The Seven-transmembrane-spanning Receptors for Endothelin and Thrombin Cause Proliferation of Airway Smooth Muscle Cells and Activation of the Extracellular Regulated Kinase and c-J un NH2-terminal Kinase Groups of Mitogen-activated Protein Kinases*

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In airway smooth muscle cells ligand binding to the seven-transmembrane endothelin and thrombin receptors stimulates cell growth. Rapid activation of the extracellular regulated kinase 2 and c-J un NH2-terminal kinase groups of mitogen-activated protein kinases was also observed. The results demonstrate a novel mechanism of seven-transmembrane receptor signaling involving activation of the J un kinase pathway. Receptor coupling to J un kinase activation may involve heterotrimeric G proteins since the kinase was enzymatically activated in cells treated with aluminum fluoride. The activity of Raf-1, measured by immune complex kinase assay, revealed that platelet-derived growth factor and phorbol 12-myristate 13-acetate both stimulated Raf-1 activity, while thrombin and endothelin did not appreciably stimulate Raf-1. The data suggest that endothelin and thrombin stimulate Raf-1-independent mechanisms of mitogen-activated protein kinase activation. Endothelin- or thrombin-induced activation of mitogen-activated protein kinases was significantly inhibited by activation of cyclic AMP-dependent protein kinase by forskolin. Proliferation of airway smooth muscle cells, measured by incorporation of [3H]thymidine into DNA, was also greatly attenuated by forskolin.

The endothelin and thrombin receptors are members of the seven-transmembrane receptor superfamily that are thought to transduce mitogenic information by coupling to G proteins. Airway smooth muscle (ASM)1 cells treated with endothelin and thrombin are known to activate second messenger systems, including increases in cytosolic calcium, phospholipase C stimulation, and activation of protein kinase C (PKC) (1–4). However, the intracellular molecular circuitry that transduces the mitogenic information from these receptors is not completely defined.

The MAP kinases, or ERKs, are 42- and 44-kilodalton serine/threonine kinases encoded by the ERK2 and ERK1 genes, respectively, that are enzymatically activated by tyrosine and threonine phosphorylation in response to numerous different stimuli (5–8). The ERKs are phosphorylated and activated by a dual specificity tyrosine/threonine kinase, MKK1, also called MAP or ERK Kinase (MEK) (9–12). In addition to MKK1, a second MEK isoform, MKK2, has been isolated that shares a high degree of amino acid identity with MKK1 (13). MEK is catalytically activated by phosphorylation on serines 218 and 222 by the serine kinase Raf-1 (14–16). Raf-1 is currently thought to be the primary physiological activator of MEK, although in adipocytes treated with insulin or PMA, a MEK kinase distinct from Raf-1 appears to be activated within 20 s and inactivated in 30 s (17).

The activation of the MAP kinases appears to be a common requirement for agents that induce cell growth and differentiation. For example, PC12 cell differentiation is blocked by a dominant negative MEK mutant in which both serine phosphorylation sites have been substituted with alanine (18). Furthermore, activating mutants of MEK in which the serine phosphorylation sites have been substituted with acidic residues results in constitutive kinase activation and cell transformation (19). Inhibition of MAP kinase synthesis by antisense oligonucleotides depletes MAP kinase protein and blocks the ability of insulin or serum to stimulate DNA synthesis (20).

