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Antimitogenic Effects of Trapidil In Coronary Artery Smooth Muscle Cells by Direct Activation of Protein Kinase A

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ABSTRACT

The triazolopyrimidine trapidil has been found in controlled clinical trials to prevent restenosis after vascular injury. Although trapidil is widely regarded as a platelet-derived growth factor receptor (PDGF) antagonist, its precise mode of action is still unknown. This study was designed to investigate the inhibition of mitogenesis by trapidil in cultured bovine coronary artery smooth muscle cells (SMC) and to identify major signal transduction pathways involved. Trapidil inhibited PDGF-BB-induced mitogenesis in SMC in a concentration-dependent manner. Comparable inhibitory effects were obtained after stimulation of smooth muscle cells by phorbol ester, which suggests that the action of trapidil was not restricted to PDGF receptor-mediated mechanisms. Trapidil also inhibited PDGF- and phorbol ester-induced mitogen-activated protein kinase as well as Raf-1 kinase activity. As a possible target of trapidil, stimulation of cellular protein kinase A (PKA) activity was detected. Trapidil also induced the phosphorylation of vasodilator-stimulated phosphoprotein in SMC. Antimitogenic effects of trapidil were completely abolished by PKA inhibitors. Neither a direct stimulation of cAMP formation nor a phosphodiesterase inhibition was observed at antimitogenic concentrations of trapidil. However, trapidil directly activated purified PKA holoenzyme in a cAMP-independent manner. In conclusion, trapidil exerts its antimitogenic effects on SMC by direct activation of PKA. Thus, PKA-mediated inhibition of the Raf-1/MAP kinase pathway may be involved in the antimitogenic actions of the compound.

Migration and proliferation of vascular SMC is an early event in vascular injury that eventually results in significant restenosis in 30–40% of patients undergoing PTCA (Ross and Fuster, 1996). The cellular background for these alterations are changes in morphology and function of SMC after endothelial denudation, resulting in their transformation from a contractile into a secretory phenotype that loses myofibrils, generates matrix proteins and responds to growth factors with cell proliferation (Reines and Ross, 1993).

One of these growth factors is PDGF. Binding of PDGF to its receptors stimulates intrinsic tyrosine kinase activity, eventually resulting in a mitogenic response. Several steps of PDGF-dependent signal transduction are known (Claesson-Welsh, 1994). They include Ras-dependent phosphorylation of Raf-1, activation of the MAP kinase pathway (Davis, 1993; Malarkey *et al.*, 1995) and nuclear factor κB (Obata *et al.*, 1996). Thus, inhibition of PDGF receptor binding and/or PDGF-activated signal transduction pathways seem to be promising targets to control injury-induced SMC proliferation.

Trapidil is a triazolopyrimidine with a complex spectrum of biological activities. These include coronary vasodilatation (Noguchi *et al.*, 1984), inhibition of platelet function (Mazurov *et al.*, 1984) and stimulation of vascular prostacyclin production (Nieder *et al.*, 1995). At subcellular level, inhibition of phosphodiesterases has been described (Bartel *et al.*, 1985; Heinroth-Hoffmann *et al.*, 1990). Clearly, the most exciting property of trapidil at the moment is its inhibition of PDGF-induced SMC proliferation *in vitro* (Ohnishi *et al.*, 1982; Tiell *et al.*, 1983; Cercek *et al.*, 1991) and *in vivo* (Liu *et al.*, 1990). A significant inhibition of restenosis after PTCA was also reported in clinical trials and was explained by PDGF receptor antagonism (Nishikawa *et al.*, 1992; Okamoto *et al.*, 1992; Maresta *et al.*, 1994).

The present study was designed to investigate the effects of trapidil on SMC proliferation and to elucidate possible mechanism(s) of its antimitogenic action. Stimulation of SMC proliferation by phorbol ester was used as a reference because PKC-induced mitogenesis has been shown to be inde-

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ABBREVIATIONS: SMC, smooth muscle cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, isobutylmethylxanthine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PBS-TT, phosphate buffered saline/Triton X-100/Tween 20; PDGF, platelet-derived growth factor; PGE_1, prostaglandin E_1; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PTCA, percutaneous transluminal coronary angioplasty; SDS, sodium dodecyl sulfate; VASP, vasodilator-stimulated phosphoprotein; PDGF-BB, platelet-derived growth factor, isoform BB.

pendent of the PDGF-signaling pathway (Sharma and Bhalla, 1993). Evidence is presented that trapidil inhibits SMC mitogenesis via a direct, cAMP-independent PKA activation and, possibly, by a PKA-mediated inhibition of the Raf-1/MAP kinase pathway.

Materials and Methods

Cell culture. Bovine coronary artery SMC were isolated as described previously (Grosser *et al.*, 1997). The cells were cultivated in a 80% Ham's F-12/20% Dulbecco's modified Eagle's medium, supplemented with 15% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin [all cell culture material was from Gibco (Eggenstein, Germany) or Becton Dickinson (Heidelberg, Germany)]. Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°. SMC were characterized microscopically by the typical "hill-and-valley" structure and by indirect immunostaining with a monoclonal α-actin antibody (Boehringer-Mannheim, Mannheim, Germany). The media were exchanged twice a week. Monolayers of the cells were passaged once a week using trypsin-EDTA (0.05%/0.5 mM). Passages 4–10 were used for the experiments. Cell viability was assessed by means of trypan blue exclusion and was found to be $> 95\%$.

