Platelet-derived Growth Factor Stimulates Protein Kinase A through a Mitogen-activated Protein Kinase Kinase-dependent Pathway in Human Arterial Smooth Muscle Cells*

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The abilities of platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-I) to regulate cAMP metabolism and mitogen-activated protein kinase (MAP kinase) activity were compared in human arterial smooth muscle cells (hSMC). PDGF-BB stimulated cAMP accumulation up to 150-fold in a concentration-dependent manner (EC50 ~ 0.7 nM). The peak of cAMP formation and cAMP-dependent protein kinase (PKA) activity occurred approximately 5 min after the addition of PDGF and rapidly declined thereafter. Incubating cells with PDGF and 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor) enhanced the accumulation of cAMP and PKA activity by an additional 2.5-3-fold, whereas IBMX alone was essentially without effect. The PDGF-stimulated increase in cAMP was prevented by addition of the cyclooxygenase inhibitor indomethacin, consistent with release of prostaglandins stimulating cAMP. PDGF, but not IGF-I, stimulated MAPK activity, cytosolic phospholipase A2 (cPLA2) phosphorylation, and cAMP synthesis which indicated a key role for MAP kinase in the activation of cPLA2. Further, PDGF stimulated the rapid release of arachidonic acid and synthesis of prostaglandin E2 (PGE2) which could be inhibited by a cPLA2 inhibitor (AACOCF3). Calcium mobilization was required for PDGF-induced arachidonic acid release and PGE2 synthesis but not for MAPK activation, whereas PKC was required for PGE2-mediated activation of PKA. In summary, these results demonstrate that PDGF increases cAMP formation and PKA activity through a MAP kinase-mediated activation of cPLA2, arachidonic acid release, and PGE2 synthesis in human arterial smooth muscle cells.

The proliferation of arterial smooth muscle cells is a key event in the formation and progression of lesions of atherosclerosis and in restenosis following angioplasty. This proliferation is most likely initiated and regulated by growth factors, such as the platelet-derived growth factors, PDGF-AA, PDGF-BB, and PDGF-AB. In atherosclerotic lesions, a major source of PDGF-BB is activated macrophages, although smooth muscle, endothelial cells, and other cells can also express and secrete PDGF dimers (reviewed in Ref. 1).

Many growth factor receptors, including PDGF receptors, activate a signal transduction pathway that includes conversion of inactive Ras-GDP to active Ras-GTP, activation of Raf, MAP kinase kinase (MAPKK or MEK (2)), and MAP kinase (MAPK) (for review, see Ref. 3). Activation of the MAPK cascade results in the stimulation of DNA synthesis and cell proliferation, which can be inhibited by expression of a dominant-negative MAPKK (4, 5). Conversely, expression of a constitutively active form of MAPKK can stimulate cell proliferation and transformation (4, 6). A number of nuclear and non-nuclear proteins have been identified as substrates for MAPK. Among the latter is phospholipase A2, thereby providing a potential link to arachidonic acid metabolism (reviewed in Ref. 7).

Activation and translocation of the cytosolic 85-kDa phospholipase (cPLA2), which catalyzes the release of arachidonic acid from the sn-2 position of phospholipids in the plasma membrane, is an important signal leading to prostaglandin synthesis (reviewed in Refs. 8 and 9). This enzyme can be distinguished from the low molecular weight forms of PLA2 by insensitivity to disulfide-reducing agents or inhibition by arachidonic acid analogues (for reviews, see Refs. 10 and 11). Regulation of cPLA2 appears to be critically dependent on the integration of multiple signals, including intracellular calcium, protein kinase C (PKC), and phosphorylation by MAP kinase (12-15). However, in some cells, calcium-independent forms of cPLA2 (16) and PKC-independent mechanisms of cPLA2 activation have also been observed (15).

