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Prostacylin Receptor Activation Inhibits Proliferation of Aortic Smooth Muscle Cells by Regulating cAMP Response Element-Binding Protein- and Pocket Protein-Dependent Cyclin A Gene Expression

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ABSTRACT

The prostanoid prostacyclin (PGI_2) inhibits aortic smooth muscle cell proliferation by blocking cell cycle progression from G_1- to S-phase. However, the mechanism of this inhibition is poorly understood. We report here that the PGI_2 mimetic, cicaprost, inhibits the induction of cyclin A and activation of the cyclin A promoter in primary and established rodent aortic smooth muscle cells. The inhibition of cyclin A gene expression is associated with a block in cyclin E-cdk2 activity and phosphorylation of both the retinoblastoma protein and p107. Inactivation of pocket proteins with human papilloma virus protein E7 partially, but not completely, restored cyclin A promoter activity in cicaprost-treated cells. Complementary studies showed that occupancy of the cAMP response element (CRE) is required for efficient activation of the cyclin A promoter in aortic smooth muscle cells, that the CRE is primarily occupied by the CRE-binding protein (CREB) and phospho-CREB, and that cicaprost blocks the binding of CREB and phospho-CREB to the cyclin A promoter CRE. Treatment with pertussis toxin reversed the inhibitory effects of cicaprost on CRE occupancy, cyclin E-cdk2 activity, and S phase entry, suggesting the involvement of G_i signaling in cicaprost action. We conclude that PGI_2 inhibits proliferation of aortic smooth muscle cells by coordinately blocking CRE- and pocket protein-dependent cyclin A gene expression.

Cardiovascular diseases such as atherosclerosis and restenosis involve injury to the endothelium and smooth muscle cell proliferation. Endothelial cells normally release the prostanoid prostacyclin (PGI_2) (Doroudi et al., 2000), which can inhibit platelet adhesion and aggregation as well as smooth muscle cell proliferation (Fink et al., 1999). Injury to the endothelium, or disturbances in laminar flow, may reduce the levels of PGI_2 by inhibiting production of cyclooxygenase-2 (Topper et al., 1996), the predominant source of PGI_2 biosynthesis in humans (McAdam et al., 1999). PGI_2 signals through its interaction with IP, a heterotrimeric G-protein-coupled receptor (Coleman et al., 1994), and others have demonstrated that the deletion of IP in mice exacerbates injury-induced smooth muscle cell proliferation and neointima formation (Cheng et al., 2002), key features of restenosis and atherosclerotic plaque formation.

IP couples to adenylyl cyclase through G_s (Namba et al., 1994) but can also regulate other effector systems by coupling to G proteins such as G_i and G_q (Schwaner et al., 1995; Smyth et al., 1996; Lawler et al., 2001). Lawler et al. (2001) have reported that the mouse IP couples initially to G_s, followed by a switch in coupling to G_i and G_q, as has also been observed for the β2-adrenergic receptor (Daaka et al., 1997). This receptor switch has been attributed to protein kinase A-mediated receptor phosphorylation. However, other kinases, including protein kinase C, AKT, and casein kinase 1α, can also phosphorylate G-protein coupled receptors (Smyth et al., 1996; Lee et al., 2001; Tobin, 2002). These phosphorylation events may play important roles in receptor-mediated signaling pathways.

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D.K. and S.A.S. contributed equally to this work.

ABBREVIATIONS: PGI_2, prostacyclin; IP, prostacylin receptor; cdk, cyclin-dependent kinase; pRb, retinoblastoma protein; CRE, cAMP response element; CDE, cell cycle-dependent element; CHR, cell cycle gene homology region; CREB, cAMP response element-binding protein; ATF, activating transcription factor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; BrdU, bromodeoxyuridine; PBS, phosphate-buffered saline; IBMX, 3-isobutyl-1-methylxanthine; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; TGF, transforming growth factor.

PGI_2 inhibits the proliferation of cultured primary arterial smooth muscle cells by blocking progression from G_1 to S phase of the cell cycle (Weber et al., 1998). G_1 phase cell cycle progression is regulated by a sequential activation of cyclin-dependent kinases (cdks), namely cyclin D1-cdk4/6 and cyclin E-cdk2. The activation of cyclin D1-cdk4/6 is regulated primarily through the induction of cyclin D1, whereas the activation of cyclin E-cdk2 is regulated primarily through a decreased association of the cdk inhibitors, usually $p21^{Cip1}$ and $p27^{Kip1}$ (Sherr and Roberts, 1999). In large part, active cyclin D1-cdk4/6 and cyclin E-cdk2 are thought to regulate G_1 phase progression by phosphorylating pocket proteins (pRb, p107, and p130), thereby regulating the activation of E2F-dependent genes such as cyclin A (Takahashi et al., 2000). Cyclin A is induced at the G_1/S interface, and the consequent formation of cyclin A-cdk2 complexes marks entry into S phase of the cell cycle.

Several reports show that cyclin A gene expression is regulated by E2F-pocket protein complexes. For example, overexpression of E2F-1 and human papilloma virus E7 rescues cyclin A expression and anchorage-independent growth (Schulze et al., 1998). Regulation of the cyclin A promoter by pocket proteins seems to involve two contiguous *cis* elements: the cell cycle-dependent element (CDE) and the cell cycle gene homology region (CHR) (Zwicker et al., 1995; Liu et al., 1998). The interactions of E2Fs and pocket proteins on the cyclin A promoter and the mechanisms by which these proteins control cyclin A expression are topics of active investigation (Takahashi et al., 2000; Wu et al., 2001), but remain poorly understood.

The cyclin A promoter contains several regulatory elements in addition to the CDE/CHR, including the well documented cAMP response element (CRE) and CCAAT element (Desdouets et al., 1995; Kramer et al., 1996). The CRE, CCAAT and CDE/CHR elements are clustered within a 118-base pair region near the transcription start sites (Henglein et al., 1994; Shimizu et al., 1998). Several studies show that the CRE and CCAAT sites are necessary for the efficient activation of the cyclin A gene (Desdouets et al., 1995; Kramer et al., 1996; Bottazzi et al., 2001). The CRE-binding protein (CREB) family of transcription factors, including CREB, ATF-1, and CRE modulator, function in a variety of physiological processes and are activated by phosphorylation at Ser133 in response to cAMP, calcium, stress, and mitogenic stimuli. Several kinases have been reported to phosphorylate CREB at Ser133 (Mayr and Montminy, 2001).

In this study, we examined the mechanism by which PGI_2 inhibits aortic smooth muscle cell proliferation and G_1-S phase progression. We report that the PGI_2 mimetic cicaprost inhibits cyclin A expression in primary murine aortic smooth muscle cells and established A10 rat aortic smooth muscle cells. Cicaprost treatment blocks the activation of cyclin E-cdk2, but this effect in itself is insufficient to explain the inhibition of cyclin A gene expression. Cicaprost also inhibits the binding of CREB and phospho-CREB to the CRE element in the cyclin A promoter. The inhibitory effects of cicaprost on cyclin E-cdk2 activity, CRE occupancy, and S phase entry are reversed in the presence of pertussis toxin. Overall, our results show that PGI_2 inhibits the proliferation of aortic smooth muscle cells by coordinately blocking pocket protein- and CRE-dependent cyclin A gene expression, and that the underlying mechanism involves G_i signaling.

