Kinetics of induction of HMG-CoA reductase mRNA by four Ca²⁺-channel blockers (1 × 10⁻⁸ M) in human skin fibroblasts: amlodipine (A), verapamil (B), nifedipine (C), nitrendipine (D). Data points are average values of experiments done in triplicate with three different cell lines.

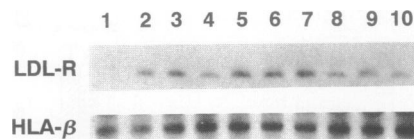


FIG. 3. Electrophoretic analysis of LDL-R mRNA in human skin fibroblasts 2 hr after addition of PDGF-BB (5 ng/ml) with and without Ca²⁺-channel blockers (amlodipine, verapamil, nifedipine, nitrendipine). Lane 1, untreated cells (control); lane 2, cells challenged only with PDGF-BB. Cells in additional lanes have been treated with Ca²⁺-channel blockers (1 × 10⁻⁸ M) for 4 hr followed by a challenge with PDGF-BB for an additional 2 hr or with Ca²⁺-channel blockers alone: lane 3, PDGF-BB plus amlodipine; lane 4, amlodipine; lane 5, PDGF-BB plus verapamil; lane 6, verapamil; lane 7, PDGF-BB plus nifedipine; lane 8, nifedipine; lane 9, PDGF-BB plus nitrendipine; lane 10, nitrendipine. HLA-β gene transcription was constitutive. Ten micrograms of total RNA was used in each lane.

alone, peaked at 2 hr, after which it slowly declined toward the basal level (Fig. 4). In contrast, when PDGF-BB and Ca²⁺-channel blockers were present concomitantly, the response was maintained at its maximum level for the duration of the experiment (48 hr).

In addition to measuring the differences in the *transcriptional* events attributable to the presence and absence of PDGF-BB with and without the drugs, the LDL-R-mRNA *translational* protein was detected on the cell plasma membrane by means of a radioimmunoassay that employed a murine monoclonal antibody specific for the LDL-R protein. Similarly, as for PDGF-BB, addition of Ca²⁺-channel blockers resulted in an increased expression of the LDL-R on the plasma membrane as a function of time of comparable potency (Fig. 5). There was no significant difference in the ability of PDGF-BB and Ca²⁺-channel blockers to affect expression of the LDL-R on the plasma membrane. The combination of both did not enhance translation of the LDL-R mRNA.

Effects of Ca²⁺-Channel Blockers on Transcription of HMG-CoA Reductase Gene. We reported earlier (4) that rPDGF-BB (10 ng/ml) stimulated transcription of HMG-CoA reductase gene in VSMC and skin fibroblasts, which peaked at 4 hr and

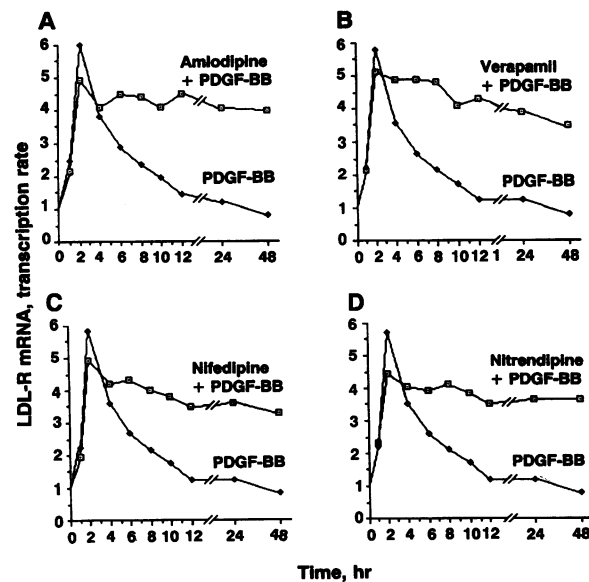


FIG. 4. Kinetics of LDL-R mRNA induced by the four Ca²⁺-channel blockers (1 × 10⁻⁸ M) in combination with PDGF-BB (5 ng/ml): amlodipine (A), verapamil (B), nifedipine (C), nitrendipine (D). Data points are average values of experiments done in triplicate with three different cell lines.