Although the coupling of hormonal and neurotransmitter receptors to cAMP synthesis is well established (reviewed in Ref. 17), the means by which growth factor receptor tyrosine kinases (eg. the PDGF receptor) regulate cAMP metabolism is less well understood. At present, there are few examples of growth factors stimulating cAMP accumulation. Before elucidation of the MAP kinase cascade, it was reported that PDGF (in the presence of phosphodiesterase inhibitors) increased cAMP formation in human arterial smooth muscle cells; PGE2, prostaglandin E2; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

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The abbreviations used are: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IBMX, 3-isobutyl-1-methylxanthine; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; IGF-I, insulin-like growth factor I; MBP, myelin basic protein; HSMC, human arterial smooth muscle cells; cPLA2, cytosolic phospholipase A2; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; PDS, plasma-derived serum; PAGE, polyacrylamide gel electrophoresis; PGE2, prostaglandin E2; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.
cAMP synthesis 6-8-fold in Swiss 3T3 cells (18). In perfused rat hearts, epididymal growth factor (EGF) was found to stimulate cAMP accumulation (19). In epithelial cells overexpressing the EGF receptor (A431 cells), EGF alone did not affect cAMP accumulation, but did potentiate an increase in cAMP in response to cAMP-elevating agents (20).

Sustained elevation of cAMP inhibits the proliferation of many cell types, including smooth muscle cells (21). This phenomenon may in part be explained by the fact that in many cell types, including human arterial SMC (hSMC), MAP kinase activation is inhibited by the cyclic AMP-dependent protein kinase (PKA) (22-26). The target for inhibition by PKA in the MAP kinase pathway may be Raf-1 (23, 27), although other unidentified targets are also likely to play an important role (28).

To further evaluate how growth regulatory molecules may regulate cAMP metabolism in hSMC, we examined the effect of two major factors known to influence hSMC (i.e. PDGF and IGF-I). We report here that PDGF rapidly stimulates cAMP synthesis through a mechanism requiring intracellular calcium, PKC activity, MAPK-dependent phosphorylation of cPLA2, and activation of prostaglandin synthesis, an effect which culminates in increased PKA activity.

**EXPERIMENTAL PROCEDURES**

**Materials—** Recombinant human PDGF-BB and PDGF-AA were gifts from Hoffman-La Roche Inc. (Basel, Switzerland). Human recombinant IGF-I was obtained from Upstate Biotechnology Inc. (UBI, Lake Placid, NY). PKI peptide (TYYADFIASGRTGRRNAIHD), a specific peptide inhibitor of PKA (29), and Leu-Arg-Arg-Ala-Leu-Gly (Kemptide) (30) were synthesized at the Peptide Synthesis Facility, Howard Hughes Medical Institute (Seattle, WA). Recombinant rat Erk2 was a gift from Dr. M. Cobb (University of Texas, Southwestern, Dallas, TX), and ascites to this protein was provided by Dr. R. Seger. Goat anti-rabbit alkaline phosphatase and anti-peptide antibodies to this protein were developed previously in this laboratory by Dr. M. Cobb (University of Texas, Southwestern, Dallas, TX), and anti-peptide antibodies to this protein were developed previously in this laboratory by Dr. M. Cobb (University of Texas, Southwestern, Dallas, TX). Recombinant rat Erk2 was a gift from Dr. M. Cobb (University of Texas, Southwestern, Dallas, TX), and ascites to this protein was provided by Dr. R. Seger. Goat anti-rabbit alkaline phosphatase and anti-peptide antibodies to this protein were developed previously in this laboratory by Dr. M. Cobb (University of Texas, Southwestern, Dallas, TX).

**Cell Cultures—** Human newborn arterial smooth muscle cells were obtained from the thoracic aorta of infants following death due to congenital heart defects or sudden infant death. The cells were isolated by the explant method and cultured as described previously (32). Subconfluent cell cultures were kept in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 micrograms/ml streptomycin, 0.25% bovine serum albumin), adjusted to pH 7.2. The cells were incubated with PDGF, IGF-I, or PMA for the indicated times. Immediately after stimulation, the cells were scraped and sonicated in buffer H [50 mM β-glycerophosphate, pH 7, 10 mM Na3VO4, 0.1 mM dithiothreitol, 25 μM aprotinin, 25 μM leupeptin, and 0.5 mM phenylmethylsulfon fluoride]. For measurement of PKA activity, cell lysates were preadsorbed on a DE52 mini column, and the phosphorylation of myelin basic protein by activated recombinant Erk2 was measured. PKA activity was determined by the incorporation of radioactive i from [γ-32P]ATP into myelin basic protein after a 30-min incubation at 37°C. MAPK activity was assayed by autoradiography. Mono Q fractions were also assayed for MAPK activity.

