Cyclic AMP Inhibition of Thrombin-induced Growth in Vascular Smooth Muscle Cells Correlates with Decreased JNK1 Activity and c-Jun Expression

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Thrombin is a potent modulator of vascular tone and vascular smooth muscle cell (VSMC) mitogenesis. Early studies from other laboratories demonstrated that cyclic AMP (cAMP) antagonizes the mitogenic effects of platelet-derived growth factor and epidermal growth factor by inhibiting the extracellular signal-regulated protein kinases (ERKs; p42, p44) group of mitogen-activated protein kinases (MAPKs) in several cell types. This report examines the role of ERKs and J un N-terminal kinase 1 (JNK1) groups of mitogen-activated protein kinases in thrombin-induced DNA synthesis in VSMCs using agents such as forskolin and dibutyryl cyclic AMP that increase intracellular cAMP levels. Both agents significantly inhibited thrombin-stimulated DNA synthesis in VSMCs. These agents, however, had no effect on thrombin induction of ERKs activation and c-Fos expression, suggesting divergence of the latter two events from the growth-signaling events of thrombin that are sensitive to inhibition by cAMP. Thrombin activated JNK1 and induced c-j un expression in VSMCs in a time-dependent manner. In contrast to ERKs and c-Fos, thrombin-induced JNK1 activation and c-j un expression were sensitive to inhibition by forskolin, suggesting an association of these events with thrombin-stimulated growth in these cells. Thrombin also increased AP-1 activity, and this response was significantly blunted by forskolin. Together, these results demonstrate a correlation between JNK1 activation and c-j un expression by thrombin and their association with the mitogenic signaling events of thrombin in VSMCs.

Besides activating platelet aggregation, thrombin also induces growth in VSMCs and fibroblasts. The coagulant and mitogenic effects of thrombin appear to be mediated by a seven-transmembrane G protein-coupled receptor that has been cloned and characterized in recent years. Thrombin activates its receptor by a unique mechanism involving cleavage of the N terminus of the receptor, generating a new N-terminus, which, in turn, acts as a tethered ligand. G protein-coupled receptors, including the thrombin receptor, do not possess intrinsic tyrosine kinase activity. Nonetheless, their ligands, such as thrombin, require protein tyrosine kinase activity to produce mitogenic effects in VSMCs.

Protein tyrosine phosphorylation events play important roles in cell proliferation and differentiation. Upon binding, peptide growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), cause their cognate receptor tyrosine kinases to undergo autophosphorylation on tyrosine residues. The autophosphorylated receptors, by interacting with Src homology 2 and Src homology 3 domain-containing proteins such as growth factor receptor-bound protein 2 and SHC, activate Ras (10–12). Ras, in turn, stimulates a cascade of kinase activities leading to activation of ERKs (13–16). Activated ERKs translocate to the nucleus and influence gene transcription by phosphorylating transcriptional factors such as ternary complex factor/Erk1 (17, 18). In a parallel pathway, Ras also activates a kinase cascade that leads to JNK activation (19–21). JNKs like ERKs modulate gene expression by translocating to the nucleus and phosphorylating transcriptional factors such as c-j un/TP-1 and ATF2 (22). Although agonists of receptor tyrosine kinases preferentially activate the "ERK" pathway (23, 24), cytokines such as tumor necrosis factor α and cellular stresses such as UV irradiation have been shown to potentiate the "JNK" pathway (23, 24).

A large body of evidence indicates that ERKs play an important role in integrating receptor tyrosine kinase-initiated mitogenic signaling events in many cell types (8, 9, 13). Because thrombin, a G protein-coupled receptor agonist, requires protein tyrosine kinase activity for its mitogenic effects in VSMCs, we have previously studied the role of ERKs in the transmission of thrombin-induced protein tyrosine phosphorylation events to cause nuclear effects in these cells. We observed a dissociation between ERKs activation and induced protein tyrosine phosphorylation events leading to DNA synthesis in VSMCs in response to thrombin (7). To understand the role of MAPKs in thrombin-induced growth in VSMCs, we have studied here the effect of cAMP on thrombin-induced DNA synthesis and ERKs, JNK1, and p38 groups of MAPKs activation in these cells. Earlier reports from several laboratories have demonstrated that cAMP inhibits the mitogenic effects of PDGF and EGF by blocking the ERK pathway at the Raf-1 level in many cell types (25, 26). In the present study, we report that forskolin and cAMP, two agents that increase intracellular cAMP levels, significantly inhibit thrombin-stimulated DNA synthesis in VSMCs without compromising the effect of thrombin on ERKs activation and c-Fos expression. On the other hand, both thrombin-induced activation of JNK1 and expression of c-j un demonstrated significantly blunted effects by the above agents in these cells. Forskolin also decreased thrombin-induced AP-1 activity. These findings thus show a correlation...
Thrombin Activates JNK1 in Smooth Muscle Cells

