Biochemical Pharmacology, Vol. 60, pp. 381–387, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00328-2

Inhibition of Platelet-Derived Growth Factor-Induced Mitogenesis by Phosphodiesterase 3 Inhibitors

ROLE OF PROTEIN KINASE A IN VASCULAR SMOOTH MUSCLE CELL MITOGENESIS

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ABSTRACT. Proliferation of vascular smooth muscle cells (SMC) in response to platelet-derived growth factor (PDGF) and other mitogens plays an important role in restenosis following coronary angioplasty. Elevation of adenosine 3′,5′-cyclic monophosphate (cAMP) concentration in SMC has been shown to inhibit SMC mitogenesis and could be obtained either directly by stimulatation of adenylyl cyclase-coupled receptors or indirectly by inhibition of cAMP-specific phosphodiesterase (PDE4) or the cyclic guanosine 3′,5′-monophosphate-inhibitable phosphodiesterase (PDE3). This study compared the effects of the selective PDE3 inhibitors trequinsin and quazinone with the selective PDE4 inhibitors Ro 20-1724 and rolipram on PDGF-induced DNA synthesis, mitogen-activated protein (MAP) kinase activation, cAMP levels, and protein kinase A (PKA) activation in SMC. Both PDE3 and PDE4 inhibitors stimulated intracellular PKA activation as seen from phosphorylation of vasodilator-stimulated phosphoprotein (VASP). However, only PDE3 inhibitors, and not inhibitors of PDE4, reduced PDGF-induced DNA synthesis and inhibited p42/p44 MAP kinase phosphorylation. At antimitogenic concentrations, the PDE3 inhibitors had only minor effects on cAMP levels. In contrast, PDE4 inhibitors increased the forskolin-induced cellular cAMP concentration 13- to 17-fold above control. These data demonstrate that inhibitors of PDE3 are potent antimitogenic agents and that a general increase in cellular cAMP levels and PKA activation *per se* are not sufficient to inhibit PDGF-induced SMC mitogenesis. BIOCHEM PHARMACOL **60**;3:381–387, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. PKA; PDGF; SMC; mitogenesis; phosphodiesterases; signal transduction

Proliferation of SMC† is a typical event in the pathogenesis of atherosclerosis [1]. It also occurs after percutaneous coronary interventions subsequent to procedure-associated endothelial injury [2]. These SMC responses are associated with their transformation from a contractile into a secretory phenotype that loses myofibrils, generates matrix proteins, and responds to growth factors with cell proliferation [1]. One of these factors is PDGF. Binding of PDGF to its receptor stimulates intrinsic tyrosine kinase activity, resulting in a mitogenic response in SMC. The PDGF-dependent signal transduction includes the activation of the MAP kinase pathway [3, 4].

Several studies have demonstrated that agents which stimulate PKA inhibit mitogen-induced phosphorylation of the p42/p44 MAP kinases (ERK-1/2) and DNA synthesis in SMC [5–9]. One possibility to stimulate PKA activity is accumulation of cAMP by inhibition of its breakdown by PDEs. At least 30 different cyclic nucleotide phosphodiesterases have now been identified in mammalian tissues and cells and can be divided into seven major families [10, 11]. Inhibition of DNA synthesis by the non-selective PDE inhibitor IBMX has been reported [12].

Since non-selective PDE inhibitors exhibit a variety of side effects in clinical settings, isoform-selective PDE inhibitors have increasingly been studied. In mammalian SMC, the breakdown of cAMP is mainly catalyzed by the cyclic guanosine 3′,5′-monophosphate (cGMP)-inhibitable isoform (PDE3) and the cAMP-specific isoform (PDE4) [12–14]. Trequinsin, quazinone, and cilostazol selectively inhibit PDE3, while rolipram and Ro 20-1724 selectively inhibit PDE4 [15–18]. Activity of both PDE subfamilies has been found in the membrane-bound as well as in the cytosolic fractions [12, 13, 17]. In bovine aortic SMC, about half of the high-affinity cAMP-hydrolyzing activity was attributed to PDE3 [12, 17]. A similar distribution between cytosolic and membrane-bound fractions was observed for PDE4 [12, 13].