**Measurement of Cyclic AMP—** Cells in two max-plates (approximately 15 × 10^6 cells/sample) were stimulated with vehicle (10 μM acetic acid, 0.25% bovine serum albumin), 1 μM PDGF-BB, or 10 μM IGF-I for 5 min. Cell extracts were prepared in Buffer H as described above and applied to a Mono Q (Pharmacia) column. The radioactive content of the column was developed with a linear gradient of 0-400 mM NaCl in the same buffer. Fractions of 1 ml were collected, and the radioactivity released prior to stimulation was subtracted from the amount released at the end of the stimulation. For measurement of PGE2 synthesis, cells in 6-well plates were washed three times in DMEM (without PDS or bovine serum albumin), and then stimulated as indicated in 1 ml of new medium. PGE2 released to the medium was measured after a 1:10–1:30-fold dilution using a PGE2 enzyme immunoassay (Amersham). The cross-reactivity of this assay is less than 7% with PGE1 and less than 5% with other related prostaglandins.

**Phosphorylation of cPLA2—** Cells in two max-plates (approximately 15 × 10^6 cells/sample) were stimulated with vehicle (10 μM acetic acid, 0.25% bovine serum albumin), 1 μM PDGF-BB, or 10 μM IGF-I for 5 min. Cell extracts were prepared in Buffer H as described above and applied to a Mono Q (Pharmacia) column. The radioactive content of the column was developed with a linear gradient of 0-400 mM NaCl in the same buffer. Fractions of 1 ml were collected, and the radioactivity released prior to stimulation was subtracted from the amount released at the end of the stimulation. For measurement of PGE2 synthesis, cells in 6-well plates were washed three times in DMEM (without PDS or bovine serum albumin), and then stimulated as indicated in 1 ml of new medium. PGE2 released to the medium was measured after a 1:10–1:30-fold dilution using a PGE2 enzyme immunoassay (Amersham). The cross-reactivity of this assay is less than 7% with PGE1 and less than 5% with other related prostaglandins.

**Measurement of MAPK Activity—** Cells in 24-well plates (approximately 3 × 10^5 cells/sample) were stimulated with PDGF, IGF-I, or PMA for the indicated times. After stimulation, the cells were scraped and sonicated in buffer H [50 mM β-glycerophosphate, pH 7, 10 mM Na3VO4, 0.1 mM dithiothreitol, 25 μM aprotinin, 25 μM leupeptin, and 0.5 mM phenylmethylsulfon fluoride]. For measurement of MAPK activity, cell lysates were preadsorbed on a DE52 mini column, and the phosphorylation of myelin basic protein by activated recombinant Erk2 was measured. MAPK activity was determined by the incorporation of radioactive i from [γ-32P]ATP into myelin basic protein after a 30-min incubation at 37°C. MAPK activity was assayed by autoradiography. Mono Q fractions were also assayed for MAPK activity.

**Immunoblotting of cPLA2—** Lysates from cells incubated with PDGF-BB or IGF-I for 5 min were obtained by harvesting the cells in a buffer containing 50 mM Hapes, pH 7.4, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 200 μM sodium orthovanadate, 10 mM tetrusodium pyrophosphate, 100 mM sodium fluoride, 3 μM p-nitrophenyl phosphate, 10 μM aprotinin, 10 μM leupeptin, and 1 mM phenylmethylsulfon fluoride. The samples were added to an equal volume of SDS-PAGE sample buffer, boiled for 5 min, and were applied to a 10% SDS-polyacrylamide gel containing 1% bisacrylamide. After electrophoresis, the gel was stained with Coomassie blue, destained, and then transferred to nitrocellulose. The membranes were incubated with polyclonal antibody to cPLA2 (1:1000) and the immunodetection was accomplished by the use of goat anti-rabbit horseradish peroxidase and enhanced chemiluminescence (ECL).