Agents that induce cellular stress, such as UV light and tumor necrosis factor α (TNF-α), activate the J NK protein kinases. The J NKs are distant relatives of the MAP kinases and were initially identified as c-J un NH2-terminal kinases (J NK) based on their phosphorylation and activation of c-J un (21–23). More recently the J NKs have also been found to phosphorylate and activate the ATF2 transcription factor (24). The human J NK1 and J NK2 genes encode 46- and 55-kDa kinases, respectively, that are distantly related to the MAP kinases and are similarly activated by phosphorylation on a single threonine and single tyrosine residue. The regulatory phosphorylation sequence of the ERKs and J NKs resides within subdomain VIII of the catalytic domain, a region thought to be involved in the regulation of many protein kinases (25, 26). The J NK regulatory sequence is Thr-Pro-Tyr, while the ERK phosphorylation sequence is Thr-Glu-Tyr (21). MKK4 is a recently identified human protein kinase with homology to MKK1 and MKK2 that activates J NK1 by phosphorylation of the Thr-Pro-Tyr sequence (27). SEK1 is the murine homolog of MKK4 that also activates J NK1 (28). Kyriakis et al. (29) have identified the rat homologs of the J NKs, referred to as stress-activated protein kinases. The J NKs may also be activated by tyrosine kinase growth factors such as epidermal growth factor in a
Ras-dependent mechanism (30).

While JNK activation is known to follow stimulation of the TNF-α receptor, as well as tyrosine kinase receptors such as the epidermal growth factor receptor, this report demonstrates that seven-transmembrane receptors also activate the JNK subgroup of MAP kinases. Activation of these kinases is inhibited by forskolin, which increases the enzymatic activity of cyclic AMP-dependent protein kinase (PKA). Furthermore, the ability of endothelin and thrombin to induce the proliferation of ASM cells is inhibited by activating PKA, suggesting that activation of the ERK and JNK kinase cascades is an essential component of the proliferative effects of endothelin and thrombin.

MATERIALS AND METHODS

Cell Culture—Rat tracheal smooth muscle cells were isolated according to Absher et al. (31).

Immunoprecipitation and Kinase Assays—To immunoprecipitate ERK2, the cells from a 60-mm plate were scraped into 0.4 ml of Nonidet P-40 immunoprecipitation buffer (1% Nonidet P-40, 10 mM Hepes, pH 7.5, 2 mM EDTA, 0.1% mercaptoethanol, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, and 0.5 mM microcystin). The lysates were clarified by centrifugation at 15,000 rpm for 10 min. Rabbit anti-ERK2 polyclonal antibody (C-14, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the clarified supernatants at 1:100 dilution. The immune complexes were incubated on ice for 1 h followed by incubation with 25 μl of protein A-agarose. The washed immune complexes were incubated in 25 μl of KB (20 mM Hepes, pH 7.5, 2 mM dithiothreitol, and 5 mM MgCl₂), 5 μCi of [³²P]ATP, and 2 μg of GST-Myc for 20 min at 30 °C.

For Raf-1 immunoprecipitation 100-mm plates were serum-starved overnight and scraped into 0.6 ml of radiolimmunoprecipitation (RIPA) buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% aprotinin, 2 mM sodium orthovanadate, and 0.5 mM microcystin). Rabbit anti-Raf-1 polyclonal antibody (C-12, Santa Cruz Biotechnology) was added to the clarified lysate at a 1:100 dilution and incubated on ice for 2 h. The washed immune complexes were incubated in 25 μl of KB, 5 μCi of [³²P]ATP, and 0.5 μg of recombinant kinase-inactive MKK1 (32) at 30 °C for 20 min.

The activity of JNK1 was assayed by immunoprecipitation with JNK1 antiserum or by affinity purification with a GST-J un(1–79) fusion protein. To immunoprecipitate JNK1, cell lysates were prepared in radiolimmunoprecipitation precipitation buffer. A rabbit polyclonal antibody raised against JNK1 (24) was added to the clarified lysates at a 1:100 dilution. The washed immune complexes were incubated with 25 μl of KB, 5 μCi of [³²P]ATP, and 2 μg GST-J un(1–79) for 20 min at 30 °C. To affinity purify JNK1 with GST-J un, cell lysates were prepared in EB (25 mM Hepes, pH 7.5, 0.3 mM NaCl, 5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 mM microcystin, and 0.2 mM sodium orthovanadate). The clarified lysates were diluted 1:2 in BB (20 mM Hepes, pH 7.5, 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 mM microcystin, and 0.2 mM orthovanadate), 20 μg of GST-J un(1–79) was added, and the complexes were incubated on ice for 2 h. The washed complexes were resuspended in 30 μl of KB and 5 μCi of [³²P]ATP for 20 min at 30 °C. The phosphorylation of each substrate protein was quantitated using densitometry. The data are expressed relative to the unstimulated control sample in each experiment.