Materials and Methods

Cell Culture. Primary aortic smooth muscle cells were isolated from wild-type C57BL/6 mice or IP-null mice by explant culture as described previously (Cuff et al., 2001) and maintained in 1:1 Dulbecco's modified Eagle's/Ham's F-12 media supplemented with 10% FBS and 2 mM L-glutamine. Experiments were performed on cells at passage 3; these cultures were ~95% positive for smooth muscle actin as determined by immunofluorescence microscopy (not shown). The A10 rat aortic smooth muscle cell line (American Type Culture Collection, Manassas, VA) was maintained in the same medium. In cell cycle experiments, near-confluent monolayers of primary or A10 smooth muscle cells were G_0-synchronized by incubation in a serum-free defined media (1:1 Dulbecco's modified Eagle's/Ham's F-12 media, 15 mM HEPES, pH 7.4, 3 mM histidine, 4 mM glutamine, 8 mM sodium bicarbonate, 10 μM ethanolamine, 10 μg/ml transferrin, 0.1 μM sodium selenite, 0.1 μM $MgCl_2$, and 2 mg/ml heat-inactivated fatty acid-free bovine serum albumin) for 48 h before stimulation with 10% FBS in the absence or presence of cicaprost. Cicaprost was obtained and used under an agreement with Schering AG (Berlin, Germany). In some experiments, cells were pretreated with 100 ng/ml of pertussis toxin (List Biological Laboratories, Campbell, CA) for the last 16 h of the 48 h serum starvation. Pertussis toxin was removed by washing; the cells were then either directly stimulated with 10% FBS ± cicaprost or trypsinized and replated in the presence of 10% FBS ± cicaprost. The viability of cicaprost- or pertussis toxin-treated cells was approximately 95% by trypan blue exclusion staining.

Immunofluorescence. Quiescent aortic smooth muscle cells and A10 smooth muscle cells (~2×10^5 cells) were added to 35-mm dishes containing autoclaved glass coverslips and incubated with 2 ml of medium in 10% FBS in the absence or presence of 200 nM cicaprost. Cells were fixed, permeabilized, and stained as described previously (Roovers et al., 1999). For detection of cyclin A, the cells were incubated for 1 h with ammonium sulfate-fractionated rabbit polyclonal antibody against cyclin A (1:100 dilution) and then for 1 h with FITC-conjugated donkey anti-rabbit antibody (1:100 dilution; Jackson Immunoresearch Labs, West Grove, PA). To monitor entry into S phase, the incubation of primary and A10 smooth muscle cells with 10% FBS was performed in the presence of BrdU (3 μg/ml; Amersham, Piscataway, NJ). Fixed cells were first incubated for 1 h with sheep anti-BrdU (1:500 dilution; Biodesign, Saco, ME) and fresh DNase (280 units/ml) and then for 1 h with FITC-conjugated donkey anti-sheep antibody (1:200 dilution; Jackson Immunoresearch Labs) in PBS with 2% bovine serum albumin. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (2 μg/ml in PBS; Sigma, Saint Louis, MO). The percentage of BrdU positive cells was assessed by counting the number of FITC-stained cells relative to 4,6-diamidino-2-phenylindole–stained nuclei using epifluorescence microscopy (100–200 cells in three or four fields of view were counted for each time point).

Measurement of cAMP Levels. Cells were seeded in 12-well plates and cultured until they reached 90% confluence. The cells were pretreated with 3 μM indomethacin (Sigma) for 16 h before stimulation. On the day of the assay, cells were treated with 1 mM IBMX, a phosphodiesterase inhibitor, for 15 min, followed by stimulation with either increasing doses of cicaprost or increasing doses of forskolin (Sigma), in the absence or presence of 200 nM cicaprost, for 10 min. Cellular cAMP was extracted in 65% ethanol and quantified by radioimmunoassay according to the manufacturer's instructions (Amersham TRK432).

Western Blotting. Quiescent A10 smooth muscle cells were trypsinized, replated at subconfluence (10^6 cells in 10 ml medium per 100-mm culture dish), and incubated in 10% FBS in the absence or presence of 200 nM cicaprost. Collected cells were lysed, and total protein concentration was determined by Coomassie binding (Bio-Rad, Hercules, CA). Equal amounts of protein (80 μg) were fractionated on reducing 12% acrylamide gels and analyzed by Western

blotting as described previously (Roovers et al., 1999) using antibodies specific for the following proteins: pRb (Zymed Labs, San Francisco, CA), p107 (Santa Cruz Biotechnology, Santa Cruz, CA), cdk4 (Santa Cruz Biotechnology or BioSource, Camarillo, CA), cyclin D1 (Upstate Biotechnology, Lake Placid, NY), cyclin E (Santa Cruz Biotechnology), and cdk2 (Upstate Biotechnology). Rabbit polyclonal cyclin A antibody was prepared in this laboratory using recombinant cyclin A as an immunogen.

In Vitro Kinase Assays. Quiescent A10 smooth muscle cells were trypsinized, replated at subconfluence (10^6 cells in 10 ml of medium per 100-mm culture dish), and incubated in 10% FBS in the absence or presence of 200 nM cicaprost. Cyclin D1-cdk4 kinase assays were performed as described previously (Welsh et al., 2001) using 200 μg of cell lysates and glutathione *S*-transferase-Rb as a substrate (Santa Cruz Biotechnology). The reaction mixtures were fractionated on reducing SDS-gels (12% acrylamide), electrophoretically transferred to nitrocellulose, and analyzed by autoradiography. Nitrocellulose membranes were also Western blotted using antibodies specific for cdk4 (BioSource) to assess the uniformity of the immunoprecipitation. Cyclin E-cdk2 kinase assays were performed as described previously (Zhu et al., 1996) using 200 μg of cell lysate, 5 μg of anti-cyclin E (Santa Cruz Biotechnology) and a 2-h incubation at 4°C with both primary antibody and protein A-agarose. The collected immunoprecipitates were washed once with lysis buffer and four times with ice-cold kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium fluoride, and 10 mM sodium orthovanadate). The washed immunoprecipitate was resuspended in 50 μl of kinase buffer containing 5 μg of histone H1 (Upstate Biotechnology), 20 μM ATP, and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The kinase reaction was incubated for 30 min at room temperature and stopped by the addition of 2× SDS sample buffer and heating the sample for 2 min at 95°C. The reaction products were analyzed by SDS gel electrophoresis, autoradiography, and immunoblotting with anti-cdk2 (Upstate Biotechnology) as described above. Kinase activity was quantified by PhosphorImager analysis and ImageQuant software (Amersham Biosciences).

Electrophoretic Mobility Shift Assays. Nuclear extracts and electrophoretic mobility shift assays (EMSAs) were prepared as described previously (Bottazzi et al., 2001). Double-stranded oligonucleotides that were used in these studies contained either the wild-type cyclin A CRE (5′-TGAATGACGTCAAGGCCGCGAG-3′) or wild-type CCAAT element (5′-CGAGCGCTTTCATTGGTCCATTTC-3′). For supershifts, the nuclear extracts were preincubated (30 min at room temperature) with 5 μl of antibodies against CREB, ATF-1, ATF-2, c-Jun, and c-Fos (all from Santa Cruz Biotechnology) or phospho-CREB (Upstate Biotechnology) before the addition of the radioactive probe. Protein-DNA complexes were fractionated on a 5% polyacrylamide gel in 90 mM Tris, 90 mM Boric acid, 20 mM EDTA, pH 8.3, at 175 V for 3 h at 4°C. The gels were dried and analyzed by autoradiography.

Expression Vectors and Luciferase Constructs. The human cyclin A promoter-firefly luciferase constructs used here were described previously (Kramer et al., 1996). The p434/cyclin A promoter construct contains 434 bases spanning from −270 to +164 of the cyclin A promoter upstream of the luciferase reporter. The p284/cyclin A promoter construct (called +CRE) contains 284 bases from −120 to +164 of the cyclin A promoter. Sequences upstream of the CRE have been deleted in +CRE. The p225/cyclin A promoter construct (called −CRE) contains 225 bases from –61 to +164 of the cyclin A promoter and is a construct in which the CRE site from the core promoter region, as well as all sequences upstream of the CRE have been deleted. The human papilloma virus-type 18 E7 expression vector was the generous gift of Lou Laimins (Northwestern University, Chicago, IL).

