Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 5478–5482, May 1996
Medical Sciences

Ca^{2+} channel blockers modulate metabolism of collagens within the extracellular matrix

(atherosclerosis/metalloproteinases/tissue inhibitor of metalloproteinase)

MICHAEL ROTH*, OLIVER EICKELBERG*, ELEONORE KÖHLER*, PAUL ERNE†, AND LUTZ H. BLOCK‡§

*Department of Research, University Hospital of Basel, CH-4031 Basel, Switzerland; †Department of Cardiology, Kantonsspital, CH-6016 Luzerne, Switzerland; and ‡Department of Medicine, University of Vienna, A-1090 Vienna, Austria

Communicated by Tadeus Reichstein, University of Basel, Basel, Switzerland, January 18, 1996 (received for review November 9, 1995)

ABSTRACT **The extracellular matrix (ECM) is an intricate network composed of an array of macromolecules capable of regulating the functional responsiveness of cells. Its composition greatly varies among different types of tissue, and dysregulation of its metabolism may contribute to vascular remodeling during the pathogenesis of various diseases, including atherosclerosis. In view of their antiatherosclerotic effects, the role of Ca^{2+} channel blockers in the metabolism of ECM was examined. Nanomolar concentrations of the five Ca^{2+} channel blockers amlodipine, felodipine, manidipine, verapamil, or diltiazem significantly decreased both the constitutive and platelet-derived growth factor BB-dependent collagen deposition in the ECM formed by human vascular smooth muscle cells and fibroblasts. The drugs inhibited the expression of fibrillar collagens type I and III and of basement membrane type IV collagen. Furthermore, Ca^{2+} channel blockers specifically increased the proteolytic activity of the 72-kDa type IV collagenase as shown by gelatin zymography and inhibited the transcription of tissue inhibitor of metalloproteinases-2.**

Cell biological changes during the development of sclerotic lesions are characterized by an increased deposition of extracellular matrix (ECM) proteins such as laminin, fibronectin, and collagens (1–3). The latter represent a family of proteins with at least 14 different members, which form the major structural part of the ECM (4). The ECM has been previously considered to be a relatively inert mass of proteins. However, recent reports have shown that it is subjected to a continuous turnover that accounts for a remodeling of ≈5–15% of total ECM per day (5). The ECM influences cell functions and is essentially involved in tissue-type-specific gene expression and proliferation (6–8). It is able to affect the differentiation state of a cell, and, consequently, alterations of the composition of the ECM modulate the responsiveness of cells to physiological stimuli such as growth factors, hormones, and cytokines (7). In addition, changes in the relative proportions of components of the ECM are apparently of crucial relevance for the pathogenesis of various diseases such as atherosclerosis (6, 9, 10). Here, the prominence of collagen as a major element of atherosclerotic plaques is well established, taking into account that 90% of the total collagen found in these lesions is formed by collagens type I and III (1). Furthermore, an increased expression of collagens IV, V, and VI was reported in human atherosclerotic plaques (1, 2).

ECM metabolism is tightly regulated by a complex network of interactions, including (*i*) the *de novo* synthesis of compounds by interstitial cells; (*ii*) the degradation of existing ECM molecules by the action of various proteases, including members of the matrix metalloprotease (MMP) family; and (*iii*) the inhibition of protease activities by specific endogenous antagonists such as the tissue inhibitors of metalloproteinases (TIMPs) (11–13). The MMPs are generally characterized by their need to bind Zn^{2+} as a cofactor and their secretion as inactive zymogen forms. Activation of MMPs is achieved by removal of a conserved aminoterminal propeptide with the subsequent exposition of the catalytic domain (11, 12).

Ca^{2+} channel blockers, which are widely used in hypertension therapy, are known to slow down the progression of existing atherosclerotic lesions and to prevent the development of new lesions in experimental animal models (14–16). We have shown earlier that Ca^{2+} channel blockers are capable of modulating the expression of various genes involved in the regulation of cellular cholesterol biosynthesis (17) and inflammation (18). To gain further insight into their protective efficacy, we investigated the potential effects of Ca^{2+} channel blockers on platelet-derived growth factor (PDGF) BB-induced and constitutive expression of collagens type I, III, IV, and TIMP-2. In addition, gelatinolytic activity of MMPs secreted by vascular smooth muscle cells (VSMCs) and fibroblasts was assessed by zymography.