There is still controversy regarding the role of the

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† *Abbreviations:* SMC, smooth muscle cells; PDGF, platelet-derived growth factor; PKA, protein kinase A; MAP kinase, mitogen-activated protein kinase; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; cAMP, adenosine 3′,5′-cyclic monophosphate; VASP, vasodilator-activated phosphoprotein; and Rp-cAMPS, Rp-isomer of adenosine 3′,5′-cyclic monophosphorothioate.

Received 1 September 1999; accepted 9 February 2000.

specific PDE isoenzymes for control of DNA synthesis. Both PDE3 and PDE4 inhibitors prevented fetal bovine serum-induced [^{3}H]thymidine incorporation [9] in porcine aortic SMC, whereas in mesangial cells no significant inhibition of mitogenesis was found [19]. In clinical trials, the selective PDE3 inhibitor cilostazol was shown to reduce restenosis after angioplasty [20, 21].

In the present study, we investigated the role of the PDE isoforms 3 and 4 on PDGF-induced mitogenesis in cultured bovine SMC in relation to changes in total cAMP levels and PKA activity, respectively. The comparison of PKA activation caused by isoform-selective PDE inhibitors in SMC might contribute to elucidate the role of distinct cAMP/PKA compartments in preventing mitogenesis in SMC.

MATERIALS AND METHODS

Cell Culture

Bovine coronary artery SMC were isolated as described previously [9]. The cells were cultured in 80% Ham's F-12/20% Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin. Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°. Cell culture material was purchased from Life Technologies or Becton Dickinson. Six- and twenty-four-well plates with subconfluent SMC were used for experiments. In 24-well plates, the cell number was about 1–2 $\times$ 10^5 per well, and in 6-well plates about 4 $\times$ 10^5.

DNA Synthesis

SMC were seeded in 24-well plates with serum-free medium for 3 days (about 50% confluency). The PDE inhibitors trequinsin, quazinone, Ro 20-1724, and rolipram (Biomol) were incubated for 10 min and the cells then stimulated with 10 ng/mL of PDGF-BB (Sigma). When the PKA inhibitors Rp-cAMPS (Rp-isomer of adenosine 3′,5′-cyclic monophosphorothioate, Biomol) and H89 ({*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]5-isoquinolinesulfonamide, HCL} Calbiochem) were used, they were added another 10 min prior to addition of the PDE inhibitors. After 20 hr of stimulation, 0.5 μCi/well of [^{3}H]thymidine (Du Pont) was added. At the end of the total incubation period of 24 hr, the media were removed. The cells were washed twice with 1 mL 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 measured by liquid scintillation counting. All measurements were done in triplicate.

p42/p44 MAP Kinase Phosphorylation

SMC, kept in serum-free medium as described above, were stimulated with PDGF-BB (10 ng/mL) for 10 min. When indicated, the PDE inhibitors were added 10 min prior to the mitogen. The p42/p44 MAP kinase phosphorylation was detected by Western blotting using phospho-specific antibodies (New England Biolabs) according to the manufacturer's protocol. Briefly, proteins were harvested into SDS sample buffer (6.25 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). For immunodetection, membranes were probed with phospho-specific MAP kinase antibodies (1:1000), followed by incubation with peroxidase-conjugated secondary antibodies (1:3000; Dianova). Blots were stained with amidoblack to control loading of the lanes.

VASP Phosphorylation

SMC were harvested after a stimulation period of 10 min with the compound to be studied. SDS–PAGE and the following steps were carried out as described above, with the VASP antibodies purchased from Santa Cruz Biotechnology. Both dephospho-VASP (p46) and phospho-VASP (p50) were detected by the antibody. Blots were stained with amidoblack to control loading of the lanes.

Cellular cAMP Concentration

Cells were seeded into 6-well plates in serum-free medium (about 50% confluency). Then, the cells were washed twice with 2 mL of a balanced salt solution (in mM: 130 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 0.8 $MgCl_2$; 5.5 glucose; 20 HEPES; pH 7.3). PDE inhibitors and forskolin (Sigma) were added for 10 min. When forskolin was combined with PDE inhibitors, the PDE inhibitors were incubated for 10 min before forskolin was added for another 10 min. This time point was chosen because after 10-min incubation of forskolin, the intracellular cAMP concentration reaches its maximum under our assay conditions. The reaction was stopped by removing the buffer and replacing with ice-cold ethanol (96%). After the ethanol was evaporated, intracellular cAMP levels were determined by radioimmunoassay as previously described [22]. All experiments were carried out in duplicate.