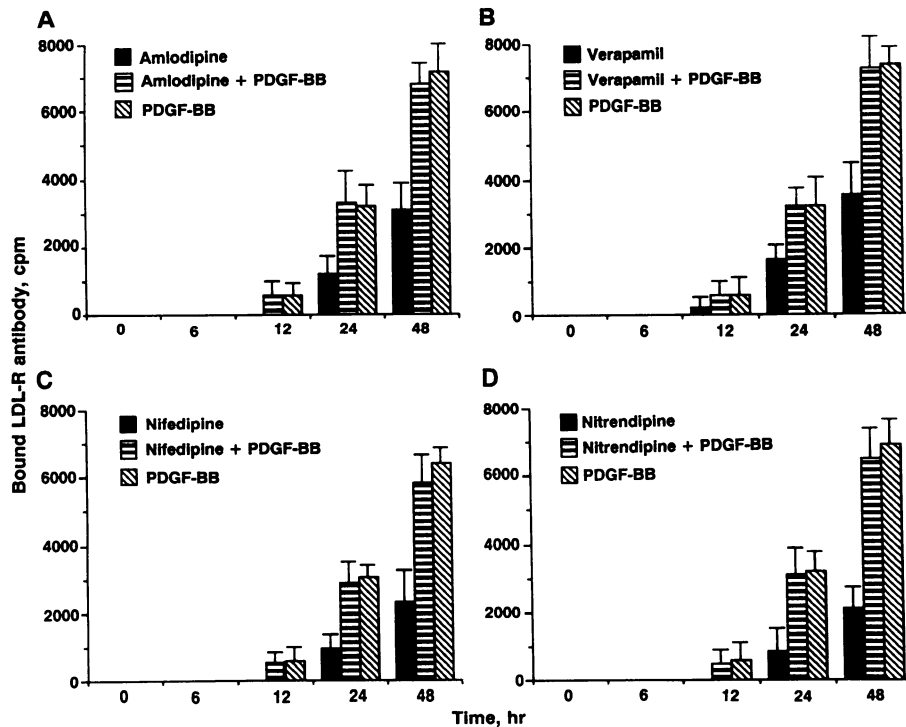


FIG. 5. Expression of LDL-R on the plasma membrane of human skin fibroblasts. Cells were challenged with either Ca^{2+} -channel blockers alone [amlodipine (A), verapamil (B), nifedipine (C), nitrendipine (D)] or Ca^{2+} -channel blockers (1×10^{-8} M) together with 5 ng of PDGF-BB per ml or PDGF-BB alone. LDL-R protein was detected by a murine anti-human monoclonal ^{125}I -labeled antibody. Experimental data represent the mean of triplicate procedures obtained with three different cell lines (\pm the standard deviations).

declined to the basal level thereafter. In contrast, the current data indicate that this effect was inhibited by Ca^{2+} -channel blockers either as a function of time (Fig. 6) or as a function of concentration; IC_{50} values were as follows: verapamil, 1×10^{-11} M; amlodipine, 6×10^{-11} M; nifedipine and nitrendipine, 8×10^{-11} M.

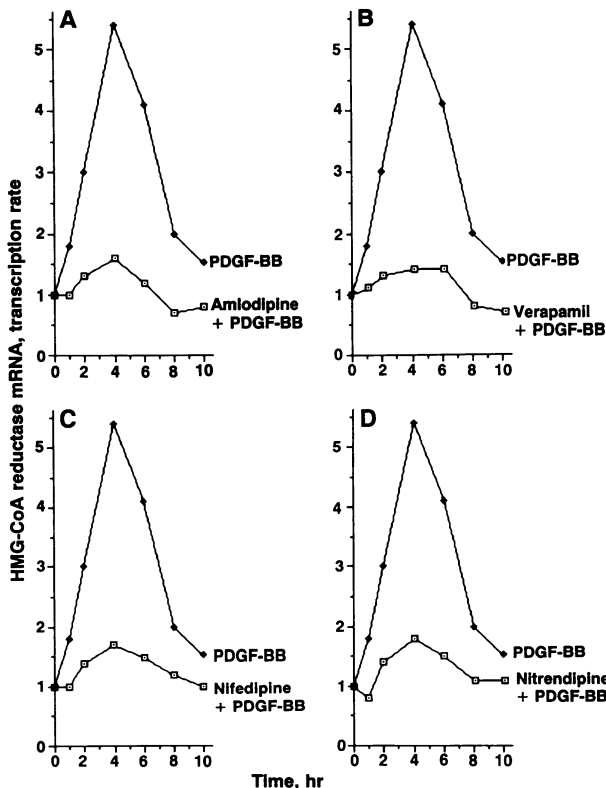


FIG. 6. Inhibitory effect of Ca^{2+} -channel blockers [amlodipine (A), verapamil (B), nifedipine (C), nitrendipine (D)] at 1×10^{-8} M on PDGF-BB-dependent induced transcription of the HMG-CoA reductase gene as a function of time. Data represent the mean of experiments done in triplicate with three different cell lines.

Effects of Ca^{2+} -Channel Blockers on PDGF-BB-Induced Expression of HMG-CoA Reductase and *c-fos* Genes. The effect of PDGF-BB on HMG-CoA reductase and *c-fos* gene expression has already been shown to involve the action of PKC (4). In addition, PKC activity was reported to be inhibited by Ca^{2+} -channel blockers in VSMC (8). To determine whether PDGF-BB-stimulated mRNA translation of HMG-CoA reductase- and *c-fos* genes was affected by Ca^{2+} -channel blockers, fibroblasts were treated with both substances concomitantly. Ca^{2+} -channel blockers prevent expression of both genes at similar concentrations, which were 2–3 orders of magnitude lower than those required for inhibiting [^3H]thymidine incorporation (data not shown).

