Adenylyl Cyclase 3 Mediates Prostaglandin E₂-induced Growth Inhibition in Arterial Smooth Muscle Cells*

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Arterial smooth muscle cell (SMC) proliferation contributes to a number of vascular pathologies. Prostaglandin E₂ (PGE₂), produced by the endothelium and by SMCs themselves, acts as a potent SMC growth inhibitor. The growth-inhibitory effects of PGE₂ are mediated through activation of G-protein-coupled membrane receptors, activation of adenylyl cyclases (ACs), formation of cAMP, and subsequent inhibition of mitogenic signal transduction pathways in SMCs. Of the 10 different mammalian AC isoforms known today, seven isoforms (AC2–7 and AC9) are expressed in SMCs from various species. We show that, despite the presence of several different AC isoforms, the principal AC isoform activated by PGE₂ in human arterial SMCs is a calmodulin kinase II-inhibited AC with characteristics similar to those of AC3. AC3 is expressed in isolated human arterial SMCs and in intact aorta. We further show that arterial SMCs isolated from AC3-deficient mice are resistant to PGE₂-induced growth inhibition. In summary, AC3 is the principal AC isoform activated by PGE₂ in arterial SMCs, and AC3 mediates the growth-inhibitory effects of PGE₂. Because AC3 activity is inhibited by intracellular calcium through calmodulin kinase II, AC3 may serve as an important integrator of growth-inhibitory signals that stimulate cAMP formation and growth factors that increase intracellular calcium.

Proliferation of arterial smooth muscle cells (SMCs) contributes to several cardiovascular diseases such as atherosclerosis (1, 2). The intracellular second messenger cAMP markedly inhibits proliferation of SMCs and antagonizes growth factor-stimulated activation of the extracellular signal-regulated kinase pathway and the S6 kinase 1 (S6K1) pathway and activation of cyclin-dependent kinases (3). Synthesis of cAMP from ATP is catalyzed by adenylyl cyclases (ACs), which are, with some exception, transmembrane enzymes activated by receptors coupled to the stimulatory G-protein Gs (4–7). To date, at least 10 different isoforms of ACs (AC1 through AC10) have been cloned and identified in a wide array of vertebrate tissues. Most tissues express several AC isoforms, which exhibit remarkable diversities in their sensitivities toward signaling molecules such as different subunits of G-proteins, calcium/calmodulin, protein kinases, and phosphatases (4–7). Although stimulation through the α subunit of Gs is the principal mechanism whereby ACs are activated (4–7), the activity of certain AC isoforms is also regulated by β subunits of Gs, Gαq, and Gαi (8), G-protein βγ subunits (9), cAMP-dependent protein kinase phosphorylation (10), protein kinase C isoforms (11–13), changes in membrane potential (14), and calcium (15–17). Calcium regulates several AC isoforms directly or indirectly though other proteins. Increases in calcium though IP3 receptors can lead to protein kinase C activation, which in turn can activate AC1, AC2, AC3, AC5, and AC7 (7). Calcium binding to calmodulin can directly stimulate AC1 and AC8 (15–17), can activate phosphatase 2B-sensitive AC9 (18), and can inhibit AC1 (19) and AC3 (20, 21) via activation of calmodulin kinase IV (CaM KIV) and CaM KII phosphorylation, respectively.

The expression of AC isoforms in mammalian tissues is also diverse. Some isoforms exhibit extremely broad patterns of expression, such as AC2 and AC9, whereas expression of other isoforms appears to be tissue-specific, such as the neurospecific expression of AC1 (22). Although AC3 was originally thought to be expressed only by the olfactory neuroepithelium, it is now known to be expressed in multiple tissues (23). Clearly, the expression of multiple AC isoforms in a cell provides an intricate system for cross-talk and fine tuning of signals increasing cAMP formation.

We show here that normal human and murine arterial SMCs express AC3 and that the principal AC isoform activated by PGE₂ in these cells is a calcium-inhibited AC with pharmacological characteristics of AC3. Furthermore, arterial SMCs isolated from AC3-deficient mice are resistant to PGE₂-mediated growth inhibition. Thus, AC3 mediates the growth-inhibitory effects of PGE₂ in arterial SMCs.