DNA synthesis. DNA synthesis was measured as described previously (Grosser *et al.*, 1997). SMC were seeded in 24-well plates (5×10^4 cells/well) and cultivated for 72 hr under standard conditions. For the subsequent 24 hr, cells were exposed to serum-free medium to allow defined stimulation with mitogens. All further incubations were carried out in serum-free medium, supplemented with 3 μM indomethacin (Luitpold Pharma, Munich, Germany) to avoid interactions with endogenously synthesized prostaglandins. If indicated, trapidil (0.1–100 μM; UCB GmbH, Kerpen, Germany), SCH 13929 (10 μM; provided by Dr. D. E. Mullins), or calphostin C (100 nM; Sigma, Deisenhofen, Germany) were preincubated for 15 min. Cells were then stimulated with PMA (10 nM; Fluka, Buchs, Switzerland) or PDGF (20 ng/ml; Boehringer Mannheim, Germany) in triplicate wells. After 20 hr, [^{3}H]thymidine (0.5 μCi/well) (Du Pont, Bad Homburg, Germany) was added. At the end of the total incubation period of 24 hr, the media were removed and the cells were washed twice with 1 ml of ice-cold PBS, 0.3 ml of ice-cold perchloric acid (0.3 M) and again with cold PBS. The cells were solubilized with 0.3 ml NaOH (0.1 M) for 30 min at 37°. [^{3}H]Thymidine incorporation was quantified by liquid scintillation counting. Protein concentration was measured using the Bio-Rad colorimetric protein assay (Bradford, 1976) (Bio-Rad, Munich, Germany).

MAP kinase phosphorylation. SMC, grown to subconfluency, were fasted for 24 hr in serum-free medium and then stimulated with PDGF-BB (20 ng/ml). If indicated, trapidil was added 2 min before the mitogen. MAP kinase phosphorylation was detected by Western blotting using phospho-specific antibodies (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Briefly, proteins were harvested into SDS sample buffer [62.5 mM Tris·HCl, pH 6.8, 2% SDS (w/v), 10% glycerol, 50 mM dithiothreitol], separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). For immunodetection, membranes were probed with phospho-specific MAP kinase antibodies (1:1000) followed by incubation with peroxidase-conjugated secondary antibodies (1:3000; Dianova, Hamburg, Germany). Band were visualized by electrochemiluminescence (Amersham Buchler, Braunschweig, Germany).

Raf-1 kinase activity. Quiescent SMC were stimulated with PDGF-BB (20 ng/ml) in the absence or presence of trapidil (1–100 μM). Thereafter, cells were lysed in lysis buffer (30 mM Tris, pH 7.5, 0.2 mM EDTA, 0.1% Triton X-100, 0.3% mercaptoethanol, 10% glycerol, 0.5 M KCl, 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°. The lysates were sonicated for 5 sec on ice and centrifuged for 30 min at 4° and $10{,}000 \times g$. The supernatants were precleared with protein A sepharose for 3 hr. Raf-1 was immunoprecipitated with a polyclonal antibody against c-Raf-1 (Santa-Cruz, Heidelberg, Germany) for 12 hr at 4° and incubated with protein A sepharose for 1 hr. Resulting complexes were washed twice in lysis buffer with 1 M KCl, twice in lysis buffer with 0.5 M KCl, and twice in lysis buffer containing 0.1 M KCl. For Raf-1 kinase assays, the immunocomplexes were washed once more in reaction buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100). For kinase reaction, immunoprecipitates were incubated in 50 μl of kinase buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton-X-100, 10 mM $MnCl_2$, 2 mM dithiothreitol), 5 μg of histone 1 (Boehringer, Mannheim, Germany) and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 5 min at 37°. The reaction was terminated with 2 × Laemmli buffer and proteins were separated on SDS-PAGE (12%). Histone 1 bands were cut off and radioactivity was determined by liquid scintillation counting. In additional experiments, Raf-1 kinase activity was also measured using a commercial c-Raf-1 immunoprecipitation kinase cascade assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions.

PKA activity. PKA activity in SMC was determined as described by McKenzie and Pouysségur (1996) with some modifications. Subconfluent SMC were rendered quiescent as described above. Trapidil (0.1–100 μM) or PGE_1 (10 nM) were added for 10 min. Stimulation was stopped by rapid aspiration of the media followed by rinsing with ice-cold PBS and addition of lysis buffer (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 10 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 0.1 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, 1% Triton X-100). Cells were then scraped off from the plate and centrifuged briefly at $12{,}000 \times g$ and 4° in a benchtop centrifuge to pellet nonlysed cells. Samples of cell extracts (20 μg of protein) were then assayed for PKA activity in the presence of 100 μM kemptide (LRRASLG; Sigma, Deisenhofen, Germany) as substrate. Reaction was started by adding 4 μCi of [γ-^{32}P]ATP. After a 15-min incubation at 25°, aliquots were placed on a 2 × 4 cm piece of Whatman P 51 paper, dried, and washed three times with 150 μM H_3PO_4. Then, radioactivity was quantified by liquid scintillation counting. Total PKA activity was determined in the presence of 10 μM cAMP. PKA activity was referred to that sensitive to 0.5 μM PKA inhibitor (McKenzie and Pouysségur, 1996). In addition, PKA activity was also measured in a cell-free system with a non-radioactive protein kinase assay kit (Calbiochem, San Diego, CA, USA) using purified PKA holoenzyme from bovine heart (Sigma, Deisenhofen, Germany). Phosphorylation of a synthetic PKA pseudosubstrate (RFARKGSLRQKNV) by trapidil or cAMP was monitored by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

VASP phosphorylation. Quiescent SMC were stimulated with trapidil or PGE_1 for the indicated times, washed with ice-cold PBS, scraped into 1× Laemmli buffer and heated at 95° for 5 min. Aliquots of cell homogenates, containing 100 μg of protein, were subjected to SDS-PAGE using a 3.5% stacking gel/9% separating gel and transferred to membranes as described by Halbrügge *et al.* (1990) with some minor modifications. After transfer, nitrocellulose sheets were blocked in PBS-TT (0.05% Tween-20, 0.3% Triton X-100, 0.01% NaN_3) supplemented with 1% hemoglobin for 1 hr at room temperature. Membranes were washed with PBS-TT and then incubated for another hour with a rabbit anti-VASP antibody (1: 800) (kindly provided by Prof. Dr. U. Walter). Radiolabeling was performed by incubating the sheets with ^{125}I-labeled protein A (0.1 μCi/ml) (ICN, Eschwege, Germany) in PBS-TT for 1.5 hr. After washing and drying, dephospho-VASP (46 kDa) and phospho-VASP (50 kDa) were detected by autoradiography.