EXPERIMENTAL PROCEDURES

Materials—Aprotinin, ATP, bovine myelin basic protein (MBP), dADP, EGTA, forskolin, β-glycerophosphate, leupeptin, phenylmethylsulfonylfluoride (PMSF), sodium deoxycholate, sodium fluoride, sodium pyrophosphate, and sodium vanadate were obtained from Sigma (St. Louis, MO). Anti-ERK1, ERK2, c-Fos, JNK1, and p38 MAP kinase polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-c-Jun rabbit polyclonal antibody was purchased from Oncogene Science (Uniondale, NY). AP-1 consensus doubled-stranded oligonucleotide and T4 polynucleotide kinase were purchased from Promega (Madison, WI). [methyl-3H]Thymidine (70 Ci/mmol) and [γ-32P]ATP (8000 Ci/mmol) were obtained from Du Pont NEN.

Cell Culture—VSMCs were isolated from the thoracic aortae of 200–250-g male Sprague-Dawley rats by enzymatic digestion as described earlier (7). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere.

DNA Synthesis—VSMCs were plated onto 60-mm dishes, allowed to grow to 70–80% confluence, and then treated with 10% (v/v) heat-inactivated calf serum for 72 h. Growth-arrested VSMCs were exposed to thrombin (0.1 unit/ml) in the presence and absence of various concentrations of forskolin and washed once with phosphate-buffered saline. Cells were then harvested by scraping into Eppendorf tubes in 1 ml of phosphate-buffered saline. Cells were pelleted by centrifugation at 3500 rpm for 5 min at 4°C, and nuclear extracts were prepared according to the method described by Dignam et al. (27). In brief, the cell pellet was suspended in 50 μl of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and repelleted by centrifugation as described above. The nuclei were resuspended in 80 μl of buffer A containing 0.1% (w/v) Triton X-100 and incubated on ice for 10 min. Nuclei were pelleted by centrifugation as described above. The nuclei pellet was then suspended in 50 μl of buffer C (20 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and incubated for 30 min on ice. Cell debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C, and the protein concentration of the nuclear extract was determined as described above. Nuclear extracts were stored at −70°C. Protein-DNA complexes were formed by incubating 5 μg of nuclear protein in a total volume of 20 μl consisting of 15 mM Hepes, pH 7.9, 3 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 4.5 μg of bovine serum albumin, 2 μg of poly(dI-dC), 15% glycerol, and 100,000 cpm of [32P]-labeled oligonucleotide probe for 20 min at 30°C. Protein-DNA complexes were resolved on a 4% polyacrylamide gel using 0.25 TBE buffer (1× TBE = 50 mM Tris, 190 mM Na2HPO4, 2.5 mM EDTA), double-stranded oligonucleotide (AP-1, 5′-CGGCTTGATGAGTCGCGGAA-3′) was labeled with [γ-32P]ATP using a T4 polynucleotide kinase kit per the supplier’s protocol (Promega). Unincorporated nucleotides were removed by chromatography in a G-25 spin column (Bio-Rad).