**RESULTS**

PDGF Stimulates a Transient Increase in cAMP and PKA Activity—Incubation of hSMC with PDGF-BB (1 μM) stimulated a rapid increase in cAMP accumulation (approximately 50-fold) that was detected as early as 2.5 min after the addition of the growth factor and reached a maximal level by 5 min (Fig. 1A). The PDGF-stimulated increase in cAMP did not require the addition of phosphodiesterase inhibitors. In parallel with
cAMP, PDGF-BB increased PKA activity up to 7–8-fold as determined by Kemptide phosphorylation (Fig. 1A). The PDGF-stimulated increase in cAMP and PKA activity was transient and disappeared within 15 min with no further increase in cAMP detected up to 1 h after PDGF addition. These results were confirmed in different isolates of human newborn arterial smooth muscle cells (data not shown). Incubation of hSMC with the phosphodiesterase inhibitor, IBMX (500 μM), increased the PDGF-stimulated PKA activity by an additional 2-fold, whereas incubation with IBMX alone only slightly elevated the PKA activity (data not shown). Similar results were found with IBMX on cAMP accumulation and suggested that PDGF was stimulating cAMP synthesis, rather than inhibiting cAMP breakdown.

The stimulation of cAMP accumulation was dose-dependent, with up to a 150-fold increase over basal levels (10–50 pmol/ml) seen at the highest concentration (10 nM) of PDGF-BB (Fig. 1B). The PDGF isoform, PDGF-AA, also increased cAMP levels in hSMC, although not to the same extent as PDGF-BB. This is likely a reflection of the 10-fold lower number of PDGF receptor α subunits compared to PDGF receptor β subunits in these cells (32). Interestingly, despite the fact that hSMC also contain functional IGF-I receptors (similar in number to PDGF-α receptors), IGF-I, at concentrations as high as 10 nM, did not increase cAMP synthesis, even in the presence of the phosphodiesterase inhibitor IBMX (Fig. 1, A and B, and data not shown).

Connection between cAMP Synthesis, MAP Kinase Activation, Intracellular Calcium, and PKC Activity—PDGF-BB-induced cAMP formation, but not MAPK activation is dependent on calcium mobilization and PKC. Human SMC in 10-cm plates were incubated for 5 min with 1 nM PDGF-BB, 1 nM IGF-I or for 20 min with 100 nM PMA. In some cells, PKC was down-regulated by a 20-h preincubation with 1 μM PMA, or intracellular calcium stores were depleted by a 1-h preincubation with 300 nM thapsigargin. Levels of cAMP (A) were measured by the cAMP immunoassay as in Fig. 1, and MAPK activity (B) was measured as phosphorylation of MBP for 15 min at 30 °C. These results are shown as mean ± S.E. of duplicate samples. These experiments were repeated twice with similar results.

Since calcium is a key second messenger in the regulation of cAMP metabolism in many cell types (reviewed in Refs. 35 and 36), we investigated whether calcium was required for the stimulation of cAMP synthesis by exposing cells to the tumor promoter, thapsigargin. Thapsigargin stimulates the release of...
calcium from intracellular stores, resulting in an initial increase of intracellular calcium and later (at approximately 1 h), as the calcium is transferred to the extracellular space, a depletion of calcium from intracellular stores (37). Addition of PDGF-BB to hSMC rapidly increases intracellular calcium, an event that can be inhibited by prior incubation with 300 nM thapsigargin for 1 h. Preincubation with thapsigargin (300 nM, 1 h) eliminated the synthesis of cAMP stimulated by PDGF-BB, demonstrating a requirement for intracellular calcium in this process (Fig. 2A). The effect of this compound on the activation of MAPK by PDGF was examined. Neither an extended (1-h) (Fig. 2B) nor a brief (5-min) (data not shown) exposure of cells to thapsigargin affected the basal or PDGF-stimulated levels of MAP kinase activity in these cells (Fig. 2B). However, a brief incubation with thapsigargin (5 min) did increase PKA activity above basal levels (thapsigargin, 404 ± 16 pmol/min/ml; basal, 80 ± 2 pmol/min/ml) and cAMP accumulation (data not shown).

Protein kinase C (PKC) has also been implicated in the regulation of cAMP formation in many cell types (reviewed in Ref. 36). Therefore, the requirement for protein kinase C (PKC) activity in PDGF-stimulated cAMP synthesis was investigated. Incubating hSMC with PMA (1 μM, 20 h) resulted in the complete loss of PMA-stimulated MAPK and MAPKK activity and in keeping with a known effect of this procedure for down-regulation of PKC activity (data not shown). Down-regulation of PMA-sensitive PKC activity inhibited the PDGF-stimulated increase in cAMP by >90% (Fig. 2A), without a significant influence on the activation of MAPK by the growth factor (Fig. 2B). Similarly, addition of the PKC inhibitor (1 μM), bisindoylmaleimide (GF109203X) prevented the formation of cAMP by PDGF without inhibiting that stimulated by forskolin (data not shown). Interestingly, PMA alone did not increase cAMP accumulation, although it did activate MAP kinase as expected (Fig. 2B).