Cyclic AMP. Cells were grown to subconfluence in 6-well plates and exposed to serum-free medium for another 24 hr. Then, the cells were washed twice with 2 ml of a balanced salt solution (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM glucose and 20 mM HEPES, pH 7.3). If indicated, balanced salt solution, containing 0.5 mM IBMX, was preincubated for 10 min at 37°, PGE_1 and/or

trapidil were added and the incubation was continued for another 10 min at 37°. The reaction was stopped by addition of ice-cold ethanol (96%). Ethanol was evaporated and intracellular cAMP levels were determined by radioimmunoassay as previously described (Schröder and Schrör, 1993).

Statistics. Data are mean ± standard error of n independent experiments, performed in duplicate or triplicate as indicated. Group differences were calculated by Student's t test (two-tailed) for unpaired samples. $p < 0.05$ was considered significant.

Results

Inhibition of PDGF- and PMA-stimulated DNA synthesis by trapidil. Addition of PDGF to quiescent SMC resulted in a 3–4-fold increase in [^{3}H]-thymidine incorporation. Preincubation with trapidil caused a concentration-dependent inhibition of PDGF-stimulated [^{3}H]thymidine incorporation (Fig. 1). Significant inhibition of DNA synthesis was observed at ≥ 10 μM trapidil. A similar inhibition was seen when cell proliferation (cell number after 48 hr) was measured (not shown).

The phorbol ester PMA was used as a receptor-independent mitogen. Addition of PMA (10 nM) resulted in a 7–8-fold increase in [^{3}H]thymidine incorporation. In contrast to PDGF, these mitogenic effects of PMA were completely abolished by the PKC inhibitor calphostin C (100 nM), indicating that PKC was not involved in PDGF-stimulated SMC mitogenesis (not shown). Interestingly, trapidil also inhibited PMA-stimulated DNA synthesis (Fig. 2) as well as cell proliferation (not shown) in a comparable concentration-dependent manner.

We have also compared the actions of the selective PDGF-antagonist SCH 13929 with that of trapidil after stimulation of mitogenesis with PDGF and PMA, respectively. As expected, trapidil and SCH 13929 (1 μM) inhibited PDGF-induced [^{3}H]thymidine incorporation (Fig. 3). In contrast, PMA-stimulated DNA synthesis was not affected by SCH 13929 and the PDGF antagonist did not alter the inhibition of PMA-induced mitogenesis by trapidil (Fig. 3).

In additional experiments, antimitogenic actions of trapidil were also found in thrombin- and lipopolysaccharide-stimulated SMC, respectively (not shown). Thus, antimitogenic actions of trapidil are not restricted to PDGF-induced mitogenesis.

Inhibition of MAP kinase by trapidil. The data so far indicate that trapidil antagonizes PDGF-induced mitogenic responses but might have additional effects, probably downstream of the PDGF receptor, that are involved in its inhibitory action on cell proliferation. One of the central mitogenic signaling pathways is the MAP kinase cascade. Therefore, we have studied the possible inhibition of MAP kinase activity by trapidil. After stimulation with PDGF, maximal phosphorylation of MAP kinase was seen 10 min after addition of the agonists (not shown). Therefore, this time point was chosen for the experiments. Preincubation of SMC with trapidil resulted in a concentration-dependent inhibition of PDGF-stimulated MAP kinase phosphorylation (Fig. 4). Inhibition of MAP kinase by trapidil was also seen in SMC stimulated with PMA, thrombin or LPS (not shown).

Inhibition of Raf-1 kinase activity by trapidil. In addition to MAP kinase, the effects of trapidil on Raf-1 kinase activity were investigated. After stimulation with PDGF, maximal Raf-1 kinase activation was seen 10 min after addition of the agonists (not shown). Therefore, this time point was chosen for the experiments. Similarly to MAP kinase, trapidil also inhibited Raf-1 kinase activity as determined by histone 1 phosphorylation assay of immunoprecipitated Raf-1 (Fig. 5). Raf-1 kinase activity was also measured using a kinase cascade assay kit based on sequential phosphorylation of MAP kinase kinase, p42 MAP kinase, and myelin basic protein by immunoprecipitated Raf-1. Using this method, similar inhibitory effects of trapidil on Raf-1 kinase activity were observed (Fig. 5).

Stimulation of PKA activity and VASP phosphorylation by trapidil. As a possible mediator of trapidil-induced inhibition of Raf-1 kinase activity, the effects of trapidil on PKA were studied. PGE_1, a known cAMP-dependent activator of PKA, was used as a control. Trapidil concentration-dependently stimulated cellular PKA activity with a maximal effect similar to that of PGE_1 (10 nM) (Fig. 6). At this concentration, PGE_1 exhibited antimitogenic effects comparable with trapidil (100 μM) in bovine coronary artery SMC (not shown).