Cytotoxicity Assays

Possible cytotoxic effects of the PDE inhibitors were studied using ethidium homodimer-1 and calcein AM (AM: bis[(acetyloxy)methyl]ester) fluorescence assays (LIVE/DEAD Viability/Cytotoxicity Kit for animal cells, Molecular Probes) according to the manufacturer's instructions.

Statistics

Data are means ± standard error of N independent experiments, performed in duplicate or triplicate as indicated. Statistical evaluation was done using the Student's *t*-test for unpaired samples, $P < 0.05$ being considered significant.

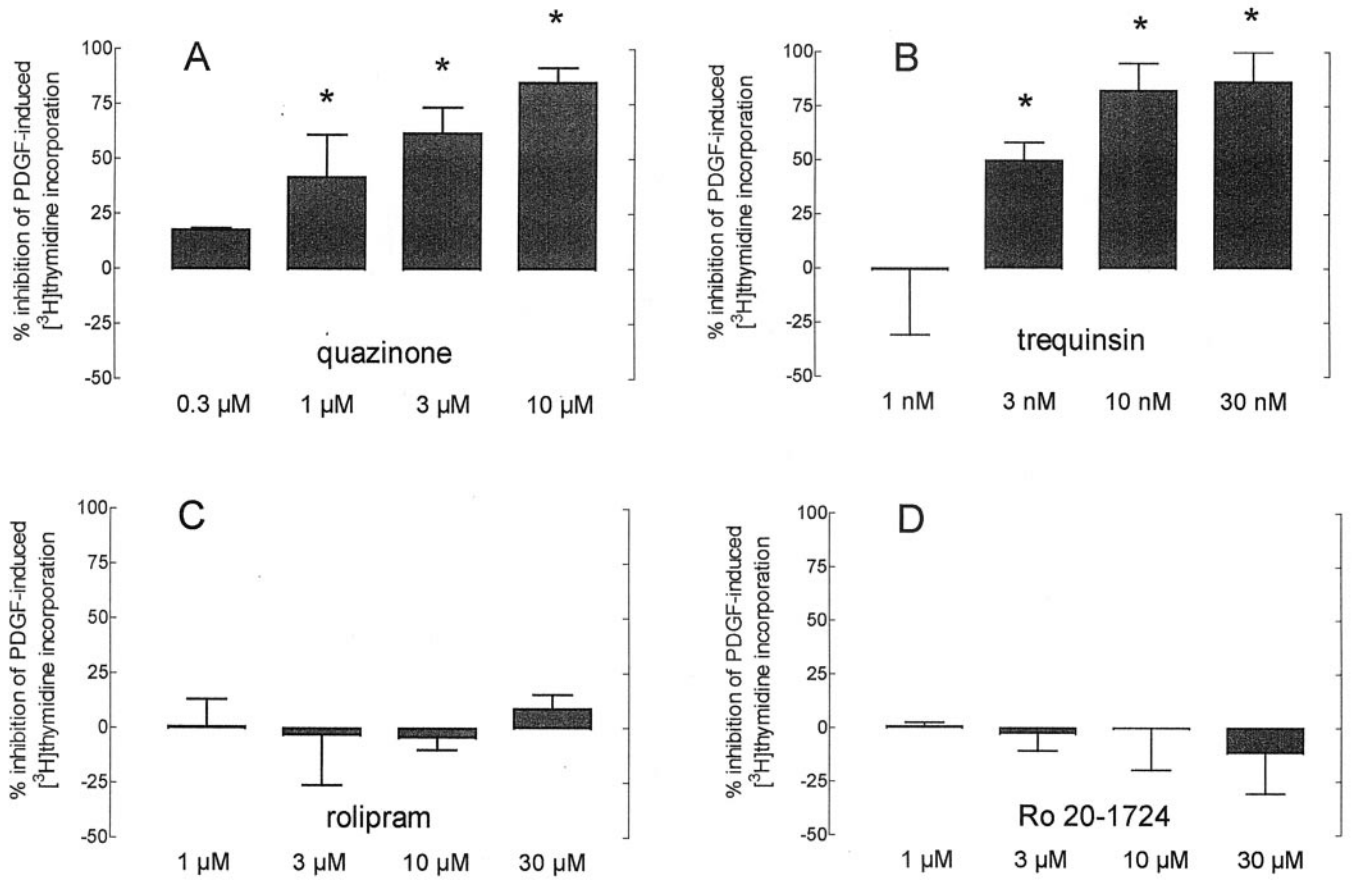

FIG. 1. Inhibition of PDGF-BB (10 ng/mL)-induced DNA synthesis by quazinone (A), trequinsin (B), rolipram (C), and Ro 20-1724 (D) in SMC. The PDE inhibitors were preincubated 10 min prior to stimulation with PDGF. Data are means ± standard error of N = 3 (PDE3 inhibitors) or 4 (PDE4 inhibitors) independent experiments performed in triplicate. *, $P < 0.05$ versus PDGF.

RESULTS

Effects of PDE Inhibitors on PDGF-Induced [^{3}H]Thymidine Incorporation

PDGF-BB stimulated DNA synthesis in SMC 9 ± 2-fold above control (N = 20). This effect was concentration dependently inhibited by the PDE3 inhibitors quazinone and trequinsin (Fig. 1, A and B). In contrast, the inhibitors of PDE4, Ro 20-1724 and rolipram, had no effect (Fig. 1, C and D). DNA synthesis was also inhibited by the non-selective PDE inhibitor IBMX and by the direct PKA activator trapidil (data not shown).

Figure 2 demonstrates that the antimitogenic effects of the PDE3 inhibitors were blocked by the PKA-specific inhibitors Rp-cAMPS (10 μM) and H89 (0.1 μM), suggesting that the inhibition of PDGF-induced DNA synthesis by trequinsin and quazinone was PKA-mediated.