MATERIALS AND METHODS

Cell Culture. Primary cultures of human pulmonary fibroblasts and VSMCs were established as described (17, 18). Fibroblasts were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum and 8 mM stabilized L-glutamine; VSMCs were cultivated in DMEM supplemented with 5% colostrum, 20 mM Hepes, and 8 mM L-glutamine. Subconfluent cultures (80% confluence) were used between passages two and six. Cells were synchronized by starvation for 48 hr in low-serum medium (0.1% fetal calf serum), replacing the medium every 24 hr. Quiescent cells were challenged with human recombinant PDGF BB (10 ng/ml; GIBCO/BRL) in the presence or absence of the drugs.

Extraction of Total RNA and Northern Blot Analysis. Total RNA was extracted using TRIZOL reagent (GIBCO/BRL) following the instructions of the distributor, and 10 μg of heat-denatured RNA per sample was fractionated on a 1% agarose/7% formaldehyde gel. Northern blotting and hybridization analyses were performed according to standard procedures using [α-^{32}P]dCTP random-primed cDNAs specific for COL1α1 (ATCC no. 61322), COL3α1 (ATCC no. 61324), COL4α1 (ATCC no. 61486), TIMP-2 (ATCC no. 79068), and HLA-β (ATCC no. 57474). Quantitative analysis of transcription signals was performed using an image-analyzing system supported on a Macintosh computer (17, 18).

Western Blot Analysis. Collagen synthesis was determined on the protein level by Western blot analysis on a ready to use gradient SDS/PAGE (4-10%; Bio-Rad). Equal amounts of protein were applied to each lane and size fractionated by electrophoresis. After electroblotting on nylon membranes (Appligene,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ECM, extracellular matrix; MMP, metalloproteinase; PDGF, platelet-derived growth factor; TIMP, tissue inhibitor of metalloproteinase; VSMC, vascular smooth muscle cell.

§To whom reprint requests should be addressed at: Department of Internal Medicine IV, Währinger Lürtel 18-20, A-1090 Vienna, Austria.