EXPERIMENTAL PROCEDURES

Reagents—PGE₂, 8-bromo-cAMP, 3-isobutyl-1-methylxanthine (IBMX), and forskolin were obtained from Biomol (Plymouth Meeting, PA) and were dissolved in ethanol, distilled water, and Me₂SO, respectively. The calcium ionophore A23187 was from Calbiochem-Novabiochem Corp. Human recombinant platelet-derived growth factor-BB (PDGF-BB) and a polyclonal anti-Gα₁α antibody generated against the peptide RMRHLQYELL of bovine Gα₁α were from Upstate Biotechnology (Lake Placid, NY). Anti-AC3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and a monoclonal anti-CaM KII antibody was from Transduction Laboratories (Lexington, KY). [2,6-³H]Adenine

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¶¶ The abbreviations used are: SMC, smooth muscle cell; AC, adenylyl cyclase; CaM K, calmodulin kinase; PDE, cyclic nucleotide phosphodiesterase; DMEM, Dulbecco’s modified Eagle’s medium; P3K, phosphatidylinositol 3-kinase; PDS, plasma-derived serum; PDGF, platelet-derived growth factor; PGE₂, prostaglandin E₂; IBMX, 3-isobutyl-1-methylxanthine.

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(20–40 Ci/mmol) was obtained from PerkinElmer Life Sciences. High glucose DMEM and calcium-free DMEM were from Life Technologies, Inc.

**AC3-deficient Mice**—The AC3 gene has recently been disrupted in mice by the laboratory of D. R. Storm (24). A colony of AC3+/− mice on a C57/D2 background was obtained from M. Dep� (22) and bred to generate age- and sex-matched wild-type (AC3+/+), AC3−/−, and AC3−/+ mice for this study. Disruption of the AC3 locus in AC3+/− and AC3−/+ mice and SMCs was confirmed by polymerase chain reaction analysis of tail biopsies or cultured arterial SMCs. The two primers (5′-CTTGGATAAGTTGCTTACCT-3′ and 5′-GGTTAAGAAAGGAGAAGACA-3′) that hybridize to sequences within the deleted region of the AC3 locus were used to identify the presence of the wild-type allele. The mutant allele was revealed by a forward primer (5′-CCCTGTGGCTCTAAGTGTTACCG-G3′) that hybridizes to the reverse complement of the 5′ region of the neomycin cassette and a second primer (5′-TGTGAATTGGTGTTCATCCTG-3′) that hybridizes 230 base pairs downstream of the forward primer as described by Wong et al. (24).

**Tissues and Cells**—Human fetal aortas were obtained from the Central Laboratory for Human Embryology at the University of Washington. Eleven aortas with a gestational age of 74–145 days (mean value of 102 days) were obtained. A segment of each aorta was fixed in methyl Carnoy's fixative for immunohistochemical detection of AC3.

Mouse SMCs were isolated from the thoracic aortas of wild-type (AC3+/+), AC3−/−, and AC3−/+ mice. The mice were killed by carbon dioxide, and the thoracic aorta was immediately dissected and cleaned from extraneous tissue, blood, and fat. The aorta was then transferred to a 35-mm dish with 4 ml of enzyme solution (2 mg/ml bovine serum albumin, 1 mg/ml collagenase type 1 ( Worthington Biochemical Corp.), 0.375 mg/ml soybean trypsin inhibitor (Worthington Biochemical Corp.), and 0.125 mg/ml elastase type III (Sigma) in DMEM for 30 min at 37 °C. Incubation of the aorta in this medium allows separation of the smooth muscle layer and the adventitia and removes endothelial cells. The adventitia was carefully separated from the smooth muscle layer using watchmaker forces, and the smooth muscle layer was then minced and incubated in the above medium for 2 h at 37 °C on a shaker. After incubation, the cells were centrifuged, washed with 10% fetal bovine serum, and plated onto 25-cm 2 dishes and grown in DMEM, 10% fetal bovine serum. The medium was changed to DMEM, 1% PDS for 2 days when the cell cultures were confluent. The SMCs were then incubated in DMEM containing 1% human plasma-derived serum (PDS). For immunoprecipitation of AC3, SMCs were harvested —