Cell Transfections and Promoter-Luciferase Assays. Transient transfections of primary aortic and A10 smooth muscle cells with promoter-luciferase vectors were performed using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) as described previously (Bottazzi et al., 1999) using ~2×10^5 cells, 1 μg of cyclin A promoter-luciferase plasmid(s), 1 μg of the human papilloma virus-type 18 E7 expression vector (or empty vector), and 0.1 μg of a *Renilla reniformis* luciferase expression plasmid (pRL-CMV; Promega, Madison, WI) to control for transfection efficiency. The transfection efficiency for both cell types was approximately 25% as determined by transient transfection of an enhanced green fluorescent protein expression vector. The final amount of DNA transfected was brought to 2.1 μg for all samples by addition of the empty vector. After an overnight recovery, the cells were serum-starved for 1 day in Dulbecco's modified Eagle's medium and 1 mg/ml heat-inactivated fatty acid-free bovine serum albumin. The serum-starved transfectants were directly stimulated with 10% FBS-Dulbecco's modified Eagle's medium in the absence or presence of 200 nM cicaprost or 10 ng/ml transforming growth factor-β (TGF-β). Cells were washed with PBS, collected, lysed, and analyzed for luciferase and *R. reniformis* luciferase activity using the Dual-Luciferase reporter assay system (Promega, Madison, WI). Cyclin A promoter-driven luciferase activity was then normalized to a constant activity of *R. reniformis* luciferase to correct for variations in transfection efficiency.

Statistical Analysis. Data are presented as mean ± S.E.M. Statistical analyses were carried out using one-tailed *t* test. Differences were considered significant at $p < 0.01$.

Results

Cicaprost Inhibits S-Phase Entry and Cyclin A Induction in Aortic Smooth Muscle Cells. To study the effects of prostacyclin in regulating cell cycle progression in aortic smooth muscle cells, we conducted experiments with the stable PGI_2 mimetic, cicaprost. This compound is a highly specific ligand for IP (K_i ~ 10 nM in Chinese hamster ovary cells expressing the mouse IP) compared with other prostanoid receptors such as EP_1 (K_i ~ 1.3 μM) and EP_3 (K_i ~ 170 nM) (Narumiya et al., 1999). We first used BrdU labeling to assess the effects of cicaprost on S-phase entry in aortic smooth muscle cells. Primary aortic smooth muscle cells were serum-starved and then stimulated with serum at subconfluence in the presence of BrdU. The results showed that a majority of the primary aortic smooth muscle cells entered S phase within 48 h of serum stimulation and that cicaprost strongly inhibited S-phase entry (Fig. 1A). Dose response curves showed that cicaprost caused the expected increase in intracellular cAMP levels in smooth muscle cells (EC_{50} ~ 40 nM; Fig. 1B, IP +/+), and that similar concentrations of cicaprost inhibited progression through G_1 phase (IC_{50} ~ 50 nM; Fig. 1C, IP +/+). The EC_{50} is slightly higher than others (Kam et al., 2001) have reported for cAMP production (EC_{50}, 5–10 nM), perhaps because we have studied endogenous IP rather than overexpressed IP. The effects of cicaprost on both cAMP production and G_1 phase progression were lost in primary aortic smooth muscle cells from IP −/− mice (Fig. 1, B and C). Cicaprost also inhibited activation of the cyclin A promoter in aortic smooth muscle cells transiently transfected with a cyclin A promoter-luciferase expression vector (Fig. 2A) and blocked the induction of cyclin A protein as determined by immunofluorescence (Fig. 2B).

Cicaprost Inhibits Pocket Protein Phosphorylation and Cyclin E-cdk2 Activity in Aortic Smooth Muscle Cells. To confirm the effects observed with primary smooth muscle cells and develop a system amenable to a mechanistic analysis, we examined the effect of cicaprost on G_1 phase cell cycle progression in the A10 rat aortic smooth muscle cell line. Cicaprost inhibited S phase entry in these cells (Fig.

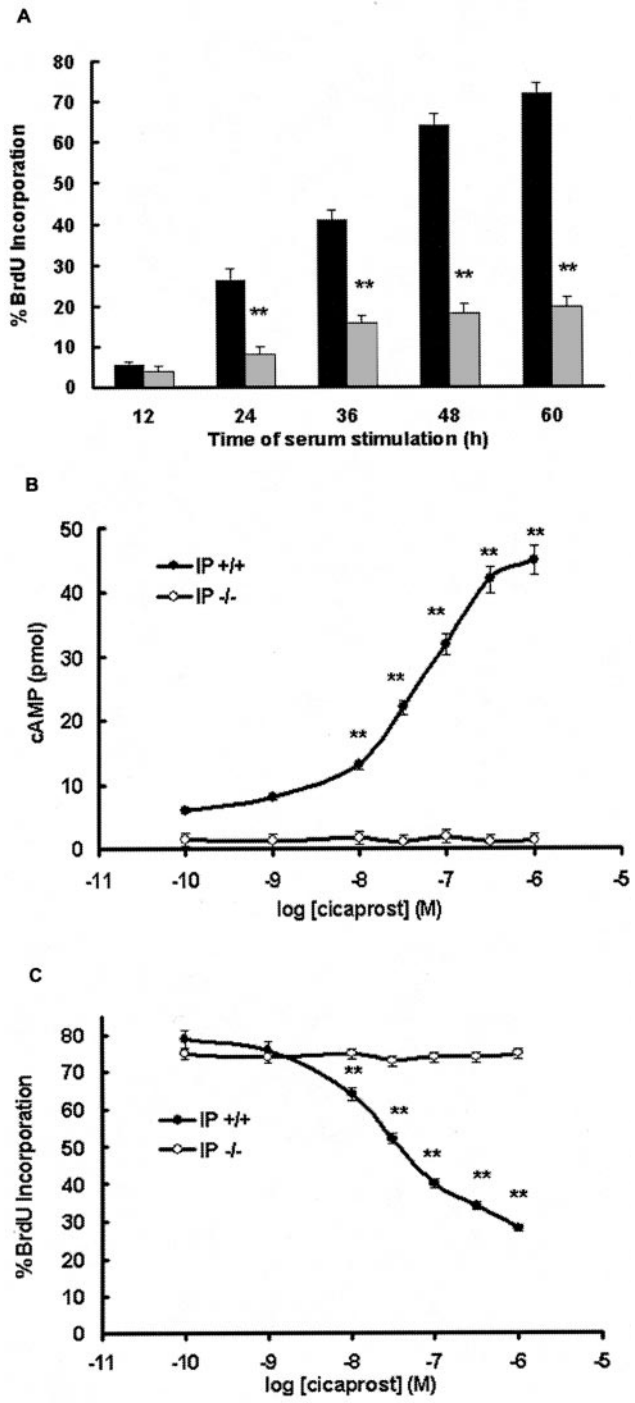

Fig. 1. Cicaprost inhibition of S-phase entry in aortic smooth muscle cells is dependent on IP. Quiescent primary aortic smooth muscle cells were trypsinized, added to 35-mm dishes containing coverslips, and stimulated with 10% FBS in the absence or presence of cicaprost. A, the cells were incubated with BrdU in the presence (▨) or absence (■) of 200 nM cicaprost for the times indicated, and BrdU incorporation into nuclei was determined by immunofluorescence microscopy. Results show the mean ± S.E.M., $n = 3$; **, $p < 0.01$ compared with cells treated with 10% FBS alone. B, primary smooth muscle cells from IP +/+ (●) and IP −/− (○) mice pretreated with IBMX and then stimulated with increasing doses of cicaprost for 10 min. Cells were collected, and cAMP levels were quantified by radioimmunoassay. Results show the mean ± S.E.M., $n = 3$; **, $p < 0.01$. C, quiescent primary aortic smooth muscle cells [IP +/+ (●) and IP −/− (○)] were stimulated for 48 h with 10% FBS in the presence of BrdU and increasing doses of cicaprost. BrdU incorporation into nuclei was determined by immunofluorescence microscopy. Results show the mean ± S.E.M., $n = 3$; **, $p < 0.01$.

