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Complex actions of protein kinase A inhibitors on mitogenesis of bovine coronary artery smooth muscle cells

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Abstract

This study investigates the possible modulation of platelet-derived growth factor-(PDGF, 20 ng/ml)-induced DNA synthesis in bovine coronary artery smooth muscle cells by the protein kinase A inhibitors Rp-adenosine-3′,5′-cyclic phosphorothioate (Rp-cAMPS, 0.03–10 μM) and {*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl} (H-89, 0.01–1 μM). Rp-cAMPS concentration dependently enhanced PDGF-induced DNA synthesis. In contrast, no potentiation of PDGF-induced DNA synthesis was seen with H-89. However, H-89 but not Rp-cAMPS, inhibited p42/p44 mitogen-activated protein kinase phosphorylation. Thus, Rp-cAMPS, but not H-89, unmasks inhibitory actions of protein kinase A on PDGF-induced mitogenesis of vascular smooth muscle cells. Low specificity may limit the use of H-89 as protein kinase A inhibitor.

Keywords: Protein kinase A; Rp-cAMPS; H-89; Mitogenesis

1. Introduction

Proliferation of vascular smooth muscle cells is an important factor in the pathogenesis of atherosclerosis and restenosis (Schwartz and Reidy, 1996). Smooth muscle cell mitogenesis is regulated by growth factors, such as platelet-derived growth factor (PDGF). Binding of this growth factor to its receptor stimulates intrinsic tyrosine kinase activity, resulting in a Ras-dependent phosphorylation of Raf-1 and activation of the p42/p44 mitogen-activated protein kinase pathway (Davis, 1993; Malarkey et al., 1995). Thus, inhibition of PDGF-activated signal transduction pathways is important in preventing uncontrolled smooth muscle cell growth. One possible inhibitory modulator of PDGF-induced mitogenesis is the protein kinase A, which has been shown to inhibit mitogenic signaling pathways (Graves et al., 1993; Cook and McCormick, 1994). We have shown that direct activation of protein kinase A by the triazolopyrimidine trapidil inhibits PDGF-induced p42/p44 mitogen-activated protein kinase phosphorylation and proliferation of bovine coronary artery smooth muscle cells (Bönisch et al., 1998). Recently, a concomitant activation of stimulatory (p42/p44 mitogen-activated protein kinases) and inhibitory (protein kinase A) pathways by PDGF has been demonstrated in aortic smooth muscle cells (Graves et al., 1996). However, the functional consequences of PDGF-induced protein kinase A activation in smooth muscle cells are not known.

The present study investigates the effects of two protein kinase A inhibitors Rp-cAMPS Rp-adenosine-3′,5′-cyclic phosphorothioate and H-89 {*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl} on PDGF-induced DNA synthesis in bovine coronary artery smooth muscle cells.

2. Materials and methods

2.1. Materials

Cell culture media were from Gibco-Life Technologies (Eggenstein, Germany). [^{3}H]thymidine was from DuPont NEN (Dreieich, Germany). Phospho-specific p42/p44 mitogen-activated protein kinase antibodies were from New England Biolabs (Beverly, MA, USA). H-89 and Rp-cAMPS were from Calbiochem (Bad Soden, Germany),

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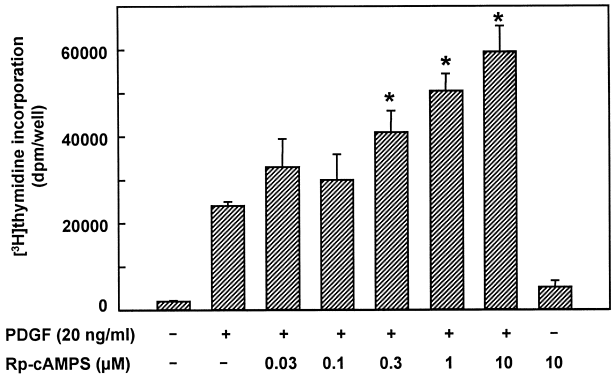

Fig. 1. Effects of Rp-cAMPS (0.03–10 μM) on platelet-derived growth factor-BB (PDGF, 20 ng/ml)-induced [^{3}H]thymidine incorporation in bovine coronary artery smooth muscle cells. Means ± S.E.M., $n = 6$, * $P < 0.05$ vs. PDGF.

myristolated protein kinase inhibitor peptide (PKI) were from Biomol (Hamburg, Germany). All other chemicals were from Sigma (Deisenhofen, Germany).