DISCUSSION

The current data indicate that Ca^{2+} -channel blockers prevent induction of the HMG-CoA reductase gene, which is achieved by physiological concentrations of PDGF-BB so as to reduce cellular cholesterol biosynthesis; on the other hand, Ca^{2+} -channel blockers enhance PDGF-BB-dependent induction of the LDL-R gene and expression of LDL-R at the plasma membrane so as to accelerate the uptake of exogenous sterol. Both effects are concomitant with inhibition of PDGF-dependent proliferation of cells. The effective concentrations of Ca^{2+} -channel blockers are well within the range required for functional changes *in vivo*—e.g., lowering of blood pressure.

Cholesterol metabolism of cells such as fibroblasts is governed by their ability to procure LDL cholesterol from their environment either via the LDL-R pathway or by synthesis *de novo*, the rate-limiting step that is catalyzed by HMG-CoA reductase. Recently, we have shown that the corresponding genes are stimulated uniquely by PDGF-AA, -AB, or -BB (4). This suggests that PDGF has a potential regulatory role in cellular cholesterol biosynthesis that is usually controlled by end-product repression (18, 19). To clarify the effect of the cholesterol-lowering drug *mevinolin*, a HMG-CoA reductase inhibitor, Ma *et al.* (20) reported that it induced expression of LDL-R genes in the livers of hamsters and rabbits. The pharmacological effect was asso-

ciated with a profound decrease in plasma levels of cholesterol. In contrast, Ca²⁺-channel blockers are not known to accomplish the same reduction of cholesterol levels, although they have been shown to increase binding of LDL to corresponding receptors (5). Etingin and Hajjar (21) have suggested, however, that Ca²⁺-channel blockers may act primarily at the cellular level by stimulating acid cholesterol esterase activity, with a resultant decrease in cytosolic free and esterified cholesterol concentrations in VSMC.

How can the dual effects of the Ca²⁺-channel blockers on PDGF-BB-induced expression of the LDL-R and HMG-CoA reductase genes be explained at the molecular level? The mechanism of action of PDGF includes its binding to specific cell surface receptors, which is followed by their autophosphorylation and phosphorylation of tyrosine residues of certain cytoplasmic substrates (9). In addition, PDGF stimulates PI turnover, including a rise in the [Ca²⁺]; and an activation of PKC (22, 23). We reported earlier that PDGF isomers have the ability to induce transcription of LDL-R mRNA, and PDGF-BB, specifically, stimulates expression of the HMG-CoA reductase gene. Although the latter effect was channeled apparently through PKC, the former occurred independently of the enzyme. This agrees with the findings of Eckardt *et al.* (17), who showed that synthesis of LDL-R is unaffected by the PKC agonist phorbol 12-myristate 13-acetate using skin fibroblasts; moreover, participation of Ca²⁺ in LDL-R gene expression was excluded by the following: modulation of extracellular concentration of Ca²⁺ was without an effect, and addition of the Ca²⁺-ionophore A23187 or the Ca²⁺-antagonist TMB-8, thought to block intracellular Ca²⁺ mobilization, failed to alter the rate of LDL-R synthesis. This suggests that the established effects of Ca²⁺-channel blockers—i.e., inhibition of Ca²⁺ influx via potential-operated channels—may not be relevant to their stimulatory influence on the LDL-R gene.

In view of the ability of Ca²⁺-channel blockers to intercalate into the plasma membrane, however, they may inhibit PDGF-dependent phosphorylation mechanisms that otherwise promote LDL-R gene expression. Furthermore, it has been reported that regulation of LDL-R and HMG-CoA reductase genes involves action of the "negative regulatory protein" (24); thus, macrophages treated with cycloheximide have an increased expression of both genes. In that Ca²⁺-channel blockers have an inhibitory effect on DNA synthesis, it is possible that they act primarily by increasing expression of the LDL-R gene through a negation of the effect of the negative regulatory protein. In contrast to the LDL-R gene, HMG-CoA reductase gene expression is controlled apparently by the action of PKC. Thus, the PDGF-BB-induced expression of the latter was inhibited by Ca²⁺-channel blockers that were reported to prevent PDGF-dependent activation of the enzyme (8).

Taking all of these factors into account, Ca²⁺-channel blockers have a potential antiatherosclerotic activity apart from their established ability to block Ca²⁺ influx via potential-operated channels (25); interestingly, their effects on

gene activation occur at concentrations near to their K_d for receptor binding (25). Further studies are needed to unravel their importance in gene activation, which appears to be involved in regulation of cellular cholesterol metabolism.

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