3A), and the IC_{50} (50 nM ± 2, $n = 3$, $p < 0.01$) was similar to that of the primary cells (see above). Cicaprost also inhibited activation of the cyclin A promoter (Fig. 3B) and induction of cyclin A protein (Fig. 3B, inset) in A10 smooth muscle cells.

We then used A10 smooth muscle cells to determine the effect of cicaprost on the G_1 phase cyclin-cdks. Cicaprost did not affect the expression of cyclin D1 (Fig. 4A), cdk4 (Fig. 4A), or cyclin D1-cdk4 activity (Fig. 4B). The lack of effect on cyclin D1-cdk4 was unexpected because the induction of cy-

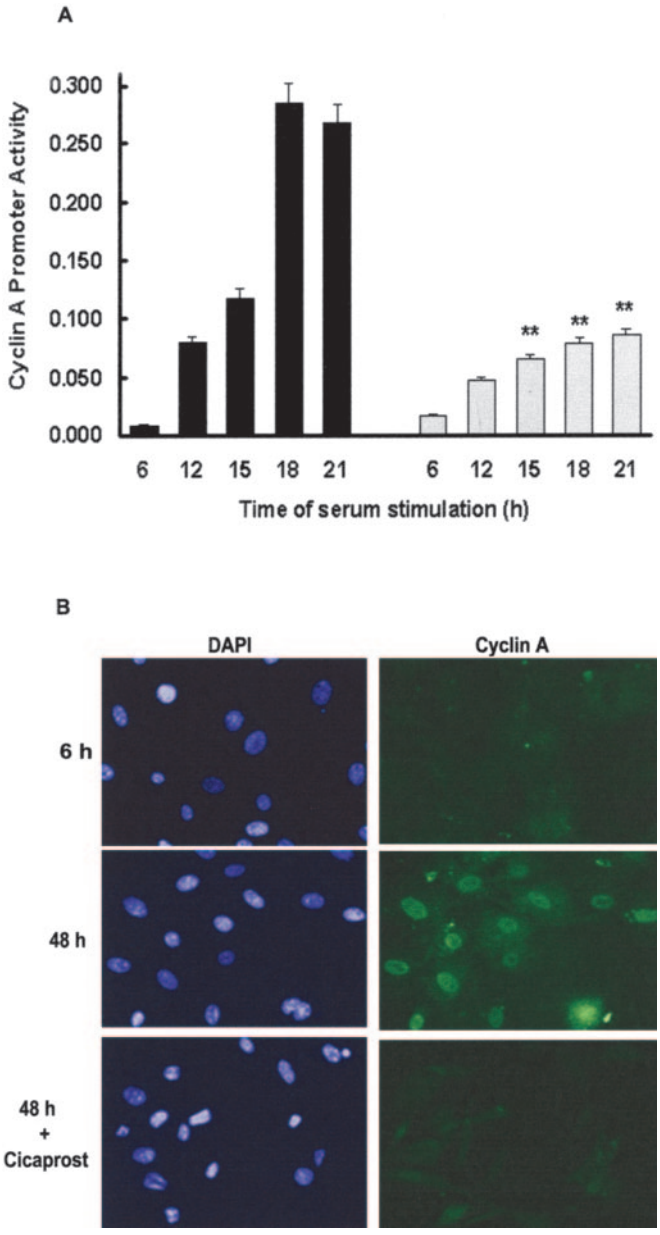

Fig. 2. Cicaprost inhibits cyclin A expression in aortic smooth muscle cells. A, primary aortic smooth muscle cells were transiently transfected with the p434/cyclin A promoter-luciferase plasmid and pRL-CMV *R. reniformis* luciferase vector, serum starved, and then stimulated with 10% FBS in the presence (▨) or absence (■) of cicaprost for 6 to 21 h. The cell lysates were analyzed for luciferase activity. Cyclin A promoter-luciferase activity is plotted relative to *R. reniformis* luciferase activity. Results show the mean ± S.E.M., $n = 3$; **, $p < 0.01$ compared with cells treated with 10% FBS alone at 15 to 21 h. B, quiescent primary aortic smooth muscle cells were incubated for 6 or 48 h with 10% FBS in the absence or presence of 200 nM cicaprost before fixation and analysis of cyclin A expression by immunofluorescence microscopy.

clin D1 is commonly blocked when cells arrest in G_1 phase. However, this result was supported by other experiments in A10 cells (data not shown), which demonstrated that cicaprost had no effect on mitogen-activated protein kinase kinase/extracellular signal-regulated kinase or phosphatidyl inositol-3 kinase/AKT signaling, the major pathways that regulate cyclin D1 expression (Diehl et al., 1998; Roovers and Assoian, 2000). Although cicaprost was also without effect on the levels of cyclin E or cdk2 (Fig. 4C), it strongly inhibited the activation of cyclin E-cdk2 (Fig. 4D). Curiously, cicaprost did not increase the expression of $p21^{Cip1}$, nor did it lead to a consistent increase in the expression of $p27^{Kip1}$ (not shown). Therefore, the mechanism by which cicaprost inhibits cyclin E-cdk2 activity may be complex and involve coordinate effects on $p27^{Kip1}$, cdk activating kinase, and cdc25A (see *Discussion*). Consistent with the strong inhibition of cyclin E-cdk2 activity, cicaprost blocked the hyperphosphorylation of both pRb and p107 (shown by the absence of the slower migrating form of these proteins; Fig. 4E).

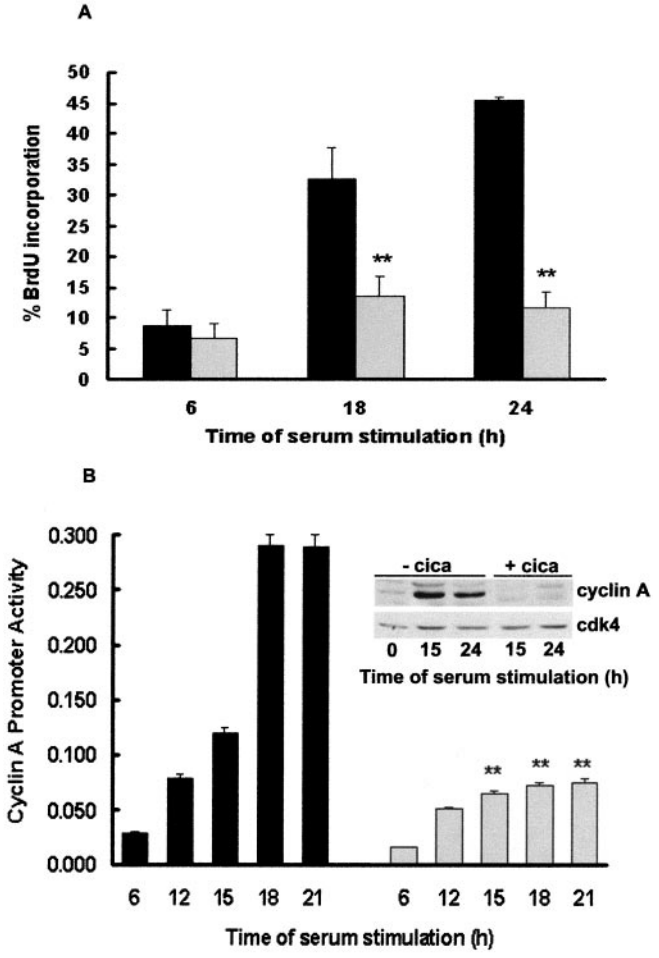

Fig. 3. Cicaprost inhibits progression into S-phase and cyclin A induction in A10 smooth muscle cells. The effect of cicaprost on BrdU incorporation (A) and cyclin A promoter activity (B) in serum-stimulated A10 smooth muscle cells was determined as described under *Materials and Methods*. ■, absence of cicaprost; ▨, presence of cicaprost. Results in A and B show the mean ± S.E.M., $n = 3$; **, $p < 0.01$ compared with cells treated with 10% FBS alone. (B, inset) Quiescent A10 smooth muscle cells were trypsinized, reseeded at subconfluence, and stimulated with 10% FBS in the presence or absence of 200 nM cicaprost for the times indicated. Collected cells were lysed and analyzed by Western blotting for the expression of cyclin A and cdk4 (loading control).