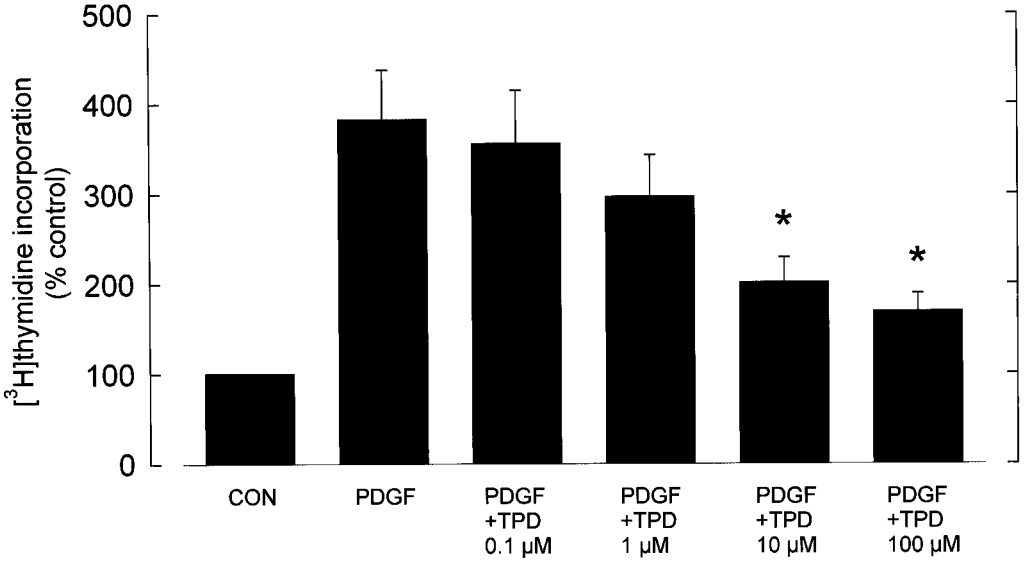

Fig. 1. Inhibition of PDGF (20 ng/ml)-induced DNA synthesis by trapidil (*TPD*, 0.1–100 μM) in vascular SMC. SMC were serum-deprived for 24 hr and subsequently stimulated with PDGF-BB. Trapidil was preincubated for 15 min prior to stimulation with PDGF. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine 20–24 hr after stimulation. Data are mean ± standard error of $n = 6$ independent experiments performed in triplicate. *, $p < 0.05$ versus PDGF.

To elucidate whether PKA activation by trapidil also results in substrate phosphorylation, the phosphorylation of VASP was studied. Trapidil caused a time- (maximum of 10 min after stimulation) and concentration-dependent phosphorylation of VASP as seen from the shift of VASP from 46 kDa to 50 kDa. As a positive control, the effects of PGE_1 on VASP phosphorylation were studied and gave similar results. A representative Western-blot is shown in Fig. 7.

Reversal of trapidil actions by PKA inhibition. To elucidate if PKA activation by trapidil is responsible for the antimitogenic actions of the compound, additional experiments have been carried out using the cell-permeable PKA inhibitor H89. In the concentration used (0.1 μM), H89 did not affect basal or PDGF-stimulated DNA synthesis in SMC. However, the inhibitory effects of trapidil on SMC DNA synthesis were completely abolished in the presence of H89 (Fig. 8). The antimitogenic effects of trapidil were also prevented by the Rp-isomer, triethylammonium salt of adenosine 3′,5′-cyclic monophosphothioate, another cell-permeable PKA inhibitor (not shown).

Similar results were obtained when the inhibition of PDGF-induced MAP kinase phosphorylation by trapidil was studied. Again, H89 completely abolished the inhibitory effects of trapidil (Fig. 9). From these data, the conclusion can be drawn that activation of PKA is causally related to the inhibition of SMC mitogenesis by trapidil.

Effect of trapidil on cellular cAMP. To elucidate the mechanism of PKA activation by trapidil, cAMP levels were measured in the absence and presence of the non-selective phosphodiesterase inhibitor IBMX (0.5 mM). Trapidil at 10 μM and 100 μM did not modify basal cAMP levels in unstimulated SMC whereas IBMX increased cAMP by 2-fold. This reflects cAMP turnover in the presence of active phosphodiesterases. Stimulation of the cells with PGE_1 (100 nM) without phosphodiesterase inhibition did not alter cAMP compared with basal values, probably because of fast degradation of generated cAMP. However, in the presence of IBMX, there was a 5-fold increase in cAMP levels by PGE_1. Trapidil at 10–100 μM did not influence cAMP in PGE_1-stimulated cells as compared with PGE_1 alone. The data are shown in Fig. 10.

Direct activation of PKA holoenzyme by trapidil. Because cAMP seems not to be the mediator of trapidil-induced PKA activation, we hypothesized that trapidil might activate PKA directly, in a cAMP-independent manner. Therefore, possible direct effects of trapidil on PKA were studied in a cell-free system using purified PKA holoenzyme. cAMP and PGE_1 were used as controls. As expected, there was an activation of PKA by cAMP, and PGE_1 was not active in this