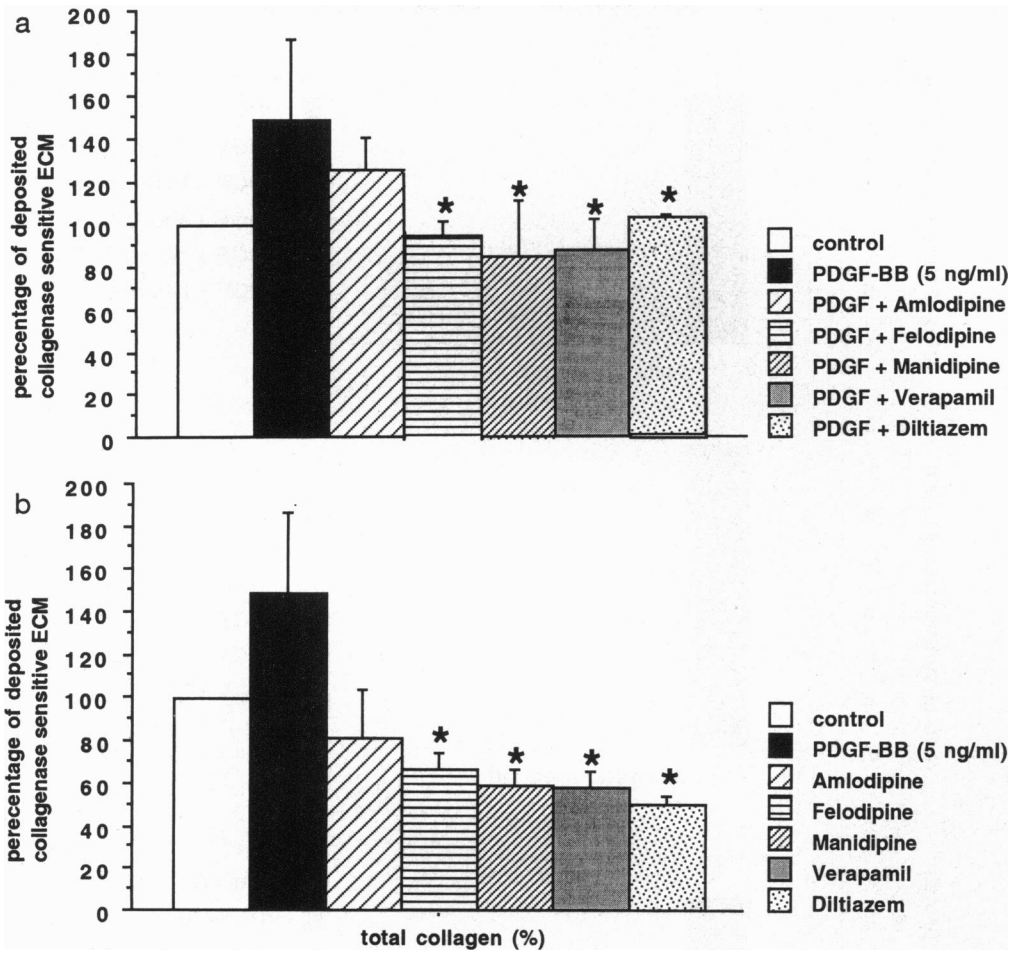

FIG. 1. Effect of Ca^{2+} channel blockers on deposition of total collagen in cultivated human VSMCs. (*a*) Subconfluent quiescent cells were stimulated with PDGF BB (10 ng/ml) for 3 days in the presence or absence of Ca^{2+} channel blockers (amlodipine, felodipine, manidipine, diltiazem, and verapamil). (*b*) To evaluate the effect of the Ca^{2+} channel blockers alone on total collagen deposition, cells were incubated for 3 days in the presence or absence of one of the Ca^{2+} channel blockers. Bars represent mean ± SD. Similar data were obtained in human fibroblasts. Asterisks indicate statistical significant reduction ($P < 0.001$) of collagen deposition when compared with PDGF-stimulated cells (*a*) or to control cells (*b*).

Strasbourg, France), the different collagens were identified by using monoclonal antibodies specific to collagen I, III, and IV (Boehringer Mannheim) (19).

Zymography. The activity of MMPs released from stimulated and unstimulated cells, in the presence or absence of Ca^{2+} channel blockers, was determined using zymographic analysis

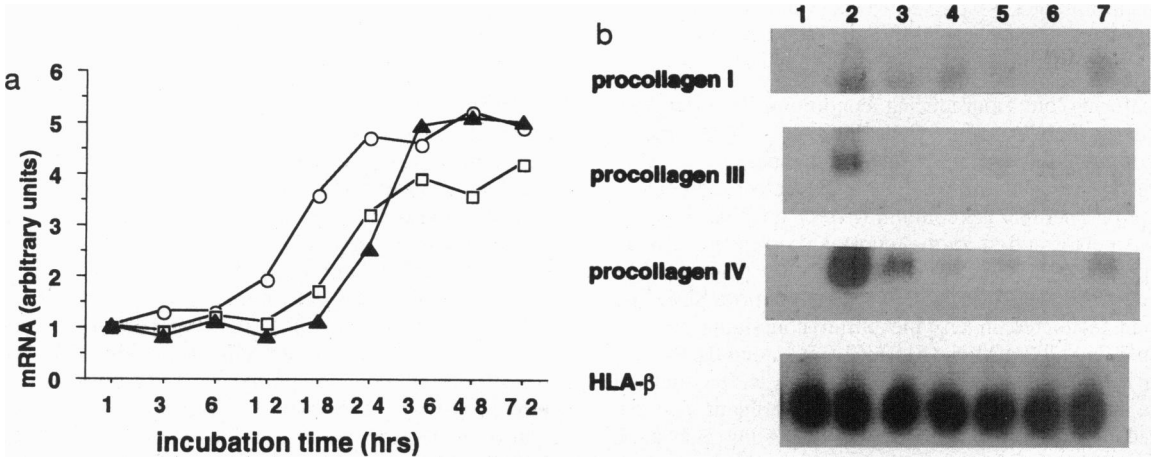

FIG. 2. Kinetics of PDGF BB-induced transcription of procollagen genes (*a*) and the effect of Ca^{2+} channel blockers on this PDGF BB-induced transcription (*b*). (*a*) Quiescent VSMCs were stimulated with PDGF BB (10 ng/ml), and total RNA was isolated at various times (0, 3, 6, 12, 24, 36, 48, and 72 hr). Northern blots were evaluated by an optical densitometric analyzing system. Lines represent typical kinetics of gene transcription of procollagens I, III, and IV in the cell line VSMC 0058. Similar results were obtained with all other cell lines. (*b*) Northern blot analysis of the effect of Ca^{2+} channel blockers on PDGF BB-induced transcription of procollagens I, III, and IV genes at 36 hr in human VSMCs. Lane 1, unstimulated VSMCs; lane 2, cells stimulated with PDGF BB (10 ng/ml); and lanes 3–7, cells stimulated with PDGF BB in the presence of amlodipine, felodipine, manidipine, diltiazem, and verapamil at 1×10^{-8} M, respectively. HLA-β was used as a constitutive control gene.