**RESULTS**

**PGE2 Activates AC3 in Human SMCs**—To investigate the characteristics of the AC isofrom(s) activated by PGE2 in SMCs, normal human aortic SMCs were stimulated with 2 μM PGE2. Forskolin, which activates all known AC isoforms except AC9, was used as a control. PGE2-induced stimulation of AC activity was observed at 1 min (80% increase over basal) and was maximal at 30 min (data not shown). Therefore, subsequent experiments were performed using a 30-min stimulation with PGE2. As shown in Fig. 1A, PGE2 induced an approximate 20-fold increase in AC activity in human SMCs. This stimulation was blocked by 65% by an increase in intracellular calcium levels induced by the calcium ionophore A23187. The results shown in Fig. 1B demonstrate that A23187 did not inhibit PGE2-induced AC activation in the absence of extracellular calcium. An extracellular calcium concentration of 0.5–10 mM was required for the inhibitory effect of A23187 (Fig. 1B). Thus, the effect of A23187 was dependent on increases in intracellular calcium levels. The inhibition of PGE2-induced AC activation by the calcium ionophore was completely reversed by co-
incubation with the CaM KII inhibitor KN-62 (Fig. 1A). KN-62 alone had no effect on AC activity (data not shown). AC3 is the only AC isoform known to be inhibited by CaM KII, indicating that the main isoform activated by PGE2 in human SMCs is AC3. Expression of CaM KII in human SMCs was verified using Western blot analysis (data not shown). When four different experiments were summarized, the A23187- and CaM KII-sensitive AC3 component of PGE2-stimulated AC activation was 62.4 ± 3.8% (data not shown). Forskolin (50 μM), on the other hand, induced an approximate 80-fold stimulation of AC activity in these cells, and this effect was not significantly inhibited by increasing intracellular calcium levels (Fig. 1A). This finding is consistent with results that show that several AC isoforms that are not inhibited by calcium are expressed in human SMCs. Together, the results show that AC3 is the principal AC isoform activated by PGE2 in human arterial SMCs.

**Human and Murine Arterial SMCs Express AC3**—To investigate whether AC3 is indeed expressed in arterial SMCs, we used Western blot and immunohistochemical analyses. To verify that AC3 is expressed in human arterial SMCs in vivo, we used human fetal aortas for immunohistochemical detection of AC3 (Fig. 2, A and B). These studies demonstrated a clear expression of AC3 in smooth muscle of fetal human aorta sections at gestational days 74 and 84 (data not shown) with much more labeling demonstrated at day 127 (Fig. 2A). Labeling was completely blocked by including AC3 peptide antigen in the reaction (Fig. 2B). AC3 was also expressed in cultured human arterial SMCs as shown by immunoprecipitation and subsequent Western blot analysis. The AC3 antibody was found to precipitate a band of ~170 kDa, corresponding to the glycosylated form of AC3 (Fig. 2C). This band was efficiently blocked by the AC3 antigen used to generate the antibody (Fig. 2C). Thus, AC3 is expressed in isolated human arterial SMCs in culture and in vivo.

Next we took advantage of an AC3-deficient mouse that was recently developed in the laboratory of D. R. Storm (24) to investigate the role of AC3 in PGE2-induced signaling. SMCs were isolated from the thoracic aortas of wild-type (AC3+/+), heterozygous mouse (AC3+/−), and AC3 knockout (AC3−/−) mice and were characterized as SMCs by the expression of smooth muscle α-actin. Expression of AC3 in membrane fractions from these cells was studied by Western blot analysis. SMCs from wild-type mice showed two prominent bands at ~170 and 180 kDa corresponding to glycosylated forms of AC3 (Fig. 2D). These bands were present in the membrane fraction and were absent from the soluble fraction (data not shown). SMCs from AC3−/− mice, on the other hand, were devoid of AC3 expression (Fig. 2D). AC3+/− mice showed an ~50% reduction of AC3 protein expression (24). To investigate if there is compensatory up-regulation of expression of Gα1 in SMCs from AC3−/− mice, Western blot analyses were performed. These studies showed that the expression of Gα1 was similar in SMCs from wild-type and AC3−/− mice (data not shown). PGE2 Preferentially Activates AC3 in Murine Arterial SMCs—As shown in Fig. 3A, PGE2 induced a 5-fold stimulation of AC activity in murine aortic SMCs. This activation was completely inhibited in the presence of A23187 and was nearly normalized by the CaM KII inhibitor KN-62, indicating that in murine aortic SMCs, like in human arterial SMCs, AC3 is the principal AC isoform activated by PGE2. Forskolin (10 μM), on the other hand, induced a stimulation of AC activity about 50-fold higher than that of PGE2 (data not shown), indicating that forskolin-sensitive AC isoforms other than AC3 were expressed by these cells. SMCs were also isolated from AC3+/−...
mice that retain ~50% of the AC3 expression level found in wild-type littermates (24). We used AC3+/− animals rather than AC3−/− animals in several of our experiments because of the low number of adult AC3−/− mice available. Aortic SMCs from AC3+/− mice showed only half of the PGE2-induced AC activation found in SMCs from wild-type mice (Fig. 3A). As in SMCs from wild-type mice, the PGE2-induced AC activity in SMCs from AC3+/− mice was completely blocked by increasing intracellular calcium concentrations and normalized by co-incubation with the CaM KII inhibitor KN-62 (Fig. 3A).