Inhibition of Pocket Protein Phosphorylation Cannot Fully Account for the Inhibitory Effect of Cicaprost on the Cyclin A Promoter. Given the effect of cicaprost on cyclin E-cdk2 activity and pRb and p107 phosphorylation, we considered the possibility that cicaprost blocks cyclin A promoter activity by interfering with pocket protein function. Primary and A10 smooth muscle cells were cotransfected with the p434/cyclin A promoter-luciferase reporter construct and a human papilloma virus-type 18 E7 expression vector [which sequesters pocket proteins and mimics the effect of cdk-mediated phosphorylation (Vousden, 1993)]. Quiescent transfectants were stimulated with serum for 18 h before analysis of cyclin A promoter activity. The expression of E7 caused cyclin A promoter activity to increase 5-fold, presumably because of enhanced pocket protein sequestration and E2F release (Fig. 5, ■). Cicaprost inhibited activation of the cyclin A promoter, and E7 rescued promoter activity partially, but not completely, in both primary and A10 smooth muscle cells treated with cicaprost (Fig. 5, ▨). The incomplete rescue was not caused by a partial effect of E7, because inhibition of cyclin A gene expression by TGF-β [which occurs typically through a pocket protein-dependent mechanism (Reynisdottir et al., 1995)] was completely overcome by expression of E7 (Fig. 5, □). Because cicaprost inhibited the cyclin A promoter despite pocket protein sequestration by E7, we reasoned that it was regulating cyclin A expression via a pocket protein-independent as well as -dependent pathway.

Cicaprost Inhibits Cre-Dependent Cyclin A Gene Expression. To address potential effects on pocket protein-independent cyclin A gene expression, we examined the effect of cicaprost on occupancy of the CCAAT or CRE sites in the cyclin A promoter. EMSAs showed that cicaprost did not inhibit occupancy of the CCAAT site but that it decreased occupancy of the CRE (Fig. 6A). Supershift EMSAs showed that CREB and phospho-CREB bind to the cyclin A CRE (Fig. 6B). A smaller amount of ATF-1 (relative to CREB) was also observed, but the binding of other potential CRE-binding proteins, such as ATF-2, c-fos, and c-jun, was undetectable (Fig. 6B). These results raised the possibility that cicaprost-dependent inhibition of CRE occupancy could account for the pocket-protein independent effect of cicaprost on cyclin A gene expression.

Because IP signaling stimulates cAMP production, we expected that it might stimulate the phosphorylation of CREB at Ser133 rather than block CRE occupancy. Cicaprost did indeed stimulate CREB phosphorylation at Ser133, but the effect was transient (~30 min) and insufficient to sustain CREB phosphorylation for the several hours needed to induce the cyclin A gene (data not shown). In fact, under our experimental conditions (cells treated with serum ± cicaprost), our data (Fig. 6B) show that serum alone supports CREB phosphorylation in late G_1 phase.

Complementary Effects of Cicaprost on Pocket Protein and CRE-Dependent Cyclin A Gene Expression in Aortic Smooth Muscle Cells. To assess the composite effects of cicaprost on CRE- and pocket protein-dependent cyclin A gene expression, we transiently cotransfected both primary and A10 smooth muscle cells with the E7 expression vector and the cyclin A promoter-luciferase reporter constructs, "+CRE" (Fig. 7A) and "−CRE" (Fig. 7B). Quiescent transfectants were stimulated with serum in the presence or

absence of cicaprost. In the absence of E7, cicaprost inhibited luciferase activity whether or not the cyclin A promoter contained the CRE. This result supports our data showing that cicaprost inhibits cyclin E-cdk2 activity and pocket protein phosphorylation (Fig. 4, D and E). However, in the presence of E7, cicaprost inhibited luciferase activity only when the promoter contained the CRE. This result, which supports our finding that cicaprost inhibits CRE occupancy (Fig. 6A), demonstrates that the combined effects of cicaprost on pocket protein- and CRE-dependent transcription can fully account for its inhibitory effect on the cyclin A promoter.

Inhibition of G_i Reverses the Inhibitory Effects of Cicaprost. Activation of IP by cicaprost rapidly increases levels of cAMP (Fig. 1B), and 8-bromo-cAMP inhibits S-phase entry of A10 smooth muscle cells (not shown). These results suggest a role for G_s in mediating the antimitogenic effect of