Prostaglandin E2 Synthesis Is Required for cAMP Formation—Previously, Rozengurt reported that PDGF stimulates cAMP synthesis through a prostaglandin-dependent pathway in Swiss 3T3 cells (18). Since the rate-limiting step in prostaglandin synthesis is believed to be the cPLA2-catalyzed release of arachidonic acid (8, 9), the ability of PDGF and IGF-I to stimulate arachidonic acid release in hSMC was examined. PDGF-BB stimulated the release of [3H]arachidonic acid within 2.5–5 min of addition, after which the accumulation leveled off (between 10 and 20 min) (data not shown). As shown in Fig. 3A, both PDGF-BB and PDGF-AA (less potently) stimulated arachidonic acid release, whereas IGF-I was without effect. Incubation of hSMC with thapsigargin for 1 h prior to PDGF abolished the PDGF-stimulated increase of arachidonic acid release (Fig. 3A). Previously, it was shown that cPLA2 activity could be inhibited by a class of arachidonic acid analogues (39, 40). To confirm that the release of [3H]arachidonic acid was due to cPLA2 activation, we incubated hSMC with the analogue AACOCF3, or the noninhibitory analogue AACOCH3 prior to the addition of PDGF. The stimulation of [3H]arachidonic acid release by PDGF was significantly inhibited by AACOCF3 (>50%), whereas the analogue AACOCH3 was without effect (Fig. 3A).

One of the major products of arachidonic acid metabolism in hSMC is prostaglandin E2 (PGE2) (38), which is formed by the action of cyclooxygenases on arachidonyl precursors. To investigate the potential role of prostaglandin release in cAMP formation, hSMC were incubated with the cyclooxygenase inhibitor, indomethacin, prior to the addition of PDGF-BB. As seen in Table I, indomethacin (10 μM, 30 min) completely inhibited the PDGF-stimulated increase in cAMP and PKA activity and slightly inhibited the basal levels of cAMP. Incubation with indomethacin did not affect the activation of MAPK, suggesting that the effects of this compound were specific to inhibition of cyclooxygenase activity. These results demonstrated that prostaglandin synthesis was required for PDGF-stimulated cAMP synthesis.

To specifically investigate whether PGE2 was involved in stimulating the increase in cAMP, PGE2 formation was measured in response to PDGF. Within 1 min of addition, PDGF increased PGE2 synthesis in hSMC, which continued to accumulate with extended exposure to PDGF (Fig. 3B). PGE2 increased PKA activity by 330% as early as 1 min after addition (187.6 ± 0.6 to 622.6 ± 12.0 pmol/min/ml). The peak of PKA activity was determined in a 5-min stimulation with 1 nM PDGF-BB, 1 nM PDGF-AA, 1 nM IGF-I, and was measured. In some instances, intracellular calcium stores were depleted by preincubation with 300 nM thapsigargin, or cPLA2 activity was inhibited by a 30-min preincubation with 30 μM AACOCF3. The AACOCF3 analogue AACOCH3 (30 μM) was used as a control. In B, the cells were washed three times with fresh DMEM without [3H]arachidonic acid, and release of [3H]arachidonic acid to the medium during a 5-min stimulation with 1 nM PDGF-BB, 1 nM PDGF-AA, 1 nM IGF-I was measured. In some instances, intracellular calcium stores were depleted by preincubation with 300 nM thapsigargin, or cPLA2 activity was inhibited by a 30-min preincubation with 30 μM AACOCF3. The AACOCF3 analogue AACOCH3 (30 μM) was used as a control. In B, the cells were washed three times with fresh DMEM and then stimulated with 1 nM PDGF-BB or vehicle (C). The results are expressed as mean ± S.D. of triplicate samples. The experiment was repeated twice with similar results.
activity occurred within 5 min of PGE2 addition, in good agreement with the ability of PDGF to rapidly increase PKA activity through PGE2 release (Table I). PDGF-induced PGE2 release was dependent on intracellular calcium. Thus, both arachidonic acid release and PGE2 release stimulated by PDGF were inhibited by depletion of intracellular calcium stores using thapsigargin (Fig. 3A and data not shown).