We next investigated whether AC isoforms other than AC3 could be activated by high concentrations of PGE2. For these experiments, SMCs isolated from AC3+/− mice and wild-type littermates were stimulated with concentrations of PGE2 up to 50 μM (Fig. 3B). The highest concentrations were well above receptor saturating concentrations. We showed that increasing concentrations of PGE2 could not compensate for the 50% reduction in AC activation seen in SMCs from AC3+/− mice, indicating that other AC isoforms did not efficiently couple to PGE2 receptors (EP2 and/or EP4 receptors) even at supra-threshold concentrations and thus were not involved in the reduced ability of PGE2 to stimulate cAMP accumulation in AC3+/− SMCs (Fig. 3).

**Fig. 3.** PGE2 stimulates AC3 activity in murine arterial SMCs. A, murine SMCs were isolated from the thoracic aorta of wild-type (WT) (AC3+/+) and age- and sex-matched AC3+/− littermates by enzymatic digestion of the smooth muscle layer. The cells were characterized as SMCs by positive staining for smooth muscle α-actin and by morphological criteria. SMCs in six-well plates were preincubated with IBMX and KN-62 and then stimulated for 30 min with PGE2 and/or A23187 as described in the legend of Fig. 1. AC activity was measured in intact cells according to Wayman et al. (20) after chromatographic separation of cAMP, ATP, ADP, and AMP according to Salomon et al. (26) and was expressed as the percentage of cAMP of total ATP + ADP + AMP. B, SMCs from wild-type and AC3+/− mice were stimulated with the indicated concentrations of PGE2 for 30 min. The results are expressed as mean ± S.E. of triplicate samples. The experiments were repeated three times with similar results.

**TABLE I**

| DNA synthesis | Cell number |
|---------------|-------------|
| cpm/well | (cells/well) |
| Vehicle | 6,263 ± 181 | 94 ± 2 |
| PDGF-BB | 11,455 ± 821 | 149 ± 0 |
| PGE2 | 3,712 ± 180 | 53 ± 3 |
| PGE2 + PDGF-BB | 7,736 ± 733 | 74 ± 3 |

Their AC3 expression, this expression level did not appear sufficient to efficiently mediate the growth-inhibitory effects of PGE2 receptors (Fig. 4, A and C). It is likely that a reduced expression of AC3, which is normally a protein present at low levels, results in inefficient cAMP formation and subsequent inhibition of DNA synthesis and cell replication following exposure of AC3+/− SMCs to PGE2. This concept is supported by the reduced ability of PGE2 to stimulate cAMP accumulation in AC3+/− SMCs (Fig. 3).

Changes in DNA synthesis were reflected by changes in the number of cells. Whereas PGE2 (10 μM) gave an approximate 70% inhibition of PDGF-BB-induced proliferation, SMCs from AC3−/− mice were completely resistant to the growth-inhibitory effects of PGE2 (Fig. 4B). Similar results were obtained when the SMCs were stimulated to proliferate by 10% fetal bovine serum (data not shown). Dose-response curves show that the concentration of PGE2 required to mediate half-maximal inhibition (IC50) of basal and PDGF-BB-stimulated DNA synthesis was in the range of 5 nM in SMCs from wild-type mice (Fig. 4C). This value is similar to the IC50 values for PGE2 binding to the EP2 (Kd ~5 nM) and EP4 (Kd ~1 nM) receptor subtypes (29). PGE2 resulted in only a 25–40% inhibition of basal and PDGF-BB-stimulated DNA synthesis in SMCs from AC3−/− and AC3+/− mice (Fig. 4C). Similar results were obtained when the SMCs were incubated in the presence of 100 μM IBMX, indicating that the inability of PGE2 to induce growth inhibition in SMCs from AC3-deficient mice was not because of an increased PDE activity in these cells (data not shown).

Furthermore, 10 μM forskolin induced an 85% inhibition of DNA synthesis in SMCs from AC3−/− mice, showing that the proliferation of these cells was inhibited by increased cAMP levels and that other AC isoforms were capable of inducing SMC growth arrest (data not shown).