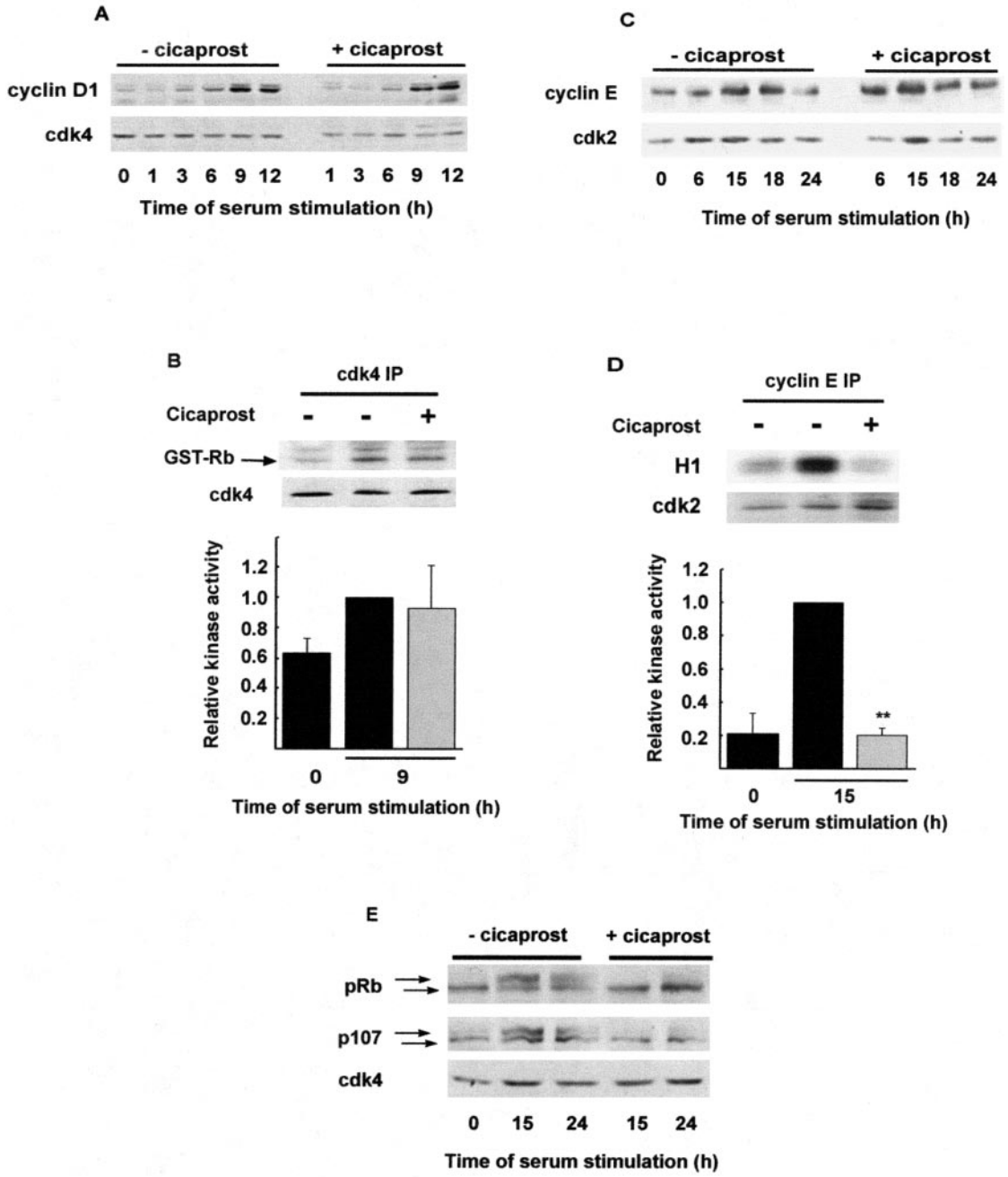

Fig. 4. Cicaprost inhibits cyclin E-cdk2 kinase activity and pocket protein phosphorylation in A10 smooth muscle cells. Quiescent A10 smooth muscle cells were trypsinized, reseeded at subconfluence, and stimulated with 10% FBS in the presence or absence of 200 nM cicaprost for the times indicated. Collected cells were lysed and analyzed by Western blotting for cyclin D1 and cdk4 (A) and cyclin E and cdk2 (C). Equal amounts of protein (200 μg) were incubated with either anti-cdk4 (B) or anti-cyclin E (D). The collected immunoprecipitates were used to assess in vitro kinase activity and then analyzed by Western blotting for associated cdk4 (B) and cdk2 (D). Kinase activity was quantified as described under *Materials and Methods* and plotted as a percentage of maximal cdk4 activity (at 9 h) and cdk2 activity (at 15 h). Results in B and D show the mean ± S.E.M., $n = 3$; **, $p < 0.01$ compared with cells treated with 10% FBS alone. Controls demonstrated that the immunoprecipitation depleted ~50% of cdk4 and ~95% of cyclin E protein. E, collected cells were lysed and analyzed by Western blotting for pRb, p107, and cdk4 (loading control). Upper and lower arrows represent the hyper- and hypophosphorylated forms of pRb and p107, respectively.

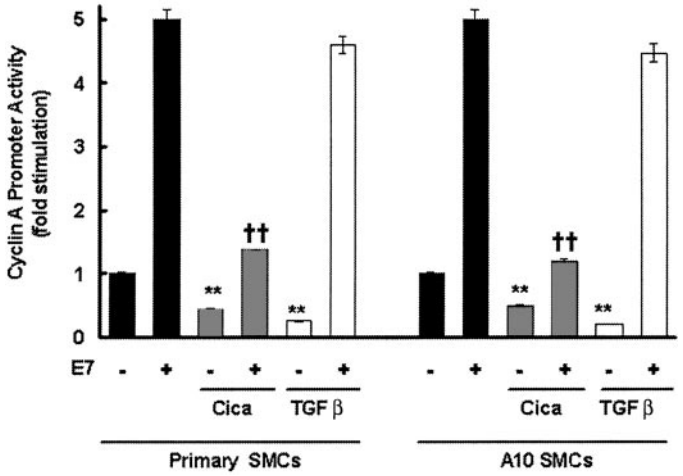

Fig. 5. Inhibition of cyclin A promoter activity by cicaprost is partially pocket protein-dependent. Primary and A10 aortic smooth muscle cells were transiently cotransfected with the p434/cyclin A promoter-luciferase plasmid, pRL-CMV *R. reniformis* luciferase vector and either empty vector (−) or the E7 expression vector (+). Quiescent transfectants were stimulated with 10% FBS in the absence or presence of 200 nM cicaprost or 10 ng/ml TGF-β for 18 h. Cyclin A promoter-luciferase activity was determined and normalized to *R. reniformis* luciferase activity. Promoter activity is plotted as -fold stimulation relative to the FBS-treated cells in the absence of E7 and cicaprost. Results show the mean ± S.E.M., $n = 3$; **, $p < 0.01$ compared with vector-transfected cells stimulated with FBS; ††, $p < 0.01$ compared with E7-transfected cells stimulated with FBS.

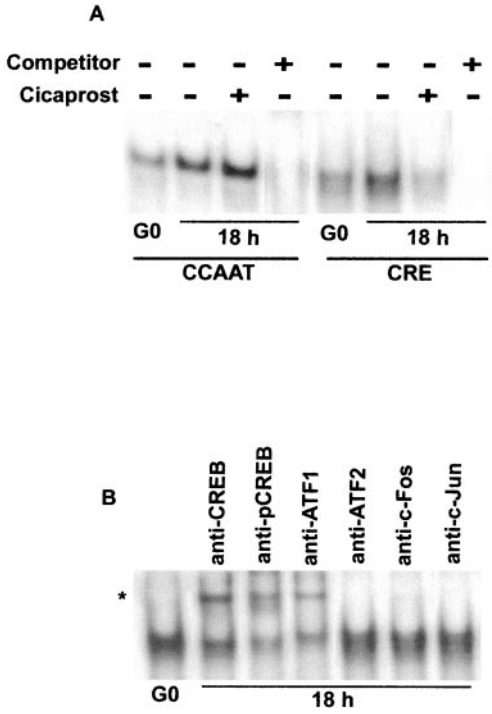

Fig. 6. Occupancy of the cyclin A CRE by CREB and phospho-CREB is inhibited by cicaprost. A, nuclear extracts were prepared from serum starved A10 smooth muscle cells (G0) or cells stimulated with 10% FBS for 18 h in the absence or presence of 200 nM cicaprost. The extracts were incubated with ^{32}P-labeled CCAAT or CRE oligonucleotides and analyzed by EMSAs. Specificity of the complexes was determined by adding a 100-fold molar excess of the respective unlabeled oligonucleotide to the extracts before addition of the labeled probes. B, supershift EMSAs were obtained by preincubating the nuclear extracts from the FBS-treated cells with antibodies to CREB, phospho-CREB, ATF1, ATF2, c-Fos, and c-Jun before addition of the labeled CRE oligonucleotide. (*, supershifted complex)

cicaprost but others have reported that the murine IP can also couple to the heterotrimeric protein G_i (Schwaner et al., 1995; Lawler et al., 2001). We found that although cicaprost stimulated cAMP levels in A10 smooth muscle cells (2.70 ± 0.40-fold; mean ± S.E.M.), it reduced cAMP levels in forskolin-stimulated cells (Fig. 8A), consistent with an IP-dependent activation of G_i as well as G_s.