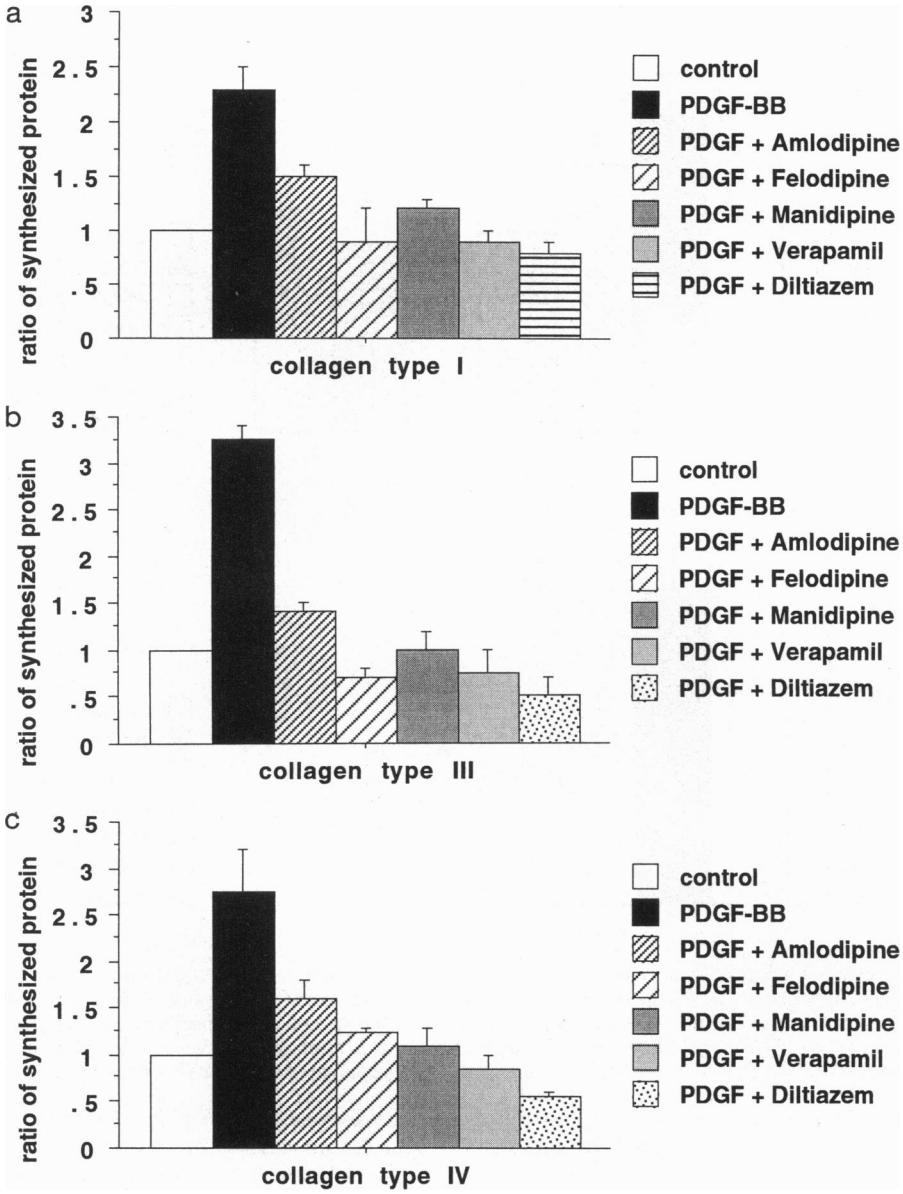

FIG. 3. Effect of the Ca^{2+} channel blockers on PDGF BB-induced protein synthesis of collagens I, III, and IV in cultivated human VSMCs. Quiescent cells were stimulated with PDGF BB (10 ng/ml) in the presence or absence of one of the Ca^{2+} channel blockers at 1×10^{-8} M. (*a*) Collagen I. (*b*) Collagen III. (*c*) Collagen IV.