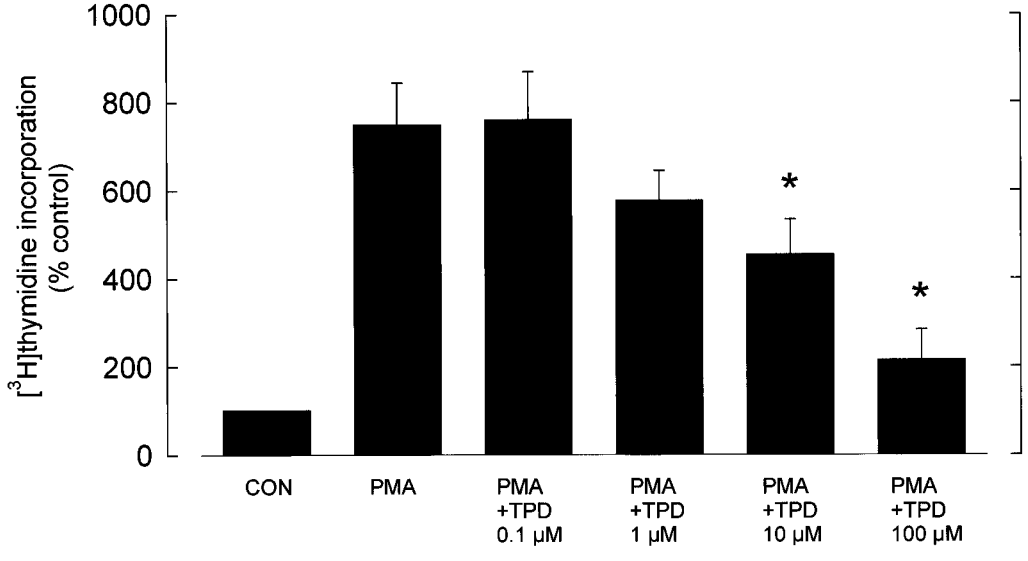

Fig. 2. Inhibition of phorbol ester (PMA, 10 nM)-induced DNA synthesis by trapidil (*TPD*, 0.1–100 μM) in vascular SMC. SMC were serum-deprived for 24 hr and subsequently stimulated with PMA. Trapidil was preincubated for 15 min prior to stimulation with PDGF. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine 20–24 hr after stimulation. Data are mean ± standard error of $n = 4$ independent experiments performed in triplicate. *, $p < 0.05$ versus PMA.

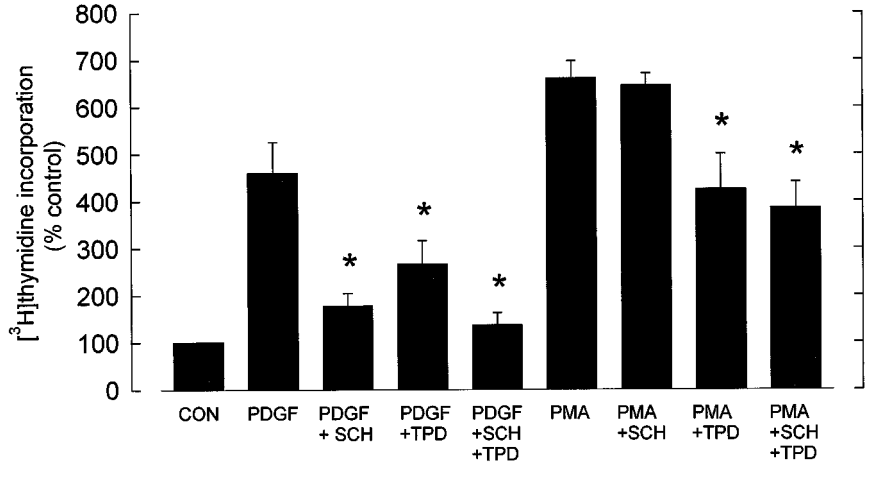

Fig. 3. Comparison of antimitogenic actions of trapidil (TPD, 10 μM) with the specific PDGF receptor antagonist SCH 13929 (*SCH*, 1 μM) in PDGF- (20 ng/ml) and PMA- (10 nM) stimulated SMC. SMC were serum-deprived for 24 hr and subsequently stimulated with PDGF-BB or PMA, respectively. Trapidil and SCH 13929, respectively, were preincubated for 15 min before stimulation. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine 20–24 hr after stimulation. Data are mean ± standard error of four independent experiments performed in triplicate. *, $p < 0.05$ versus PDGF or PMA.

system. Interestingly, there was a concentration-dependent activation of PKA by trapidil that was completely prevented by the PKA inhibitor (0.5 μM). The data are summarized in Fig. 11. Thus, trapidil stimulates PKA independently of cAMP formation.

Discussion

Several previous studies have shown antiproliferative actions of trapidil on SMC and fibroblasts *in vitro* and *in vivo* (Ohnishi *et al.*, 1982; Tiell *et al.*, 1983; Liu *et al.*, 1990; Cercek *et al.*, 1991). These data from animal experiments and/or cell culture studies were confirmed by clinical trials, demonstrating inhibition of restenosis after PTCA by the compound (Nishikawa *et al.*, 1992; Okamoto *et al.*, 1992; Maresta *et al.*, 1994). The present study demonstrates that trapidil is a potent inhibitor of PDGF-induced mitogenesis in bovine coronary artery SMC. Growth factor-induced SMC mitogenesis was concentration-dependently inhibited by trapidil. In most assays these inhibitory effects were obtained at concentrations of 10 μM trapidil. This is in the range of therapeutic plasma levels of the compound (Harder *et al.*, 1996) and suggests that the well-defined conditions of cell culture *in vitro*, used in the present study, might sufficiently reflect the more complex *in vivo* situation.

The antimitogenic actions of trapidil have been explained by an antagonism of PDGF-mediated mitogenesis, putatively at the level of the PDGF receptor (Ohnishi *et al.*, 1982; Nishikawa *et al.*, 1992; Okamoto *et al.*, 1992; Maresta *et al.*, 1994). The present study shows that the effects of trapidil on SMC mitogenesis cannot be explained by PDGF receptor antagonism. For example, inhibition of DNA synthesis by trapidil is not restricted to PDGF but can also be observed after stimulation with other receptor agonists, such as thrombin or lipopolysaccharide. We have also demonstrated that trapidil antagonizes mitogenic receptor-independent actions of PKC stimulation by PMA. Phorbolesters directly stimulate PKC with subsequent phosphorylation of Raf-1 and activation of the MAP kinase signaling pathway (Morrison *et al.*, 1988; Kolch *et al.*, 1993; Marquart *et al.*, 1994). PKC seems not to be involved in PDGF-induced mitogenic signaling in SMC (Sharma and Bhalla, 1993). In our system, the PKC inhibitor calphostin C blocked mitogenic signaling to PMA but not to PDGF. In addition, the effects of trapidil on PMA-induced mitogenesis were not affected by SCH 13929, a specific PDGF receptor antagonist (Mullins *et al.*, 1994). Interestingly, several PDGF-dependent signaling events are not affected by trapidil. Hoshiya and Awazu (1998) have recently shown that trapidil does not modify PDGF-induced