Effects of PDE Inhibitors on PDGF-Induced MAP Kinase Phosphorylation

The data so far indicate that only the PDE3 inhibitors quazinone and trequinsin, but not the PDE4 inhibitors Ro 20-1724 and rolipram, antagonize PDGF-induced mitogenic responses. One of the early central mitogenic signaling pathways is the MAP kinase cascade. After stimulation with PDGF, maximal phosphorylation of MAP kinases was seen after 10 min (not shown). Therefore, this time point was chosen for subsequent experiments. Preincubation with the selective PDE3 inhibitors trequinsin and quazinone inhibited the phosphorylation of p42/p44 MAP kinase. In contrast, the PDE4 inhibitors Ro 20-1724 and rolipram had no effect (Fig. 3).

Effects of PDE Inhibitors on cAMP Accumulation

Treatment of SMC with the PDE4 inhibitors Ro 20-1724 (30 μM) and rolipram (30 μM) increased basal cAMP levels, but only the latter had a significant effect, increasing cAMP levels 3-fold above control ($P < 0.05$). In contrast, the PDE3 inhibitors quazinone (10 μM) and trequinsin (30 nM) did not cause an increase in basal cAMP levels. In combination with the adenylyl cyclase activator forskolin, the increase of cAMP levels by 10 μM quazinone was also not significant. Trequinsin (30 nM) elevated the forskolin-induced cAMP level only 3-fold. In contrast, the two PDE4 inhibitors markedly enhanced the forskolin-induced cAMP concentration: Ro 20-1724 elevated the cAMP concentration 17 ± 5-fold, while rolipram increased it 13 ± 4-fold