RESULTS AND DISCUSSION

To determine whether forskolin and dADP inhibit thrombin-induced growth in VSMCs, growth-arrested VSMCs were treated with and without thrombin (0.1 unit/ml) in the presence and absence of forskolin (10 μM) or dADP (1 mM) for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation into trichloroacetic acid-precipitable material. Thrombin stimulated VSMC DNA synthesis 12-fold compared to untreated cells, and this response was significantly blunted by both forskolin and dADP (Fig. 1). Forskolin inhibited thrombin-induced VSMC DNA synthesis in a dose-dependent manner with 40 and 100% inhibition at 1 and 10 μM concentration, respectively (data not shown). Earlier studies from other laboratories have demonstrated that forskolin and other agents such as prostaglandin E2, which increase intracellular cAMP levels, antagonize the mitogenic effects of PDGF and EGF by inhibiting the ERK pathway at Raf-1 level in various cell types including human arterial smooth muscle cells (25, 26). Raf-1 is an upstream serine/threonine kinase, the activity of which is required for phosphorylation and activation of MEK1 and
MEK2 (13–16). MEKs (also called MKKs) are a group of dual specificity enzymes and activate, with differential specificities, ERKs, JNKs, and p38 groups of MAPKs by phosphorylating threonine and tyrosine residues (13, 15, 28). To investigate whether forskolin and dcAMP decrease thrombin-induced VSMC growth by a mechanism similar to that reported for receptor tyrosine kinase agonists (25, 26), growth-arrested VSMCs were treated with and without thrombin (0.1 unit/ml) for various periods of time in the presence and absence of forskolin (10 μM) or dcAMP (1 mM), and cell lysates were prepared. ERKs activities in the cell lysates were determined by in-gel kinase assay using MBP as a substrate. As shown in Fig. 2, thrombin activated ERKs in a biphasic manner with a first and highest peak of activity at 5 min (20-fold), followed by a second and more sustained lower peak of activity at 2 h (5-fold). Forskolin and dcAMP, surprisingly, had no effect on thrombin activation of ERKs (Fig. 2), a result that indicates that forskolin and dcAMP inhibit thrombin-induced growth by interfering with a mitogenic signaling event that is distinct from the ERKs.

Recent studies have shown that G protein-coupled receptor agonists, such as carbachol and angiotensin II, potently activate JNKs in fibroblasts and epithelial cells, respectively (29, 30). This suggests further that JNKs play an important role in signaling events elicited by G protein-coupled receptor agonists in these cells. To determine if thrombin activates JNKs and their role in the mitogenic effect of thrombin, growth-arrested VSMCs were treated with and without thrombin (0.1 unit/ml) for various periods of time as well as in the presence and absence of forskolin (10 μM), and cell lysates were prepared. JNK1 activity in the cell lysates was determined by an immunocomplex kinase assay using recombinant glutathione S-transferase-c-Jun as a substrate. Thrombin activated JNK1 in a time-dependent manner (Fig. 3). Thrombin activation of J NK1 was observed at 5 min, reached maximum (7-fold) by 10 min, and dropped almost to basal levels by 1 h (Fig. 3). Interestingly, thrombin-induced activation of JNK1 was significantly blocked by forskolin (Fig. 3). To find out whether thrombin activates the p38 group of MAPKs, and if so, its responsiveness to elevation of intracellular cAMP levels, growth-arrested VSMCs were treated with and without thrombin (0.1 unit/ml) in the presence and absence of forskolin (10 μM) for various time periods, and cell lysates were prepared. Cell lysates containing equal amounts of protein (500 μg) from each condition were immunoprecipitated with anti-p38 antibodies, and the immunoprecipitates were subjected to in-gel kinase assay using MBP as a substrate. In comparison to levels in untreated cells, no significant changes were observed in p38 MAPK activities in response to thrombin in the presence or absence of forskolin (data not shown).

ERKs via phosphorylation activate transcriptional factors such as ternary complex factor/Elk1 (17, 18), whereas JNK1 activates c-Jun/AP-1 and ATF2 (22). The role of these transcriptional factors in regulating the expression of c-Fos and c-Jun in many cell types in response to a wide variety of stimuli has been documented (13, 31). To relate the activation of ERKs and JNK1 to c-Fos and c-Jun expression by thrombin, growth-arrested VSMCs were treated with and without thrombin (0.1 unit/ml) for various periods of time as well as in the presence and absence of forskolin (10 μM) or dcAMP (1 mM). Cell extracts were prepared, and equal amounts of protein (40 μg) from each condition were analyzed for c-Fos and c-Jun by Western blotting using appropriate antibodies. Thrombin stimulated expression of c-Fos and c-Jun in a time-dependent manner in VSMCs (Fig. 4). Induced expression of both c-Fos (50-fold) and c-Jun (15-fold) occurred at 2 h and persisted for at least 4 h. In addition, whereas forskolin and dcAMP had no effect on thrombin-induced c-Fos expression, both these agents significantly attenuated the stimulation of c-Jun expression by thrombin.
To test whether decreased expression of c-Jun also results in reduced AP-1 activity, growth-arrested VSMCs were treated with and without thrombin (0.1 unit/ml) for 2 h in the presence and absence of forskolin (10 μM) or dCAMP (1 mM). Cell extracts were prepared and analyzed for c-Fos and c-Jun proteins as described in the legend to Fig. 4. These results were reproduced in three separate experiments.