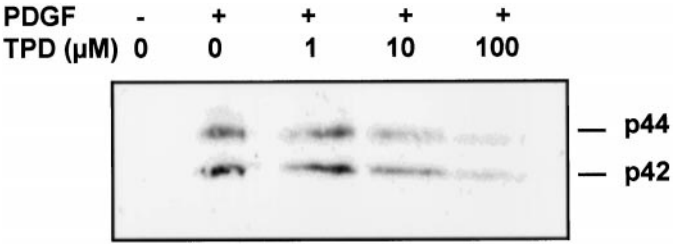

Fig. 4. Effect of trapidil (TPD, 1–100 μM) on PDGF (20 ng/ml)-stimulated MAP kinase phosphorylation. SMC were serum-deprived for 24 hr and subsequently stimulated for 10 min with PDGF-BB. Trapidil was preincubated for 10 min prior to stimulation with PDGF. MAP kinase phosphorylation was detected by Western-blotting with phospho-specific MAP kinase antibodies. The figure shows one representative experiment out of four with similar results.

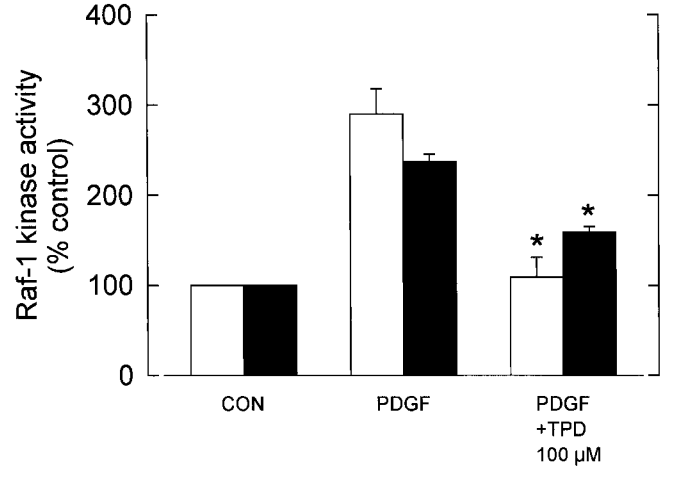

Fig. 5. Inhibition of PDGF (20 ng/ml)-induced of Raf-1 kinase activity by trapidil (*TPD*, 100 μM). SMC were serum-deprived for 24 hr and subsequently stimulated for 10 min with PDGF-BB. Trapidil was preincubated for 10 min before stimulation with PDGF. Raf-1 was immunoprecipitated and kinase activity was measured by histone 1 phosphorylation (□) or using a kinase cascade assay kit (■). Data are mean ± standard error of $n = 3$ (□ or $n = 2$ (■) independent experiments performed in triplicate. *, $p < 0.05$ versus PDGF alone.

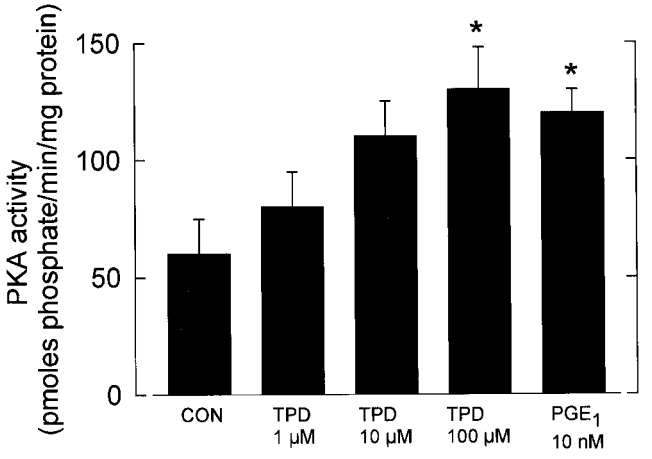

Fig. 6. Trapidil (TPD, 1–100 μM)-induced stimulation of PKA activity in SMC as compared with PGE_1 (10 nM). SMC were serum-deprived for 24 hr and subsequently treated for 10 min with trapidil or PGE_1. PKA activity was measured by kemptide phosphorylation. Data are mean ± standard error of $n = 4$ independent experiments performed in duplicate. *, $p < 0.05$ versus control (*CON*).

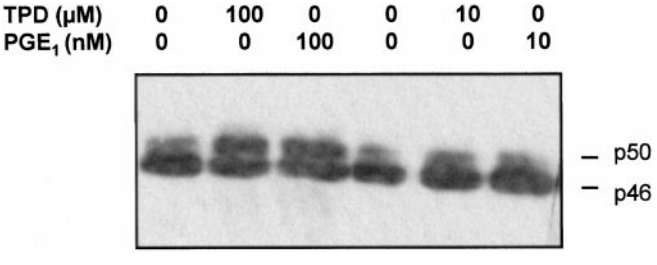

Fig. 7. Trapidil (TPD, 10–100 μM)- and PGE_1 (10–100 nM)-induced VASP-phosphorylation in SMC. SMC were serum-deprived for 24 hr and subsequently treated for 10 min with trapidil or PGE_1. Dephospho-VASP (p46) and phospho-VASP (p50) were detected by Western-blotting with anti-VASP antibodies. Shown is one representative experiment of five with similar results.