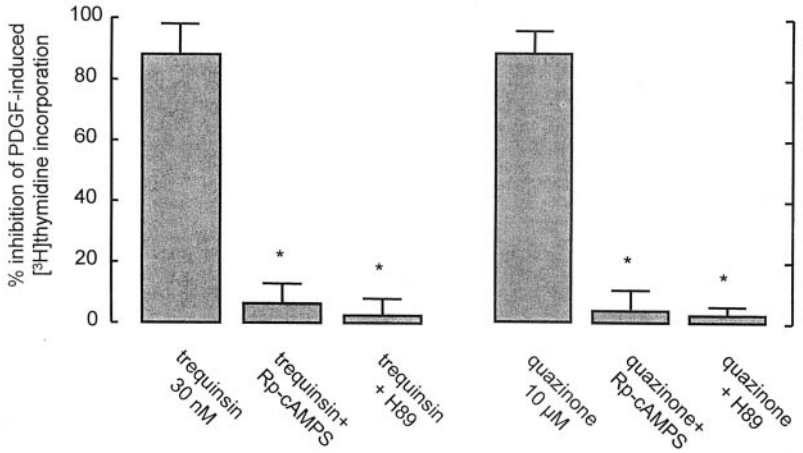

FIG. 2. Inhibition of PDGF-BB (10 ng/mL)-induced DNA synthesis by selective PDE3 inhibitors in the absence and presence of the PKA inhibitors H89 (0.1 μM) and Rp-cAMPS (10 μM). In this concentration, PKA inhibitors had no significant effect on basal and PDGF-induced DNA synthesis. The PKA inhibitors were incubated 10 min prior to trequinsin (30 nM) or quazinone (10 μM). Data are means ± standard error of N = 3 independent experiments performed in triplicate. *, $P < 0.05$ versus PDGF + PDE3 inhibitors.

above control ($P < 0.01$). A minor elevation of intracellular cAMP levels was observed when forskolin (100 nM) was used in the absence of PDE3 inhibitors. These data are summarized in Fig. 4.

Effects of PDE Inhibitors on VASP Phosphorylation

To elucidate changes in intracellular PKA activity, the PKA-dependent phosphorylation of the substrate VASP [23, 24] was studied. Forskolin was used as a positive control. Although 10 μM quazinone and 30 nM trequinsin had only a minor stimulatory effect on the intracellular cAMP concentration, they were able to phosphorylate VASP in a similar fashion to Ro 20-1724 and rolipram, respectively (Fig. 5). In both cases, the phosphorylation-dependent shift was abolished by the PKA inhibitor H89 (Fig. 3), confirming the results on inhibition of mitogenesis, i.e. that both types of inhibitors acted as activators of PKA. PGE_1 and trapidil gave similar results (not shown).

Effects of PDE Inhibitors on Cell Viability

No cytotoxic effects of PDE inhibitors (24-hr incubation with maximal concentrations) were observed using ethidium homodimer-1 and calcein AM fluorescence assays (not shown).