2.2. Cell culture

Coronary artery smooth muscle cells were isolated enzymatically from the left anterior descending coronary artery of adult cattle and cultured as previously described (Zucker et al., 1998).

2.3. DNA synthesis

DNA synthesis was measured as described previously (Bönisch et al., 1998). Smooth muscle cells were seeded in 24-well plates and cultured for 72 h under standard conditions. Cells were serum-deprived for 72 h in order to allow defined stimulation with the BB isoform of PDGF (20 ng/ml). If indicated, the protein kinase A inhibitors H-89 and Rp-cAMPS were incubated for 15 min prior to stimulation with PDGF. After 20 h, cells were pulsed with [^{3}H]thymidine (0.5 μCi/well) for 4 h. Subsequently, the media were removed and [^{3}H]thymidine incorporation was determined as previously described (Bönisch et al., 1998).

2.4. Mitogen-activated protein kinase (p42/p44) phosphorylation

Smooth muscle cells, grown to subconfluency, were serum-deprived for 72 h and then stimulated with PDGF for 10 min. If indicated, the protein kinase A inhibitors H-89 and Rp-cAMPS were incubated for 15 min prior to stimulation with PDGF. Mitogen-activated protein kinase (p42/p44) phosphorylation was detected by Western blotting using phospho-specific antibodies (New England Biolabs), according to the manufacturer's protocol. Bands were visualized by enhanced chemiluminescence (Amersham Buchler, Braunschweig, Germany).

2.5. Cytotoxicity assays

Possible cytotoxic effects of Rp-cAMPS and H-89 (24 h incubation) were studied using ethidium homodimer-1 and calcein AM fluorescence assays (LIVE/DEAD Viability/Cytotoxicity Kit for animal cells, Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. At the maximal concentration used (10 μM for Rp-cAMPS, 1 μM for H-89), no cytotoxic effects were observed.

2.6. Statistics

Data are means ± S.E.M. from n experiments. Statistical analysis was performed using two-tailed Student's t-test for unpaired data. $P < 0.05$ was considered significant.

3. Results

In the first set of experiments, the effects of the selective protein kinase A inhibitor Rp-cAMPS (0.03–10 μM) on PDGF (20 ng/ml)-induced DNA synthesis in coronary artery smooth muscle cells were studied. Rp-cAMPS did not affect basal [^{3}H]thymidine incorporation. However, Rp-cAMPS concentration dependently increased PDGF-induced DNA synthesis (Fig. 1). Thus, the specific protein kinase A inhibitor Rp-cAMPS demasked the modulatory effects of protein kinase A on PDGF-induced mitogenesis. With the highly specific protein kinase A inhibitor, PKI (0.5 and 2 μM), similar results were found (not shown).

Surprisingly, the protein kinase A inhibitor H-89 did not enhance the mitogenic actions of PDGF (Fig. 2). We hypothesized that as compared to Rp-cAMPS, H-89 might exhibit a lower specificity as protein kinase A inhibitor. We have, therefore, compared the effects of Rp-cAMPS

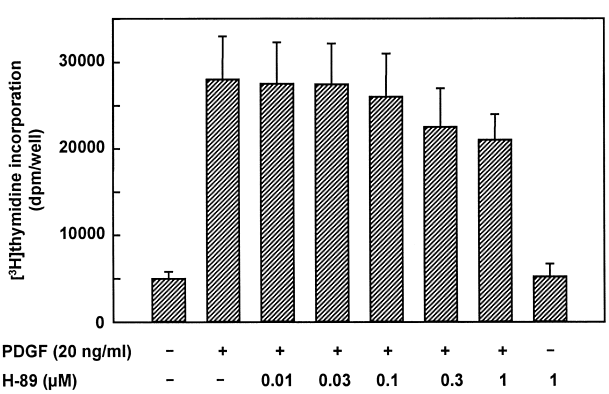

Fig. 2. Effects of H-89 (0.01–1 μM) on platelet-derived growth factor-BB (PDGF, 20 ng/ml)-induced [^{3}H]thymidine incorporation in bovine coronary artery smooth muscle cells. Means ± S.E.M., $n = 5$, * $P < 0.05$ vs. PDGF.