under denaturing but nonreducing conditions. In brief, cell supernatants were collected at various times (0, 12, 24, 36, and 48 hr) after addition of the respective Ca^{2+} channel blocker. Aliquots (10 μl) of each sample were applied onto a denaturing SDS/8% polyacrylamide gel containing 0.1% gelatine. Electrophoresis was performed at 25-mA constant current for 2 hr at room temperature, followed by equilibration in twice-distilled water containing 2.5% Triton X-100 for 1 hr to remove SDS. The gel was then incubated in enzyme buffer containing 50 mM Tris·HCl (pH 7.3), 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35 for 18 hr at 37°C. Bands of enzymatic activity were visualized by negative staining with standard Coomassie brilliant blue dye solution (20). Molecular sizes of bands displaying enzymatic activity were identified by comparison to prestained standard proteins (Bio-Rad), as well as to purified recombinant MMP-1, MMP-2, or MMP-3 (Anawa Trading, Wangen, Switzerland). To assess a possible direct effect of the Ca^{2+} channel blockers on the enzymatic activity of MMPs, identical zymograms were incubated in the presence or absence of 10^{-6} M to 10^{-8} M of each of the drugs, respectively.

Proline Incorporation Assay. Subconfluent serum-deprived cell cultures were stimulated for 48 and 72 hr with PDGF BB in the presence or absence of Ca^{2+} channel blockers, and the incorporation of [^{3}H]proline was assessed by liquid scintillation counting. In brief, cells were incubated in low-serum medium containing 1 μCi (1 Ci = 37 GBq) of [^{3}H]proline per ml and 50 μg of sodium ascorbate per ml. *De novo* deposition of total collagen was determined in ethanol-fixed ECM (70% ethanol, twice for 15 min at room temperature), after removal of the culture medium and lysis of the cells (25 mM NH_4OH for 10 min at room temperature). ECM was washed with 50 mM Tris·HCl/1 mM $CaCl_2$/1 mM proline, pH 7.5, followed by 4 hr of incubation in a buffer containing 50 mM Tris·HCl (pH 7.5), 5 mM $CaCl_2$, and 2.5 mM *N*-ethylmaleimide, either with or without collagenase (30 units/ml). Residual ECM was solubilized by overnight incubation in 0.3 M NaOH and 1% SDS. Radioactivity in supernatants or in residual matrix was determined by liquid scintillation counting. Calculations of collagen content in the ECM were as follows: (*i*) (supernatant dpm without collagenase $\times$ 100)/(supernatant dpm without

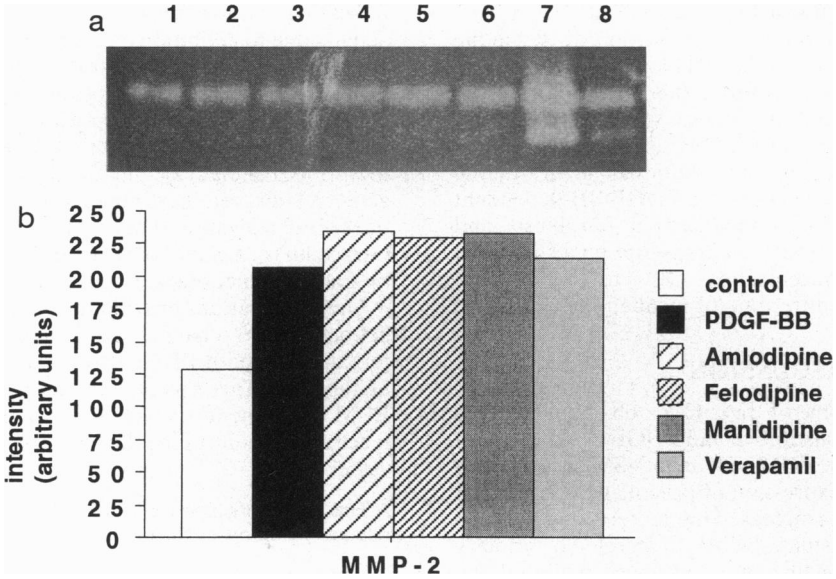

FIG. 4. Ca^{2+} channel blockers enhance the proteolytic activity of metalloproteinases. (*a*) Representative zymographic analysis of the gelatinolytic activity of MMP-2 in cell culture supernatants of a primary human VSMC line (V038) after 36-hr incubation. Lane 1, unstimulated control cells; lane 2, cells stimulated with PDGF BB (10 ng/ml); lanes 3–6, cells stimulated with 1×10^{-8} M amlodipine, felodipine, manidipine, and verapamil, respectively; and lanes 7 and 8, recombinant human MMP-2 at 20 ng/ml and 5 ng/ml, respectively. Similar results were obtained with three other VSMC lines. (*b*) Quantitative analysis of a representative zymogram obtained with culture supernatants of primary human VSMCs.

collagenase + solubilized matrix dpm without collagenase) = % background; (*ii*) supernatant dpm with collagenase − % background = ECM collagen dpm); and (*iii*) (ECM collagen dpm × 100)/[collagen dpm + 5.4 × (supernatant dpm with collagenase + solubilized matrix dpm with collagenase − ECM collagen dpm)] = % ECM collagen synthesis (21).