The important findings of this study are that: 1) thrombin activates JNK1 in growth-arrested VSMCs; and 2) this response is linked to the mitogenic effect of thrombin in these cells. The involvement of JNK1 in the mitogenic signaling...
events of thrombin in VSMCs is supported by our finding that agents that increase cAMP levels blocked both JNK1 activation and DNA synthesis induced by thrombin. A variety of growth stimulants, including the receptor tyrosine kinase agonists PDGF and EGF, have been shown to preferentially activate the ERK pathway in many cell types (13–16, 23). In addition, several studies have shown that cAMP abrogates PDGF- and EGF-induced growth in smooth muscle cells and fibroblasts, respectively, by inhibiting the ERK pathway at the Raf-1 level (25–26). On the other hand, our results indicate that thrombin, a G protein-coupled receptor agonist, potently activates both ERKs and JNK1. Nonetheless, forskolin inhibited only thrombin-stimulated JNK1 activation but not ERKs activation.

These findings, along with others, suggest that cAMP inhibits receptor tyrosine kinase- and G protein-coupled receptor-mediated growth via different mechanisms. It is possible that cAMP attenuates the receptor tyrosine kinase- and G protein-coupled receptor-mediated DNA synthesis by activating different protein kinase A isozymes: one for antagonizing Raf-1 activity and the other for inhibiting the MEK kinase activity. MEK kinase is a serine/threonine kinase and activates SEK/JNK in the JNK pathway (16, 20). SEK/JNK, in turn, by phosphorylating threonine and tyrosine residues, activates stress-activated protein kinases (19, 24, 32, 33). At least two types of protein kinase A isozymes, types I and II, have been isolated, and differential activation of these isozymes in response to different hormones has been demonstrated (34, 35). In this regard, it is important to note that type I, but not type II, protein kinase A isoform activation inhibited T-cell activation by inhibiting JNK1 activity but not ERKs activity (37). These observations are consistent with our present findings. Future studies should address whether thrombin activates the upstream kinases of JNK1, i.e. SEK/JNK, and MEK kinase in the JNK pathway and at what level cAMP blocks this pathway to inhibit growth. It is also possible that cAMP inhibition of VSMC growth is in part due to its effects on additional as yet undiscovered or unstudied kinase cascades.

c-Fos and c-jun dimerize to form the transcriptional factor AP-1 (31, 38, 39). c-jun and its related family of proteins (J un-B and J un-D) alone by homodimerization can form AP-1, whereas c-fos and its related family of proteins (Fos-B, FRA-1, and FRA-2) cannot (31, 38–40). AP-1 plays an important role in cell proliferation and differentiation (31, 38, 39). Since thrombin induced expression of the AP-1 constituents, c-Fos and c-jun, in growth-arrested VSMCs, it is likely that this transcriptional factor is an important mediator of the growth-related nuclear events of thrombin in these cells. In fact, our finding that inhibition of thrombin-induced VSMC DNA synthesis by cAMP, preceded by down-regulation of c-jun and AP-1 activity, supports a role for this transcriptional factor in the mitogenic signaling events of thrombin in VSMCs. A similar dose-dependent effect of forskolin on inhibition of AP-1 activity and DNA synthesis in VSMCs also suggests that the latter two events are related. Although AP-1 activity can be modulated by increased expression of its constituents, c-Fos and c-jun, postranslational modifications such as phosphorylation of these proteins also play a significant role in the regulation of AP-1 in response to a wide variety of growth stimuli (31, 38, 39). For example, in response to both growth factors and cytokines, JNKs are activated (23, 33, 41), and in turn, these modulate gene expression by phosphorylating and activating c-jun transactivating activity (19, 24). The role of c-jun in its autoregulation has been demonstrated (31). These findings, along with the fact that cAMP-inhibited JNK1 activation was associated with decreased expression of c-jun in thrombin-treated VSMCs, suggest that JNK1 mediates thrombin-inhibited c-jun expression in these cells. Several studies have demonstrated that in response to a variety of growth factors, ERKs phosphorylate and activate ternary complex factor/EIk1 (17, 18, 42–44), which, in turn, by association with serum response factor binds to the serum response element and induces c-Fos transcription (43, 45). Although cAMP did not block thrombin-stimulated ERKs activation and c-Fos expression in VSMCs, it remains to be studied whether ERKs play any role in thrombin-induced c-Fos expression and DNA synthesis in these cells. Nevertheless, the present findings show differential mechanisms of regulation of expression of c-Fos and c-jun by thrombin in growth-arrested VSMCs. In addition, these results also show a correlation between JNK1 activation, c-jun expression, AP-1 activity, and growth in VSMCs in response to this agonist.
Thrombin Activates JNK1 in Smooth Muscle Cells

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