We then sought to determine whether G_i-mediated signaling was involved in the inhibitory effects of cicaprost on cell cycle progression. Serum-stimulated primary and A10 smooth muscle cells were incubated with pertussis toxin, an irreversible inhibitor of G_i proteins. Neither cicaprost nor pertussis toxin affected CCAAT occupancy (Fig. 8B, lanes 1–3). However, pertussis toxin reversed the inhibitory effect of cicaprost on CRE occupancy (Fig. 8B, compare lanes 5 and

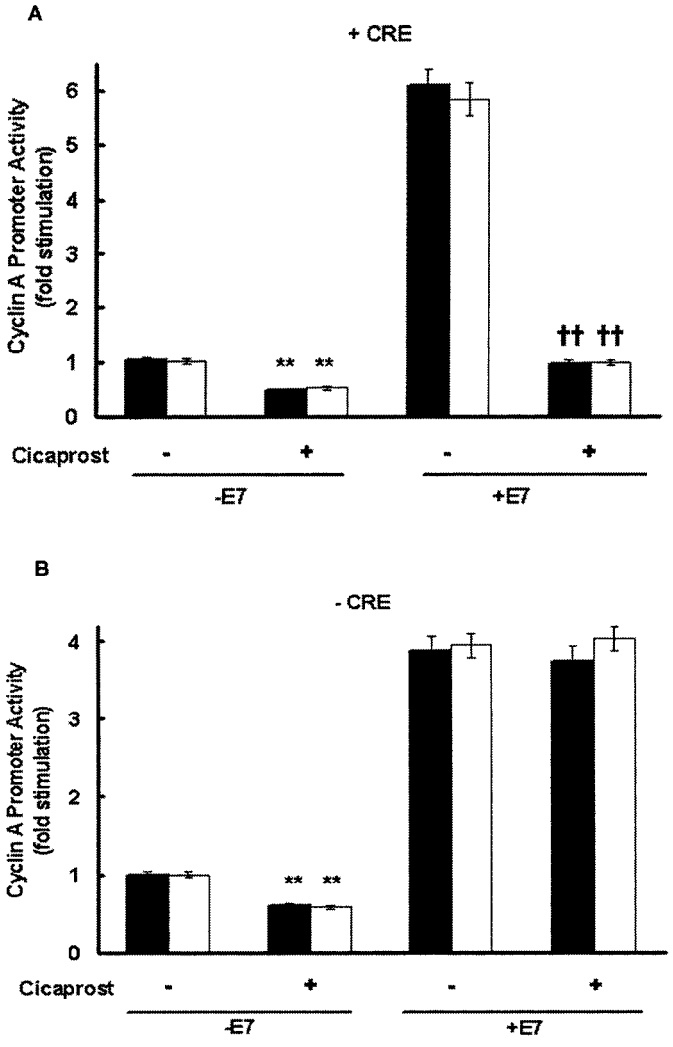

Fig. 7. Cicaprost inhibits cyclin A transcription in a pocket protein- and CRE-dependent manner. Primary (■) and A10 (□) aortic smooth muscle cells were transiently cotransfected with the +CRE (A) or −CRE (B) cyclin A luciferase reporter plasmids, 0.1 μg of pRL-CMV *R. reniformis* luciferase vector, and either empty vector (−E7) or the E7 expression vector (+E7). Quiescent transfectants were stimulated with 10% FBS in the presence or absence of 200 nM cicaprost for 18 h. Cyclin A promoter-luciferase activity was determined and normalized to *R. reniformis* luciferase activity. Promoter activity is plotted as -fold stimulation relative to the FBS-treated cells in the absence of E7 and cicaprost. Results show the mean ± S.E.M., $n = 3$; **, $p < 0.01$ compared with vector-transfected cells stimulated with FBS (+CRE, and −CRE); ††, $p < 0.01$ compared with E7-transfected cells stimulated with FBS (+CRE).

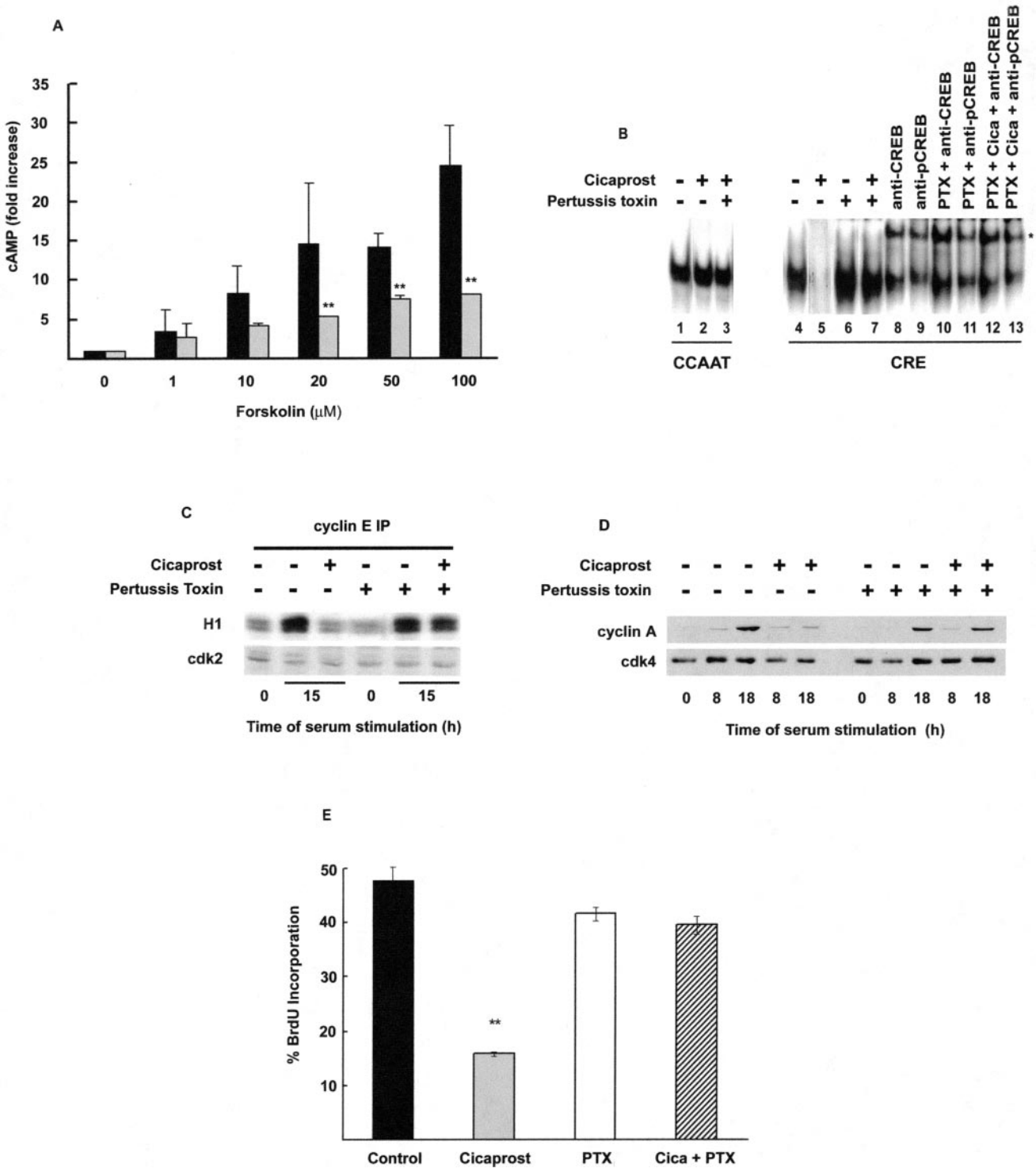

Fig. 8. Inhibition of G_i reverses the inhibitory effects of cicaprost in smooth muscle cells. A, A10 smooth muscle cells pretreated with IBMX were stimulated with increasing doses of forskolin in the presence (▨) or absence (■) of 200 nM cicaprost for 10 min. Cells were collected, and cAMP levels were quantified by radioimmunoassay. To isolate the effect of cicaprost on G_i, cAMP levels in cells treated with both forskolin and cicaprost were plotted as fold-increase relative to the cells treated with cicaprost alone (14.8 pmol of cAMP; defined as 1.0). cAMP levels in cells treated with forskolin alone were plotted as -fold increase relative to unstimulated cells (5.7 pmol of cAMP; defined as 1.0). Results show the mean ± S.E.M., $n = 2$; **, $p <$ 0.01 compared with cells treated with forskolin in the absence of cicaprost. B to E, quiescent A10 smooth muscle cells were trypsinized, seeded at subconfluence, and stimulated with 10% FBS in the absence or presence of 200 nM ± cicaprost. Some cells were pretreated with pertussis toxin (PTX) before incubation with FBS ± cicaprost. B, nuclear extracts were prepared 18 h after incubation with FBS ± cicaprost, incubated with ^{32}P-labeled CCAAT or CRE oligonucleotides, and analyzed by EMSA and supershift EMSA using antibodies to CREB and phospho-CREB. (*, supershifted complex) C, cells were collected 0 and 15 h after incubation with 10% FBS ± cicaprost, lysed, and incubated with anti-cyclin E. Immunoprecipitated complexes were used to assess in vitro kinase activity of cyclin E-cdk2 and analyzed by western blotting for cyclin E-associated cdk2. D, collected cells were analyzed by Western blotting for cyclin A and cdk4 (loading control). E, FBS-stimulated cells (control) were incubated with BrdU in the absence or presence of PTX and cicaprost for 18 h. BrdU incorporation into nuclei was determined by immunofluorescence microscopy. Results show the mean ± S.E.M., $n = 3$; **, $p < 0.01$ compared with cells treated with 10% FBS alone.