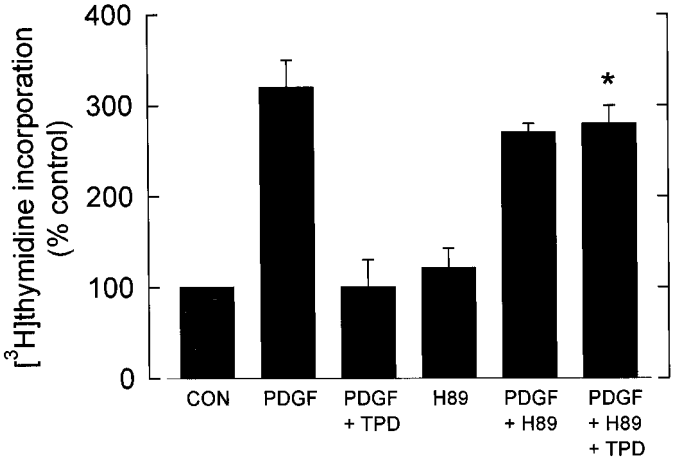

Fig. 8. Inhibition of PDGF (20 ng/ml)-induced DNA synthesis by trapidil (TPD, 100 μM) in the absence and presence of the PKA inhibitor H89 (0.1 μM). SMC were serum-deprived for 24 hr and subsequently stimulated with PDGF-BB. When indicated, H89 was preincubated for 10 min prior to stimulation with PDGF. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine 20–24 hr after stimulation. The data are mean ± standard error of $n = 3$ experiments performed in triplicate. *, $p < 0.05$ versus PDGF + trapidil.

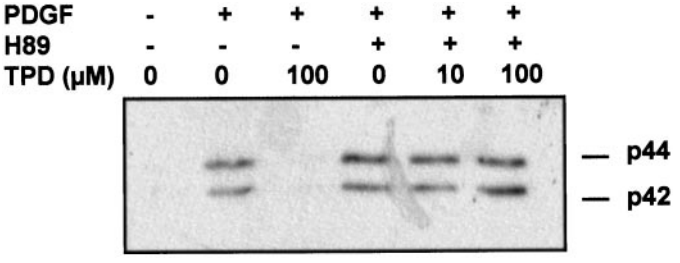

Fig. 9. Effect of trapidil (TPD, 10–100 μM) on PDGF (20 ng/ml)-stimulated MAP kinase phosphorylation in the absence and presence of the PKA inhibitor H89 (0.1 μM). SMC were serum-deprived for 24 hr and subsequently stimulated for 10 min with PDGF-BB. When indicated, H89 was preincubated for 10 min before stimulation with PDGF. MAP kinase phosphorylation was detected by Western-blotting with phospho-specific MAP kinase antibodies. Shown is one representative experiment of three with similar results.

autophosphorylation of the PDGF receptor in rat SMC. Taken together, these data suggest that antimitogenic actions of trapidil have a target that is common for both PKC- and PDGF-mitogenic signaling pathways.

One of these targets is the MAP kinase (Davis, 1993). We have found that trapidil inhibits MAP kinase activation, including activation by mitogens others than PDGF or PMA, such as LPS and thrombin. Growth factors that act on receptors with intrinsic tyrosine kinase activity, such as PDGF, are known to stimulate MAP kinase by a Ras/Raf-1-dependent mechanism (Davis, 1993). The MAP kinase cascade can also be activated by stimulation of G-protein-coupled receptors by thromboxane A_2 or thrombin via activation of phospholipase C_β, subsequent generation of diacylglycerol, PKC, and finally Raf-1 activation (Davis, 1993; Post and Brown, 1996). Alternatively, direct stimulation of PKC by phorbol esters is also followed by activation of the Raf-1/MAP kinase cascade (Sözeri *et al.*, 1992; Kolch *et al.*, 1993; Carroll and May, 1994; Marquart *et al.*, 1994). Therefore, we have investigated Raf-1 as a possible target of trapidil actions. Trapidil markedly inhibited Raf-1 kinase activity. Thus, inhibition of Raf-1 activity could explain the interruption of the MAP kinase cascade by trapidil.

The next question to be answered was how trapidil interferes with Raf-1 kinase activity. Because Raf-1 can be inhibited by PKA (Cook and Mc Cormick, 1993), we have investigated whether PKA-dependent interruption of the Raf-1/MAP kinase pathway may account for the antimitogenic actions of trapidil in SMC. We have shown that cellular PKA was activated by trapidil and that this PKA stimulation was sufficient for phosphorylation of VASP, a physiological substrate for PKA (Markert *et al.*, 1996). In addition, the inhibitory effects of trapidil on DNA synthesis and MAP kinase phosphorylation were completely abolished by PKA inhibitors. These data suggest that stimulation of PKA by trapidil interrupts the mitogenic signaling pathway in SMC.

To elucidate the mechanism of PKA activation, the effects of trapidil on cAMP levels were measured. Previous studies have shown that trapidil increases cAMP levels *in vitro* at high concentrations. This action was explained by inhibition