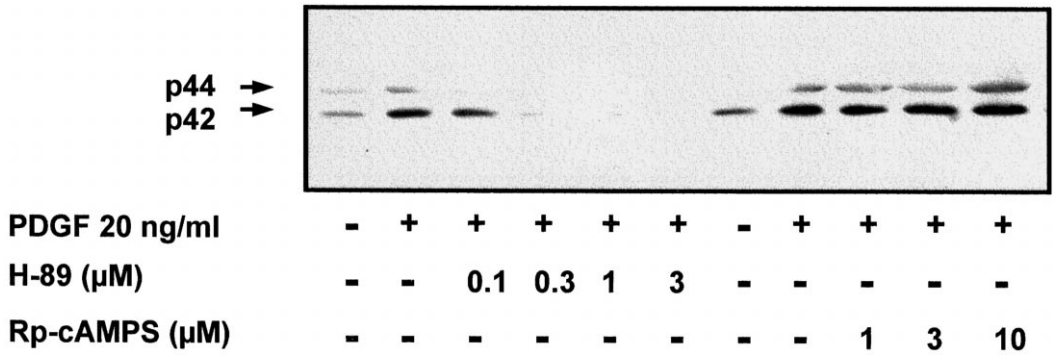

Fig. 3. Effects of H-89 (0.1–3 μM) and Rp-cAMPS (1–10 μM) on platelet-derived growth factor-BB (PDGF, 20 ng/ml)-induced p42/p44 mitogen-activated protein kinase phosphorylation in bovine coronary artery smooth muscle cells. Western blot representative for $n = 3$ independent experiments.

and H-89 on PDGF-induced p42/p44 mitogen-activated protein kinase phosphorylation. Interestingly, mitogen-activated protein kinase phosphorylation was markedly inhibited by H-89 (1 μM). At 10 μM H-89, a complete inhibition of PDGF-induced mitogen-activated protein kinase phosphorylation was observed (Fig. 3). In contrast, Rp-cAMPS (10 μM) did not affect the phosphorylation of p42/p44 mitogen-activated protein kinase by PDGF (Fig. 3).

4. Discussion

The present study is the first to demonstrate a functional role of PDGF-induced protein kinase A activation (Graves et al., 1996) in the regulation of smooth muscle cell mitogenesis. Our data provide evidence for a complex regulation of PDGF-induced smooth muscle cell mitogenesis, which involves a concomitant activation of stimulatory (e.g. p42/p44 mitogen-activated protein kinase) and inhibitory (e.g. protein kinase A) mechanisms.

In contrast to Rp-cAMPS, H-89 did not demask inhibitory actions of protein kinase A on PDGF-induced smooth muscle cell mitogenesis. In previous studies, we have established the inhibitory actions of both compounds on protein kinase A in the same cell system (Bönisch et al., 1998). The mechanisms leading to protein kinase A inhibition by Rp-cAMPS differ from those by H-89. The cAMP analogue Rp-cAMPS acts at the cAMP binding site(s) of the regulatory subunit of the enzyme (Rothermel and Parker-Botelho, 1988), while H-89 inhibits protein kinase activity of the enzyme (Snyder et al., 1992, Engh et al., 1996). Since ATP binding is essential for all protein kinases, we hypothesized that as compared to Rp-cAMPS, H-89 might exhibit a lower specificity as protein kinase A inhibitor. This might be due to the chemical structure of action of 5-isoquinolinesulfonamide (H-X) kinase inhibitors, which more or less affect nearly all kinases in smooth muscle cells. A possible kinase that might have been affected by H-89 is the p42/p44 mitogen-activated protein kinase, an important signaling molecule in PGDF-induced mitogenesis. We have demonstrated that mitogen-activated protein kinase phosphorylation was markedly inhibited by H-89. But it cannot be excluded that others might be inhibited as well. Thus, in contrast to Rp-cAMPS and PKI, H-89 appears not to be a specific inhibitor of protein kinase A. On the other hand, inhibition of protein kinase A-dependent antimitogenic actions in vascular smooth muscle cells by H-89 have been found (Chen et al., 1999). Thus, differences in the action of H-89 on mitogenesis might be due to differences in content and activity of protein kinase A, MAP and other kinases in the cell system used. In addition, discrepancies to the results of other groups on effects of H-89 on smooth muscle cells mitogenesis (Chen et al., 1999) might be due to differences in experimental conditions including cell source and cell culture media. Although with low concentrations of H-89 (0.1 μM), protein kinase A-dependent antimitogenic actions have been reversed in smooth muscle cells (Osinski and Schrör, 1999), it might have advantages to use a more specific protein kinase A inhibitor like Rp-cAMPS or PKI to avoid effects on other kinases.

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