RESULTS

Effect of Ca^{2+} Channel Blockers on Total Collagen Synthesis. Fig. 1 illustrates the effect of Ca^{2+} channel blockers on the deposition of newly synthesized collagen by determining the content of deposited collagen in the ECM of human VSMCs and fibroblasts. Both constitutive and PDGF BB-induced deposition of collagen were downregulated in the presence of Ca^{2+} channel blockers at nanomolar concentrations (1×10^{-8} M to 1×10^{-9} M). This effect of the drugs was clearly dose-dependent (data not shown). Considering the efficacy of the different drugs, we observed that manidipine was the most potent drug in inhibiting PDGF BB-stimulated deposition of collagen.

Ca^{2+} Channel Blockers Inhibit Expression of Collagen Genes. PDGF BB induced *de novo* synthesis of mRNA encoding the procollagens type I, III, and IV starting at 18, 24, and 36 hr, respectively, after the addition of the growth factor in human fibroblasts and VSMCs (Fig. 2*a*). The PDGF BB-induced transcription was abolished when cells were preincubated with actinomycin D (10 μg/ml), indicating that the growth factor-dependent increase of the respective mRNA signals was mainly due to *de novo* synthesis and not to an increased mRNA stability (data not shown). Ca^{2+} channel blockers affected this PDGF BB-induced transcription of the three procollagen genes in a dose-dependent manner. Transcription of the procollagens was significantly inhibited at concentrations of 1×10^{-9} M and completely abolished at concentrations of 1×10^{-8} M (Fig. 2*b*).

Similar inhibitory effects of Ca^{2+} channel blockers on PDGF-induced and constitutive *de novo* synthesis of collagens were obtained at the protein level when Western Blot analysis and immunofluorescence microscopy were performed. As shown in Fig. 3, the expression of collagens type I, III, and IV was significantly inhibited in the presence of the Ca^{2+} channel blockers at a concentration of 1×10^{-8} M.

Effect of Ca^{2+} Channel Blockers on Proteolytic Activity of MMPs. Fig. 4*a* shows a representative zymogram of culture supernatants of human VSMCs treated with the respective Ca^{2+} channel blockers. The zymogram revealed one major band at 72 kDa that was identified as the 72-kDa type IV collagenase by comparison with recombinant human MMP-2 (Fig. 4*a*). The data were confirmed by Western blotting with a monoclonal antibody to human MMP-2 (Anawa Trading; data not shown). Assignment of this band to the latent form of MMP-2 was carried out after *in vitro* activation by 2 mM *p*-aminophenylmercuric acetate for 2 hr at 37°C (22), which resulted in shift toward the lower molecular weight form, which was also detected in the recombinant form. Furthermore, enzymatic activity of MMP-2 was inhibited after addition of 10 mM EDTA, a specific inhibitor of MMPs. In contrast, gelatinolytic bands displayed after zymography were unaffected by incubation in phenylmethylsulfonyl fluoride, a specific inhibitor of serine proteases.

Enhanced MMP-2-dependent digestion of gelatine was observed in cell culture medium sampled after 36 and 48 hr of

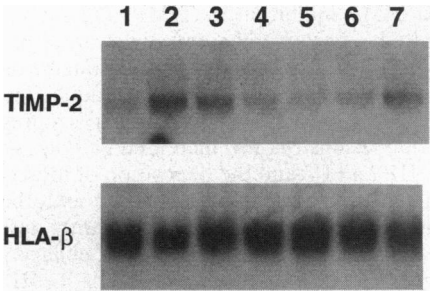

FIG. 5. Northern blot analysis of the inhibitory effect of Ca^{2+} channel blockers on PDGF BB-induced *de novo* synthesis of TIMP-2 mRNA in human VSMCs (V042). Lane 1, unstimulated VSMCs; lane 2, cells stimulated with PDGF-BB (10 ng/ml); and lanes 3–7, cells stimulated with PDGF BB in the presence of amlodipine, felodipine, manidipine, verapamil, or diltiazem at 1×10^{-8} M. HLA-β was used as a constitutive control gene. Similar results were obtained in other VSMC lines and fibroblasts.

incubation. This effect occured in the presence of all drugs tested. Fig. 4*b* shows the increased proteolytic activity of MMP-2 in the presence of the respective Ca^{2+} channel blockers.