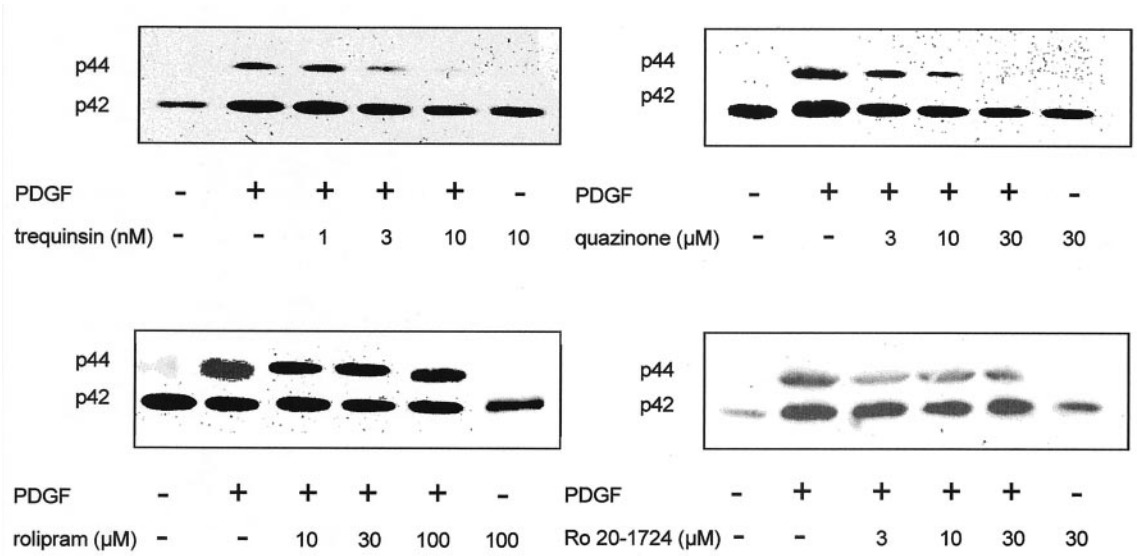

FIG. 3. Effect of selective PDE inhibitors on PDGF-BB (10 ng/mL)-induced MAP kinase phosphorylation. PDE inhibitors were preincubated 10 min prior to stimulation with PDGF. p42/p44 MAP kinase phosphorylation was detected by Western blotting with phospho-specific MAP kinase antibodies. The figure shows a representative experiment out of three with similar results for each PDE inhibitor.

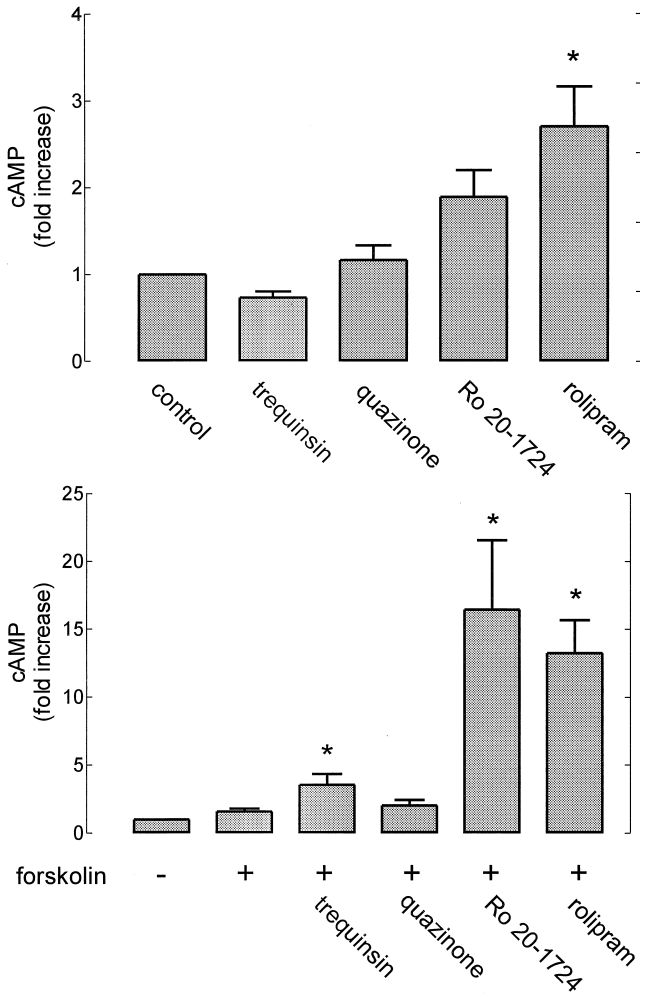

FIG. 4. Effect of trequinsin (30 nM), quazinone (10 μM), Ro 20-1724 (30 μM), and rolipram (30 μM) on cellular cAMP levels in the presence and absence of forskolin (0.1 μM). The basal cAMP concentration was 3 ± 1.3 pmol/L. Data are means ± standard error of N = 3 independent experiments performed in triplicate. *, $P < 0.05$ versus control (± forskolin) + PDE inhibitors.

DISCUSSION

The aim of this study was to investigate the role of PDE isoforms 3 and 4 on PDGF-induced mitogenesis in cultured bovine coronary artery SMC. Additionally, we studied the significance of changes in total cAMP levels for antimitogenic effects and VASP phosphorylation as an indicator for PKA activity. We have demonstrated a potent antimitogenic effect of the selective PDE3 inhibitors trequinsin and quazinone in PDGF-stimulated SMC at concentrations 2- to 10-fold above the reported IC_{50} values for PDE3 inhibition (Fig. 1), i.e. 0.3 nM for trequinsin [25] and 0.6 μM for quazinone [16]. The antimitogenic effect in terms of DNA synthesis was also seen in an early cellular mitogenic signaling pathway, i.e. MAP kinase phosphorylation. In contrast, the inhibitors of PDE4, Ro 20-1724 and rolipram, affected neither PDGF-induced MAP kinase phosphorylation nor DNA synthesis at concentrations well in the range of maximum inhibition of PDE4 activity: The IC_{50} for inhibition of PDE4 for Ro 20-1724 is 2 μM [17], the IC_{50} for rolipram 0.6–1 μM [15].