Down-regulation of PKC inhibited the ability of PGE2 to stimulate PKA activity from 6.5-fold in vehicle-treated cells to 1.4-fold in cells subjected to PKC down-regulation (Table I). In contrast, neither down-regulation of PKC nor addition of the PKC inhibitor, bisindolylmaleimide, inhibited the formation of cAMP stimulated directly by forskolin (data not shown). PKC down-regulation did not inhibit PDGF-induced PGE2 release during a 5-min stimulation (11.1 OM to 30.6 OM of PGE2 released/10^6 cells subjected to PKC down-regulation compared to 33 OM to 25.7 OM of PGE2 released/10^6 vehicle-treated cells).

PDGF Stimulates MAPK Activation and cPLA2 Phosphorylation—Since the peak of PDGF-stimulated cAMP and PKA correlated with the maximal activation of MAPK in these cells (Ref. 32 and Fig. 1), we investigated whether MAPK was directly involved in the mechanism of PKA activation. To investigate the phosphorylation of cPLA2 by MAPK, cell extracts were subjected to Mono Q chromatography and examined for their MBP phosphorylating activity. The fractions testing positive for MAPK activity were then characterized with respect to the presence of this enzyme as shown immunologically and by cPLA2 phosphorylating ability. As seen in Fig. 4A, PDGF-BB potently stimulated MAP kinase activity (MBP phosphorylation), whereas IGF-I did not increase this activity. Immunoblotting of the peak fractions from PDGF-treated cells confirmed that these samples contained phosphorylated and active MAPK (Erk1 and Erk2) as judged by band shift and further demonstrated that fractions from unstimulated or IGF-I-stimulated cells did not contain detectable MAPK activity (Fig. 4B). As shown in Fig. 4, fractions from PDGF-stimulated cells contained one major peak of MBP phosphorylating activity (Fig. 4A) that co-eluted with the peak of cPLA2 phosphorylation (Fig. 4C). Fractions from IGF-I-stimulated cells did not contain cPLA2 phosphorylating activity above basal levels (Fig. 4C). These results suggested that the deficiency in cAMP synthesis observed with IGF-I was due to the inability of this growth factor to significantly activate MAPK.

We further investigated the phosphorylation of endogenous cPLA2 under conditions that led to the activation of cAMP synthesis in hSMC. Phosphorylation of cPLA2 by MAPK results in a mobility shift on SDS-PAGE that correlates with the activation of this enzyme (13). Extracts of hSMC stimulated with PDGF or IGF-I were examined by immunoblotting for cPLA2. As seen in Fig. 5, the cPLA2 from untreated hSMC was a doublet similar to the baculovirus-expressed human recombinant cPLA2 standard which is partially phosphorylated in the SF9 cells (31). Samples from PDGF-treated cells showed that cPLA2 mobility was shifted completely relative to the untreated or IGF-I-treated samples. The lack of effect of IGF-I is consistent with the finding that IGF-I did not activate MAPK in these cells and that cPLA2-dependent release of arachidonic acid was not stimulated by IGF-I.

Influence of PDGF-stimulated PKA on MAPK Activation—We previously demonstrated that PKA could inhibit PDGF-stimulated MAPKK and MAPK activity in hSMC, an effect that was observed with forskolin or PGE2 (22). We therefore investigated whether the stimulation of PKA activity by PDGF could affect the activation of the MAP kinase cascade. Addition of indomethacin (10 OM, 30 min) prior to PDGF abolished the growth factor-stimulated increase in cAMP and PKA activity without affecting the magnitude of MAPKK or MAPK activation by PDGF (Table I and data not shown). Furthermore, addition of indomethacin did not alter the time course of MAPKK or MAPK activation in response to PDGF in hSMC (data not shown).