Ca^{2+} Channel Blockers Inhibit the Transcription of TIMP-2. PDGF BB induced the *de novo* synthesis of TIMP-2 mRNA in human fibroblasts and VSMCs, starting at 12 hr of incubation and continuing for an additional 48 hr. In the presence of Ca^{2+} channel blockers, the PDGF BB-dependent transcription of TIMP-2 was inhibited in fibroblasts and VSMCs. This inhibitory effect on transcription of TIMP-2 occured at nanomolar concentrations (10^{-8} to 10^{-9} M) and was maintained for the entire time of incubation (Fig. 5).

DISCUSSION

Nanomolar concentrations of five Ca^{2+} channel blockers significantly decreased constitutive and PDGF BB-dependent collagen deposition in the ECM of human VSMCs and fibroblasts and inhibited the expression of procollagens I, III, and IV. Furthermore, all drugs increased the proteolytic activity of the 72-kDa type IV collagenase (MMP-2) secreted by primary human VSMCs. Additionally, the substances inhibited the transcription of TIMP-2 in both fibroblasts and VSMCs.

The ECM is believed to play an important role in the regulation of functional differentiation. Its components control both the differentiation and biological responsiveness of cells (6, 7). Consequently, alterations in the tissue type-specific composition of the ECM have serious impacts in malignancies and inflammatory diseases including atherosclerosis (9, 23, 24). The major processes leading to atherosclerotic lesion development are as follows: (*i*) proliferation and migration of smooth muscle cells and macrophages, (*ii*) increased deposition of ECM compounds including collagens and proteoglycans secreted by VSMCs and fibroblasts, and (*iii*) accumulation of lipid and esterified cholesterol within the surrounding matrix (9). Considering these pathophysiological events, we have previously reported on the beneficial effect of Ca^{2+} channel blockers on intracellular cholesterol metabolism (17) and on their influence on expression of inflammatory mediators (18).

The present data suggest a new aspect of the protective effects of Ca^{2+} channel blockers because they are capable of influencing the metabolism of the ECM associated with atherosclerosis. Although Ca^{2+} channel blockers have long been known to slow down the progression of lesion development in animal models of atherosclerosis, the molecular mechanisms of these effects are not yet completely understood (14–16). Under physiological conditions, the composition of the ECM is subjected to a permanent turnover that results from balanced synthesis and degradation (5, 25). However, the precise mechanisms controlling this homeostasis, which involve *de novo* synthesis of ECM compounds, ECM degradation by MMPs, and MMP inhibition by TIMPs (12), have not been fully elucidated. Consequently, dysregulation of one of these factors may result in alteration of the components of the ECM during the formation of atherosclerotic plaques.

We have shown that PDGF BB, which is upregulated in atherosclerotic lesions (9, 26), increased *de novo* synthesis of collagens I, III, and IV and the deposition of total collagen in the ECM. These effects of PDGF BB were counterbalanced by Ca^{2+} channel blockers. The drugs significantly downregulate the PDGF BB-induced deposition of total collagen by inhibiting the gene expression of procollagens I, III, and IV. Furthermore, an increased proteolytic activity of MMP-2 was observed in the culture medium of VSMCs treated with Ca^{2+} channel blockers. In contrast, incubation of the enzymes with the Ca^{2+} channel blockers during zymography did not result in enhanced proteolysis (data not shown), excluding a direct effect of the drugs on the activity of MMP-2. Recent reports demonstrated an influence of intracellular calcium levels on the gelatinolytic activity of MMP-2 and stromelysin-1 (27–29), indicating that lower calcium levels lead to decreased MMP-2 activity due to inhibition of transcription. In contrast, Lohi and Keski-Oja (30) reported that the calcium ionophores ionomycin and A23187 decreased the enzymatic activity of MMP-2 in human fibrosarcoma cells, indicating that higher calcium levels lead to decreased MMP-2 activity. Analogously, we found that lower intracellular calcium levels lead to increased proteolytic activity. However, there may also exist a regulatory mechanism of MMP-2 activation that is not primarily linked to changes of intracellular calcium concentrations and is still brought about by Ca^{2+} channel blockers.

Our observations provide further insight into the protective actions of Ca^{2+} channel blockers on atherosclerosis by counterbalancing both PDGF BB-induced and constitutive ECM metabolism. This is due to inhibition of transcription of certain ECM components as well as to interference with regulatory mechanisms controlling the metabolism of the ECM within the vessel wall.