**DISCUSSION**

**AC3 Mediates the Growth-inhibitory Effects of PGE2 in SMCs**—At least 10 isoforms of AC (AC1 through AC10) have been cloned to date, and they are expressed in a tissue-selective manner. However, most tissues express several AC isoforms. Previous studies on arterial smooth muscle from different species have demonstrated expression of multiple AC isoforms. AC2, AC4, AC5/6, and AC7, but not AC3, have been found in bovine pulmonary artery SMCs (30, 31). Rat aortic SMCs have been shown to express AC3 and possibly AC8 but not the neuronal AC1 (32). Human SMCs also express several different AC isoforms. In addition to AC3, AC4, AC5/6, AC9, and possibly
It is becoming evident that the properties of different AC isoforms can determine the intracellular response to extracellular stimulation of G-protein-coupled receptors. It has been suggested that ACs act as coincidence detectors. For example, AC3 activity is inhibited by increases in calcium levels in intact cells. This inhibition is due to phosphorylation of Ser-1076 in AC3 by CaM KII, which is activated by calcium/calmodulin (34). AC3 activity is also inhibited by regulator-of-G-protein-signaling-2, RGS2 (35). Because RGS2 expression can be stimulated by growth-promoting factors in arterial SMCs (36), this provides another mechanism of cross-talk between growth inhibitors and growth factors. Other AC isoforms are stimulated by increases in calcium or regulated by other protein kinases and/or phosphatases (7). Previous studies have shown that specific AC isoforms may have specific biological functions. Accordingly, in NIH3T3 cells, overexpression of AC6 does not affect proliferation, whereas overexpression of AC2 leads to inhibition of cell cycle progression and inhibition of the extracellular signal-regulated kinase pathway (37). Furthermore, AC2 and several other AC isoforms are up-regulated during growth arrest and differentiation of P19 cells (38, 39).

Our results show that AC3 is the principal AC isoform activated by PGE2 in human and murine aortic SMCs despite the presence of other AC isoforms. It is possible that AC3 has a greater sensitivity to Gs-activated receptors than other ACs (20) and therefore is the preferred AC isoform activated by all

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G₃ receptor agonists. It is also possible that the extracellular and/or intracellular conditions favor activation of AC3. For example, a high cAMP-dependent protein kinase activity is likely to inhibit AC5 and AC6 activities (7). Consistent with AC3 being the principal AC isoform activated by PGE₂, we further show that AC3 mediates the growth-inhibitory effects of PGE₂ in SMCs.

**Regulation of cAMP Levels by Calcium in Human Arterial SMCs—PGE₂ is a major prostanoid secreted by endothelial cells that can be utilized to promote growth.**

The growth-inhibitory actions of PGE₂ are mediated by cAMP. Cyclic AMP inhibits proliferation of SMCs in culture (41–44) and reduces formation of neointimal lesions after arterial injury in vivo (42, 45, 46). Previous studies have shown that AC3, most likely through activation of the cAMP-dependent protein kinase, inhibits several mitogenic signal transduction pathways in SMCs. Thus, elevation of cAMP levels results in inhibition of PDGF-induced activation of the extracellular signal-regulated kinase pathway (47–49), inhibition of S6K1, and inhibition of growth factor-induced phosphorylation of PHAS-1, a translation initiation factor 4E-binding protein that regulates translation initiation (50). In rat SMCs, S6K1 activity and proliferation are inhibited by forskolin at concentrations that do not result in inhibition of the extracellular signal-regulated kinase pathway. It is thus possible that S6K1 or the upstream phosphatidylinositol 3-kinase (PI3K) is especially sensitive to the inhibitory action of cAMP (50). Interestingly, cAMP was recently found to inhibit the lipid kinase activity of PI3K in COS cells transfected with the catalytic subunit of PI3K (51). It is not known whether cAMP has several targets in the PI3K pathway in SMCs and whether inhibition of the PI3K pathway indeed mediates the growth-inhibitory actions of cAMP.

**Why is AC3 the major AC isoform selected by nature to mediate the growth-inhibitory signaling of PGE₂?**

AC3 activity is inhibited in intact cells by low concentrations of calcium/calmodulin through the phosphorylation of AC3 by CaM KII (52). Furthermore, many growth factors lead to an inhibition of the PI3K pathway indeed mediates the growth-inhibitory signaling of PGE₂? AC3 activity, which regulates translation initiation (50). In rat SMCs, S6K1 activity and proliferation are inhibited by forskolin at concentrations that do not result in inhibition of the extracellular signal-regulated kinase pathway. It is thus possible that S6K1 or the upstream phosphatidylinositol 3-kinase (PI3K) is especially sensitive to the inhibitory action of cAMP (50). Interestingly, cAMP was recently found to inhibit the lipid kinase activity of PI3K in COS cells transfected with the catalytic subunit of PI3K (51). It is not known whether cAMP has several targets in the PI3K pathway in SMCs and whether inhibition of the PI3K pathway indeed mediates the growth-inhibitory actions of cAMP.
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