**DISCUSSION**

PDGF initiates a multitude of biological effects through the activation of intracellular signal transduction pathways such as the MAP kinase cascade, phosphatidylinositol turnover, and calcium mobilization (reviewed in Ref. 41), and these effects are believed to contribute to smooth muscle cell proliferation and directed migration (32). Further, changes in eicosanoid metabolism can regulate smooth muscle cell growth and contraction through alterations in cAMP metabolism and calcium homeostasis (reviewed in Ref. 38). How these key signal transduction pathways are integrated is not presently well understood. Because of our interest in the cross-talk between the MAPK cascade and PKA, we investigated the effects of growth factors on cAMP metabolism in hSMC. We found that PDGF induces a strong and rapid formation of cAMP through a mechanism that includes MAP kinase-mediated activation of cPLA2, release of arachidonic acid, prostaglandin PGE2, and the subsequent activation of adenylyl cyclase. Although parts of these signaling

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**TABLE I**

| Treatment | [cAMP] (pmol/ml) | PKA activity (pmol/min/ml) | MAPK activity (pmol/min/ml) |
|-----------|------------------|---------------------------|---------------------------|
| Vehicle   | 50.8 ± 0.0       | 55.3 ± 3.6                | 41.4 ± 5.9                |
| Indomethacin | 11.7 ± 0.0   | 45.4 ± 5.4                | 47.8 ± 4.2                |
| PDGF-BB   | 1636.5 ± 108.3 | 463.3 ± 34.8              | 108.9 ± 0.7               |
| Indomethacin/PDGF-BB | 12.4 ± 5.5 | 42.7 ± 21.1 | 112.4 ± 3.6 |

**TABLE II**

| Treatment | PKA activity (pmol/min/ml) |
|-----------|---------------------------|
| Vehicle   | 171.5 ± 1.4               |
| PDGF-BB   | 1434.5 ± 13.6             |
| PGE2      | 11148 ± 9.0               |
| Thapsigargin | 204.6 ± 2.7       |
| Thapsigargin/PGE2 | 1034.0 ± 77.8 |
| PKC down-regulation | 362.0 ± 26.9 |
| PKC down-regulation/PGE2 | 515.8 ± 15.0 |

**Note**

Cells in 10-cm dishes (approximately 2.2 × 10^6 cells) were preincubated in the presence or absence of 10 OM indomethacin for 30 min and then stimulated with 1 nM PDGF-BB for 5 min. Levels of cAMP, PKA, and MAP kinase activities were determined as described in Figs. 1 and 2. The results are shown as mean ± S.E. of duplicate samples. The experiment was repeated several times with similar results.
occur. Several observations support these concepts. PKC, and MAP kinase activity are necessary for this event to demonstrate that at least three independent signals, specifically human arterial SMC. In addition, our studies demonstrate (3) that MAPK phosphorylation is an essential signal in the activation of cPLA$_2$, and can account for the majority of PDGF-stimulated cAMP synthesis observed in hSMC.

We examined the possibility that, in addition to effects of PDGF on prostaglandin metabolism, alternative mechanisms could facilitate the coupling of growth factor signal to cAMP synthesis in hSMC. We were unable to find a direct effect of PDGF on adenylate cyclase activity in membranes obtained from hSMC or on the phosphorylation of the α-subunit of the G-protein $G_{i,3}$. In contrast to results obtained in epidermal growth factor (EGF)-stimulated cells (44–46). Instead, our results suggest that increased prostaglandin metabolism through the activation of cPLA$_2$ can account for the majority of PDGF-stimulated cAMP synthesis observed in hSMC.

Both PDGF-BB and PDGF-AA potently stimulated MAPK activity, cPLA$_2$ phosphorylation, arachidonic acid release, and cAMP synthesis in this study. IGF-I did not influence any of these events, despite the fact that in hSMC the number of IGF-I receptors is equivalent to the number of PDGF-α receptors, and that the IGF-I receptor is coupled to phosphatidyl-inositol turnover, calcium mobilization, and chemotaxis (32). In the present study, the inability of IGF-I to elevate cAMP correlates with an absence of effect of this growth factor on MAPK activity and cPLA$_2$ phosphorylation in hSMC. These experiments confirm the findings of others (13–15), demonstrating that MAPK phosphorylation is an essential signal in the activation of cPLA$_2$. Recently, Sa et al. (47) reported that, in endothelial cells, basic fibroblast growth factor stimulates a MAPK-dependent activation of cPLA$_2$, supporting the role for MAPK in cPLA$_2$ regulation in other cell types.