These negative findings concerning antimitogenic effects of PDE4 inhibitors are at variance with some previous studies demonstrating antimitogenic effects of PDE4 inhibitors in porcine and rat vascular SMC [26, 27]. For example, Pan *et al.* [27] showed that Ro 20-1724 and rolipram ($EC_{50} > 100$ μM) attenuated the proliferation of rat aortic SMC (A10 cells). The EC_{50} values for antimitogenic activity were about 50- to 100-fold higher than the IC_{50} for PDE4 inhibition. The reasons for these different findings are unknown, but might relate to species differences and/or a different experimental design. On the other hand, our findings agree with the data of Chini *et al.* [19]. These authors reported an inhibition of mesangial cell proliferation by selective inhibition of PDE3, but not of PDE4.

At maximal antimitogenic concentrations, only rolipram significantly enhanced the cellular cAMP level (Fig. 4). When SMC were incubated with forskolin, only the PDE4 inhibitors and, to a much smaller extent, trequinsin caused a significant increase in cAMP concentration. In our study, no tight correlation between the elevation of the intracellular cAMP level by PDE4 inhibitors and antimitogenic actions was found. In contrast, inhibitors of PDE3 had no significant effect on the cAMP level in the absence of forskolin, but were able to almost completely block PDGF-induced mitogenesis. One explanation for this apparent discrepancy could be that the intracellular pool of cAMP that is metabolized by PDE4 in SMC is considerably larger than that metabolized by PDE3. In addition, some of the cAMP accumulation in the presence of PDE4 inhibitors might spill over into the cAMP/PKA compartment regulated by PDE3, perhaps accounting for the antimitogenic effect of PDE4 inhibitors found in some studies [26, 27].

Limitations of studies with cultured cells, including SMC, are potential changes in the responsiveness of the cells in intracellular signaling pathways (e.g. upregulation of PDEs and PKA) as opposed to primary cells [28]. The data presented herein support the hypothesis that PDE isoenzymes are involved in a functional and/or ultrastructural compartmentalization of the cAMP/PKA signaling pathway. Our data concerning trequinsin and quazinone agree with previously observed effects of PDE3 inhibitors [18, 26, 27]. PDEs might play a key role in the compartmentalization of the cAMP/PKA signaling pathway by preventing cAMP from leaving distinct compartments and activating PKA there. Conceivably, the cAMP pool regulated by PDE3 may activate a PKA subtype that is attached via specific A-kinase anchoring proteins (AKAPs) to different cellular ultrastructures [29]. Thus, AKAPs might determine the compartmentalization of PKA, while adenylyl cyclase-coupled receptors together with isoforms of PDEs might lead to the compartmentalization of cAMP. In mammals, PKAII is predominantly bound to AKAPs [29]. In preliminary studies, we found that PKAII-selective agonists inhibited PDGF-induced SMC mitogenesis, while