7), and the complexes seen in the cicaprost/pertussis toxin-treated cells contained CREB and phospho-CREB (Fig. 8B, lanes 12 and 13), just as the complexes did in the absence of cicaprost (Fig. 8B, lanes 8–11). Furthermore, the inhibitory effects of cicaprost on cyclin E-cdk2 activity (Fig. 8C), cyclin A induction (Fig. 8D), and S phase entry (Fig. 8E) were also reversed by pertussis toxin. This was not a generic effect because pertussis toxin did not affect the extent of S phase entry in response to serum (Fig. 8E) or reverse TGF-β–mediated inhibition of smooth muscle cell cycle progression (data not shown). Thus, G_i signaling is required for the antimitogenic effects of cicaprost in smooth muscle cells.

Discussion

The aim of our study was to elucidate the mechanism by which PGI_2 inhibits cell cycle progression of aortic smooth muscle cells. We have shown that cicaprost inhibits G_1- to S-phase progression by blocking cyclin E-cdk2 activity and pocket protein phosphorylation. Cicaprost also prevents cyclin A gene activation in a pocket protein-independent manner by inhibiting CRE occupancy by CREB and phospho-CREB. The effects of cicaprost on S-phase entry, the cyclin A promoter, and cyclin A itself have been observed in both primary and established smooth muscle cells. Thus, our studies indicate that PGI_2 inhibits proliferation of aortic smooth muscle cells by coordinately blocking both pocket protein- and CRE-dependent cyclin A gene expression.

Although we see a nearly complete inhibition of cyclin E-cdk2 activity by cicaprost in A10 smooth muscle cells, the mechanism by which this occurs is not clear. Cicaprost neither increased the expression of $p21^{Cip1}$ nor consistently increased $p27^{Kip1}$ levels. Although cdk2 activity is typically regulated by changes in the expression of these cdk inhibitors, others have reported that cdc25A and perhaps cdk-activating kinase may also regulate cyclin E-cdk2 activity (Nagahara et al., 1999). Similarly, SPARC, a matrix-associated glycoprotein, inhibits cyclin E-cdk2 activity in human arterial smooth muscle cells without affecting $p21^{Cip1}$, $p27^{Kip1}$, cyclin E, or cdk2 (Motamed et al., 2002).

PGI_2 signals through its interaction with the IP receptor, a heterotrimeric G-protein-coupled receptor (Coleman et al., 1994). It is well established that IP couples to G_s (Namba et al., 1994) and increases cAMP production. Thus, PGI_2 would be expected to stimulate the phosphorylation of CREB (a known cAMP-dependent kinase substrate). Although we could detect a rapid increase in CREB phosphorylation at Ser133 in cicaprost-treated smooth muscle cells, the effect was very transient (lasting ~30 min) and insufficient to sustain CREB phosphorylation for the several hours needed to induce the cyclin A gene. However, EMSAs performed at 18 h (when the cyclin A promoter is active) showed that cicaprost decreases the occupancy of the cyclin A CRE in serum-stimulated A10 smooth muscle cells, indicating that cicaprost is blocking the binding of CREB and phospho-CREB to the CRE. CREB can also be phosphorylated at Ser142 by casein kinase II and calcium-calmodulin kinase II, and this phosphorylation blocks the formation of CREB-CBP complexes and the activation of target genes (Sun et al., 1994). The particular kinases required for CREB phosphorylation at Ser133 and Ser142 in our system, and the mechanism of the IP effect is currently being investigated.

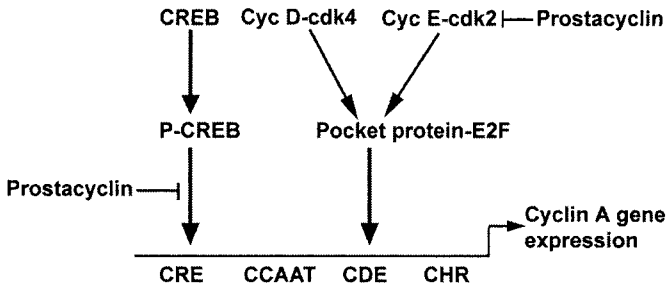

Fig. 9. Regulation of the cyclin A promoter by PGI_2. The figure shows that PGI_2 inhibits the activation of cyclin E-cdk2 and occupancy of the cyclin A CRE. These effects, which cooperate to inhibit CRE and CDE/CHR-dependent activation of the cyclin A promoter, result in decreased cyclin A gene expression and a reduced rate of cell cycle progression into S phase.

Interestingly, we find that all of the antimitogenic effects of cicaprost (i.e., the inhibition of CRE occupancy, inhibition of cyclin E-cdk2 activity, inhibition of cyclin A gene expression, and inhibition of S-phase entry) are reversed in cells treated with pertussis toxin. Thus, in both primary and A10 smooth muscle cells, the antimitogenic effect of cicaprost requires G_i signaling. Although it is possible that G_i acts alone to mediate the antimitogenic effect of cicaprost on cyclin A expression and G_1 phase progression, the antimitogenic effect of 8-bromo-cAMP suggests a role for G_s signaling. It is possible that G_s and G_i must both be activated, either sequentially (e.g., as reported by Lawler et al., 2001) or in parallel. Future studies will be directed at characterizing the relative roles of G_i and G_s in the antimitogenic effects of cicaprost.

Together with our previous studies on regulation of the cyclin A gene (Bottazzi et al., 2001), the studies described here lead to a working model for the inhibitory effect of PGI_2 on the cyclin A promoter and, consequently, the proliferation of aortic smooth muscle cells (Fig. 9). We have previously reported that serum-derived mitogens stimulate the phosphorylation of CREB (Bottazzi et al., 1999), and we now show that PGI_2 inhibits CRE occupancy by CREB and phospho-CREB. Mitogens and the extracellular matrix also act coordinately to activate cyclin D1-cdk4 and cyclin E-cdk2, but PGI_2 specifically inhibits the activation of cyclin E-cdk2, thereby preventing pocket protein inactivation. We propose that by coordinately regulating CRE- and pocket protein-dependent cyclin A gene expression, PGI_2 inhibits entry into S phase and prevents the proliferation of aortic smooth muscle cells. These effects are likely to underlie the antiproliferative effect of PGI_2 and IP in injury-induced cardiovascular disease.

Acknowledgments

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Correction to "Prostacylin receptor activation inhibits proliferation of aortic smooth muscle cells by regulating cAMP response element-binding protein- and pocket protein-dependent cyclin A gene expression"

In the above article [Kothapalli D, Stewart SA, Smyth EM, Azonobi I, Puré E, and Assoian RK (2003) *Mol Pharmacol* **64:**249–258], Daniel J. Rader should have been included as an author. His name was added in proof but was inadvertently omitted from the printed journal. The corrected author list will appear online.

We regret this error and apologize for any confusion or inconvenience it may have caused.