In addition to MAP kinase activation, PKC and PKA activity and intracellular calcium mobilization were critical for the activation of PKA by PDGF in hSMC. Since both cPLA$_2$ (13–15) and adenylate cyclase (48–50) can be regulated by calcium and PKC in other cell types, we investigated some of the mechanisms responsible for this calcium and PKC dependence. PMA did not significantly stimulate arachidonic acid release, PGE$_2$ release, or cAMP synthesis, although PMA increased both MAPK and PKC activities as expected. Thus, activation of MAPK or PKC alone is insufficient for stimulation of cAMP synthesis in hSMC. PKC down-regulation blocked both PDGF-induced and PGE$_2$-induced PKA activation, without affecting the ability of forskolin to activate the adenylate cyclase, or the ability of PDGF to stimulate PGE$_2$ release. Together, these results suggest that PKC is required for PGE$_2$ receptor signaling to PKA activation. Depletion of calcium from intracellular stores blocked PDGF-induced arachidonic acid release, PGE$_2$ release, and the subsequent PKA activation in hSMC without inhibiting MAPK activation. Further, no effect on PGE$_2$-stimulated PKA activation was seen, suggesting that inhibition of PDGF-induced PKA activation by intracellular calcium depletion is due to inhibition of cPLA$_2$ activation. In addition, transient increases in cAMP synthesis were observed in hSMC when intracellular calcium levels were increased dramatically by a short stimulation with thapsigargin or by sphingosine-1-phosphate (51), for example. This effect was independent of MAPK activation, and the concentrations of intracellular cal-

3 L. M. Graves, unpublished observations.

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**Fig. 4.** PDGF-induced MAPK activity phosphorylates cPLA$_2$ in vitro. Human SMC in 10-cm plates were incubated without addition (Δ), with 1 nM PDGF-BB (○) or 1 nM IGF-I (□) for 5 min. The samples were separated on a Mono Q column, and the fractions were measured for MAP kinase activity (A). Fractions 22, 30, 32, 34, 36, and 44 from each sample were immunoblotted for the presence of MAPK by using an anti-ERK antibody (B). The same fractions were incubated with $1 \mu$g of recombinant cPLA$_2$, and the phosphorylation of cPLA$_2$ (C) was measured as described under "Experimental Procedures." Lane A contains active Erk2 (B) or cPLA$_2$ phosphorylated by recombinant Erk2 (C). This experiment was repeated twice with similar results.

**Fig. 5.** PDGF-induced phosphorylation of cPLA$_2$ in hSMC. Lysates (50 μg) from hSMC treated with vehicle (lane 2), 1 nM PDGF-BB (lane 3), or 10 nM IGF-I (lane 4) for 5 min were immunoblotted using an antibody to cPLA$_2$, as described under "Experimental Procedures." The standard human cPLA$_2$ (5 μg) was purified from baculovirus-infected SF9 cells (lane 1). The upper band represents the phosphorylated form of cPLA$_2$. The experiment was repeated twice with similar results.
cium required for direct cAMP stimulation were higher than those obtained following stimulation with PDGF or IGF-I (51). Possibly, high intracellular concentrations of calcium may directly stimulate the adenylate cyclase types I and III (reviewed in Ref. 35).

Previously, we reported that PKA can inhibit PDGF-stimulated MAPK signaling in hSMC (22). Therefore, we examined whether the increase in cAMP and PKA activity in response to PDGF could inhibit the MAP kinase cascade in a “feedback” manner. Such a negative feedback mechanism on MAPK (Erk2) activity has recently been demonstrated by Pyne and co-workers following bradykinin-induced cAMP accumulation (52). We were unable to find an effect of the PDGF-stimulated PKA activity on the time course of MAPK or MAPK activation in response to PDGF, nor was the PDGF-stimulated increase in PKA activity sufficient to limit the magnitude of MAPK activation by this growth factor. At this time, we can only speculate whether the increase in cAMP and PKA activity in response to PDGF, nor was the PDGF-stimulated increase in PKA activity on the time course of MAPK or MAPK activation in response to PDGF, nor was the PDGF-stimulated increase in PKA activity sufficient to limit the magnitude of MAPK activation by this growth factor. At this time, we can only speculate that the PDGF-stimulated increase in PKA activity occurs too transiently to sufficiently impede the activation of MAPK in hSMC. Alternatively, the PDGF-stimulated increase in cAMP and PKA activity may be involved in PKA-mediated transcription or cytoskeletal remodeling events such as actin filament reorganization which is known to occur in response to PDGF (51).

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