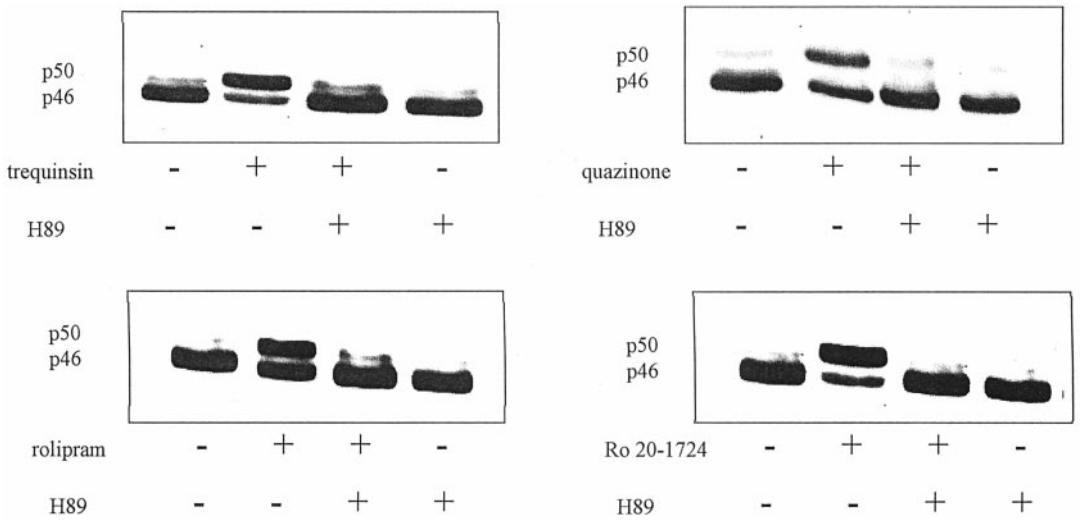

FIG. 5. VASP phosphorylation induced by trequinsin (30 nM), quazinone (10 μM), Ro 20-1724 (30 μM), and rolipram (30 μM). Cells were treated for 10 min with PDE inhibitors. When indicated, cells were preincubated with the PKA inhibitor H89 (0.1 μM). Dephospho-VASP (p46) and phospho-VASP (p50) were detected by Western blotting with VASP antibodies. Shown is one representative experiment out of three with similar results for each PDE inhibitor.

PKAI-selective agonists failed to do so [30]. Compartmentalized elevation of cAMP levels and PKA-dependent actions have also been shown in cardiac myocytes [31, 32]. For example, β_2-adrenergic modulation of cardiac contraction is mediated via compartmentalized cAMP signaling [32]. In addition, Reinitz *et al.* have shown differences in the pattern of the PKA isoform in determined regions in cardiac myocytes, which might be an important factor for the compartmentalization of cAMP/PKA signaling [31].

Possible cellular consequences of the activation of PKA by selective inhibitors of PDE3 and PDE4 were measured in terms of VASP phosphorylation (Fig. 5). Interestingly, both inhibitors of PDE3 and PDE4 phosphorylated VASP, indicating elevated intracellular PKA activity. The PKA inhibitor H89 reversed this effect. This suggests that the antimitogenic actions of trequinsin and quazinone involve PKA activation, although no significant increase in global cAMP levels was detectable. Thus, measuring the phosphorylation of VASP might be a more sensitive tool for detecting PKA activation than the determination of total cellular cAMP levels. In human platelets, PKA-dependent inhibition of platelet aggregation was observed without detectable changes in cAMP concentrations, although a concentration-dependent phosphorylation of the PKA substrate VASP was detected [33]. In our study, VASP phosphorylation was increased by selective inhibitors of both PDE3 and PDE4, indicating that VASP is present in both compartments. With the structurally different PKA inhibitors H89 and Rp-cAMPS, the inhibitory action of trequinsin and quazinone on PDGF-induced DNA synthesis was completely abolished. This is additional evidence for the hypothesis that their antimitogenic effect involves PKA stimulation.

Prostacyclin [7, 9], β_2-adrenergic agonists [8], adenosine mimetics [6], and trapidil [5] have been demonstrated to inhibit mitogenesis in SMC by activating PKA. The present results suggest that distinct subcellular compartments of cAMP/PKA might mediate these inhibitory effects. With respect to PDE, these and other data [14, 19] indicate a more specialized function of PDE isoenzymes in compartmentalizing the cAMP/PKA signaling pathway. Mitogenesis in bovine SMC appears to be regulated by a cAMP/PKA pathway directly linked to PDE3 isoenzymes. While non-selective PDE inhibitors such as IBMX have antimitogenic effects, a variety of side effects limits their use in clinical settings. In contrast, selective PDE3 inhibitors might offer a chance to prevent SMC proliferation via a more specialized PKA activation. In addition, selective inhibition of cAMP/PKA compartments, regulated by distinct PDE isoforms such as PDE3 without affecting other cAMP/PKA compartments, might be a useful pharmacological approach for other clinical indications such as allergy and asthma.

The authors are grateful to Sabine Menzel for expert technical support, Erika Lohmann for competent secretarial assistance, and Artur-Aron Weber for helpful comments. The study was supported by an educational grant from the Forschungsgruppe Herz-Kreislauf e.V. to M.T.O.

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