This work was supported by a grant from the Alfried Krupp Foundation and by the Swiss National Foundation (Grant 32-39446.93).

1. Rekhter, M. D., Zhang, K., Narayanan, A. S., Phan, S., Schork, M. A. & Gordon, D. (1993) *Am. J. Pathol.* **143,** 1634–1648.
2. Katsuda, S., Okada, Y., Minamoto, T., Oda, Y., Matsui, Y. & Nakanishi, I. (1992) *Arterioscler. Thromb.* **12,** 494–502.
3. Wissler, R. W. & the PDAY collaborating investigators (1994) *Atherosclerosis* **108,** S3–S20.
4. van der Rest, M. & Garrone, R. (1991) *FASEB J.* **5,** 2814–2823.
5. Laurent, G. J. (1987) *Am. J. Physiol.* **252,** C1–C9.
6. Grimaud, J. A. & Lortat-Jacob, H. (1994) *Pathol. Res. Pract.* **190,** 883–890.
7. Lin, C. Q. & Bissell, M. J. (1993) *FASEB J.* **7,** 737–743.
8. Sakata, N., Kawamura, K. & Takebayashi, S. (1990) *Exp. Mol. Pathol.* **52,** 179–191.
9. Ross, R. (1993) *Nature (London)* **362,** 801–809.
10. Stary, H. C. (1990) *Eur. Heart J.* **11** (Suppl. E), 3–19.
11. Nagase, H. (1994) *Contrib. Nephrol.* **107,** 85–93.
12. Woessner, J. F., Jr. (1991) *FASEB J.* **5,** 2145–2154.
13. Hatamochi, A., Mori, K. & Ueki, H. (1994) *Arch. Dermatol. Res.* **287,** 115–121.
14. Kunjara-Na-Ayudhya, R., Thomson, A. B. R. & Kappagoda, C. T. (1994) *Cardiovasc. Res.* **28,** 1089–1095.
15. Nayler, W. G. & Panagiotopoulos, S. (1993) *Am. Heart J.* **125,** 626–629.
16. Haller, H. (1993) *Am. J. Kidney Dis.* **21/6,** S26–S31.
17. Block, L. H., Matthys, H., Emmons, L. R., Perruchoud, A. P., Erne, P. & Roth, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9041–9045.
18. Roth, M., Keul, R., Emmons, L. R., Hörl, W. H. & Block, L. H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4071–4075.
19. Crabos, M., Roth, M., Hahn, A. W. A. & Erne, P. (1994) *J. Clin. Invest.* **93,** 2372–2378.
20. Kleiner, D. E. & Stetler-Stevenson, W. G. (1994) *Anal. Biochem.* **218,** 325–329.
21. Bashey, R. I., Donnelly, M., Insinga, F. & Jimenez, S. A. (1992) *J. Mol. Cell. Cardiol.* **24,** 691–700.
22. Stetler-Stevenson, W. G., Krutzsch, H. C., Wacher, M. P., Margulies, I. M. K. & Liotta, L. A. (1989) *J. Biol. Chem.* **264,** 1353–1356.
23. Galis, Z. S., Sukhova, G. K., Lark, M. W. & Libby, P. (1994) *J. Clin. Invest.* **94,** 2493–2503.
24. Schwartz, S. M., deBlois, D. & O'Brien, E. R. M. (1995) *Circ. Res.* **77,** 445–465.
25. Gardi, C., Calzoni, P., Marcolongo, P., Cavarra, E., Vanni, L. & Lungarella, G. (1994) *Thorax* **49,** 312–318.
26. Ross, R., Masuda, J., Raines, E. W., Gown, A. M., Katsuda, S., Sasahara, M., Malden, L. T., Masuko, H. & Sato, H. (1990) *Science* **248,** 1009–1012.
27. Kohn, E. C., Jacobs, W., Kim, Y. S., Alessandro, R., Stetler-Stevenson, W. & Liotta, L. A. (1994) *J. Biol. Chem.* **269,** 21505–21511.
28. Kohn, E. C., Alessandro, R., Spoonster, J., Wersto, R. P. & Liotta, L. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1307–1311.
29. Baragi, V. M., Sweet, A. M., Thompson, M. A., Hawkins, K. L., Toy, K. A. & Rosebury, W. S. (1995) *Connect. Tissue Res.* **31,** 153–160.
30. Lohi, J. & Keski-Oja, J. (1995) *J. Biol. Chem.* **270,** 17602–17609.