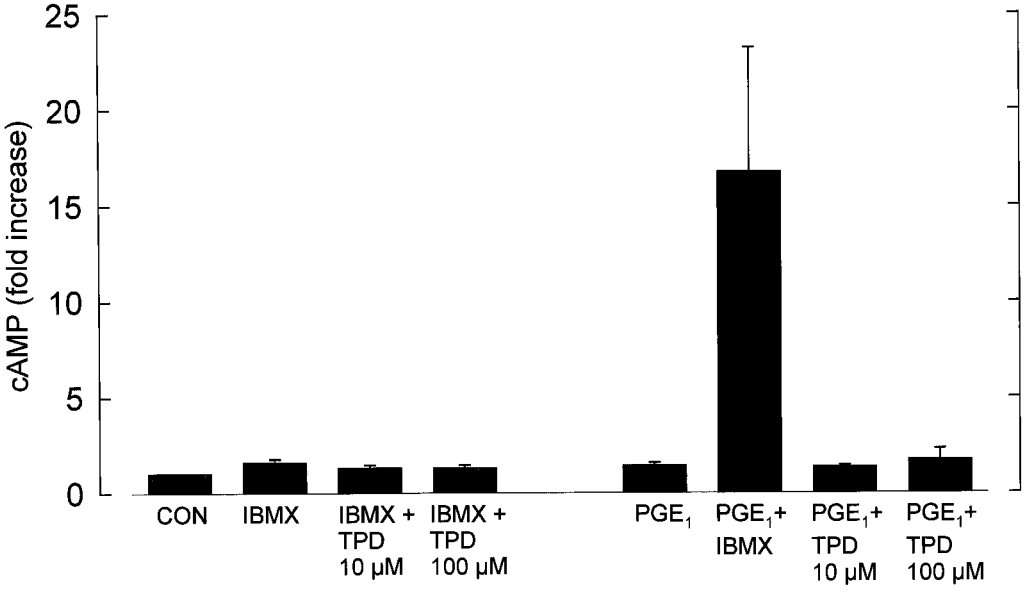

Fig. 10. Effects of trapidil (*TPD*, 10–100 μM) and PGE_1 (100 nM) on cellular cAMP levels in the presence and absence of IBMX (0.5 mM). SMC were serum-deprived for 24 hr. IBMX was preincubated for 10 min, then PGE_1 and/or trapidil were added for another 10 min. The data are mean ± standard error of $n = 3$ experiments performed in triplicate.

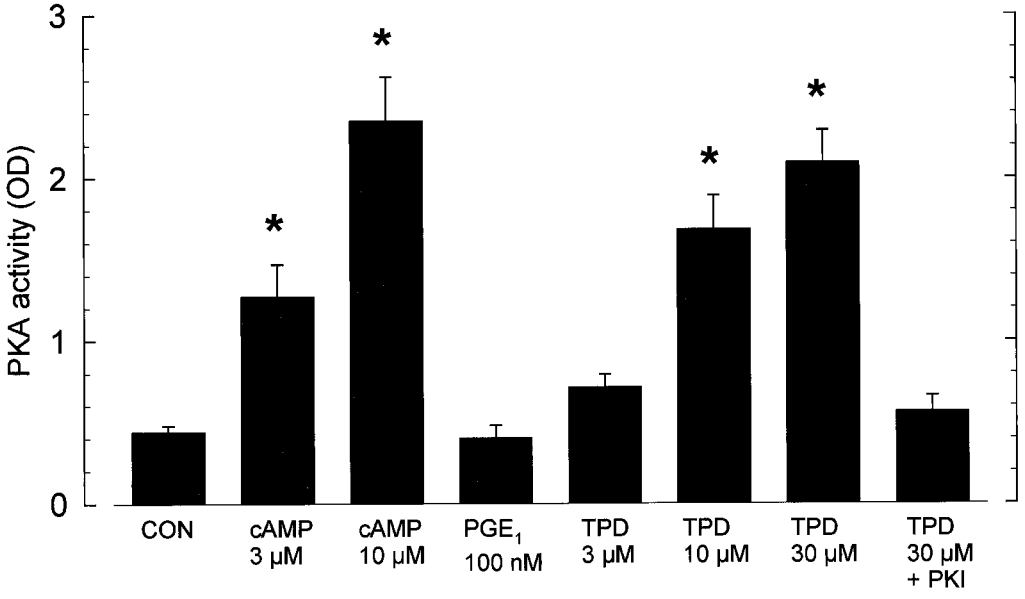

Fig. 11. Effects of trapidil (*TPD*, 3–30 μM) on purified PKA holoenzyme activity as compared with cAMP (3–10 μM) and PGE_1 (100 nM). PKA activity was measured by phosphorylation of a synthetic PKA pseudosubstrate (RFARKGSLRQKNV) and monitored by enzyme-linked immunosorbent assay. Protein kinase A inhibitor (*PKI*, 0.5 μM) was used to demonstrate PKA specificity. The data are mean ± standard error of $n = 4$ experiments performed in duplicate.

of phosphodiesterases (Bartel *et al.*, 1985; Heinroth-Hoffmann *et al.*, 1990). cAMP exerts its antimitogenic effect via stimulation of PKA with subsequent inhibition of mitogenic signaling pathways such as the Raf-1/MAP kinase pathway (Cook and Mc Cormick, 1993; Graves *et al.*, 1993; Wu *et al.*, 1993). Thus, an increase in cAMP might explain the antimitogenic action of trapidil. In agreement with this, a recent study has shown that trapidil, in a concentration of 2 mM, stimulated cAMP formation (1.3–1.9-fold) in rat aortic SMC. This was accompanied by an inhibition of PDGF-induced DNA synthesis and MAP kinase activation without alteration of PDGF signaling upstream of Raf-1 (Hoshiya and Awazu, 1998). However, we failed to demonstrate an increase in cellular cAMP at antimitogenic concentrations of trapidil ($\leq$ 100 μM). Furthermore, trapidil did not increase PGE_1-induced cAMP generation. Thus, there was no evidence for phosphodiesterase-inhibiting activity of trapidil at concentrations up to 100 μM. However, trapidil stimulates cellular PKA activity and phosphorylation of physiological substrates of PKA, such as vasodilator-stimulated phosphoprotein (VASP).

A striking and somehow unexpected finding of the present study was the demonstration of direct activation of purified PKA holoenzyme by trapidil. This novel mechanism might explain PKA stimulation in SMC by trapidil in the absence of an increase in cAMP levels. One might speculate that PKA activation with subsequent inhibition of the Raf-1/MAP kinase pathway accounts for the antimitogenic effects of trapidil. However, Raf-1 may not be the only cellular target for PKA-mediated trapidil actions. For example, we have shown that trapidil inhibits the activation of the transcription nuclear factor-κB (unpublished observations). Thus, additional mechanisms for the antimitogenic effects of trapidil may exist.

Taken together, we conclude that trapidil exerts its antimitogenic effects by direct activation of PKA that results in an inhibition of Raf-1/MAP kinase pathway. Recently, activation of PKA signaling has been shown to inhibit SMC proliferation induced by vascular injury *in vivo* (Indolfi *et al.*, 1997). Thus, the unique properties of trapidil as a direct PKA activator might explain the inhibition of restenosis subsequent to PTCA by trapidil *in vivo*.

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