DNA Synthesis—ASM cells were grown to confluence in 24-well culture dishes. The medium was changed to Dulbecco’s modified Eagle’s medium plus 0.5% fetal bovine serum for 72 h, after which the agonists were added for an additional 18–20 h in the presence of 1 μCi/ml [³H]thymidine. The cells were washed with Versene, trypsinized, and lysed in the presence of 10% trichloroacetic acid. The lysates were applied to glass microfiber filters (GF/B, Whatman), washed, and counted. Radioactivity was expressed as disintegrations/min/well. Each condition is expressed as the average of four wells.

RESULTS

Endothelin and Thrombin Activate ERK2 in Airway Smooth Muscle—The effects of endothelin and thrombin on airway smooth muscle cells include activation of phospholipase C and PKC (4). To determine if these receptors also activate the MAP kinase pathway, the catalytic activity of the ERK2 MAP kinase was assayed by immune complex kinase assay. Serum-starved ASM cells treated with PDGF (15 ng/ml), endothelin (200 nm), thrombin (40 nm), or PMA (1 μM) resulted in a large increase in ERK2 catalytic activity, as determined by phosphorylation of the MAP kinase substrate GST-Myc (Fig. 1B). Western blotting of the immunoprecipitates revealed that the samples with increased catalytic activity contained phosphorylated ERK2 (Fig. 1A, upper band). ERK2 migrates more slowly on SDS gels following phosphorylation of the regulatory tyrosine or threonine residues (10).

Pretreatment of the cells with forskolin, an agent that increases the catalytic activity of PKA, inhibited the ability of endothelin, thrombin, and PMA to activate the ERK2 MAP kinase (Fig. 1A). The ability of PDGF to activate the MAP kinase pathway appears to be independent of the activity of PKA (Fig. 1C). Endothelin and Thrombin Activate JNK1 in Airway Smooth Muscle—Activation of the JNKs occurs in cells treated with agents that induce cellular stress such as UV light, TNF-α, anisomycin, and cycloheximide. Mitogenic agonists such as epidermal growth factor have also been reported to activate the JNKs (30). The ability of endothelin or thrombin to activate the JNKs was assayed in airway smooth muscle cells. JNK1 binds to the amino terminus of the c-j un(1–79) protein and allows affinity purification of the kinase by a GST-J un fusion protein immobilized on glutathione-Sepharose. ASM cells treated with endothelin, thrombin, or TNF-α resulted in rapid activation of JNK activity, as determined by the phosphorylation of a GST-J un(1–79) fusion protein (Fig. 2). The observed phosphorylation occurred on the J un portion of the fusion protein, as there was no phosphorylation of GST alone detected in parallel experiments (not shown). Maximal activation of J un kinase was observed in the range of 10–100 nm endothelin or thrombin (Fig. 2A). The kinetics of J un kinase activation by these seven-transmembrane receptors was rapid, with the maximal activation observed at 15 min. The catalytic activity of the kinase returned to baseline by 30 min (Fig. 2B). To evaluate the impact of PKA on the J un kinase pathway, ASM cells were treated with TNF-α, endothelin, or thrombin following activa-
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**FIG. 2. Activation of the J NKs in airway smooth muscle cells.**
A, dose response of J NK activation in endothelin- or thrombin-treated ASM cells. Serum-starved ASM cells were treated with increasing doses of endothelin or thrombin. J NK activity was measured in GST-J un complex by measuring the incorporation of [32P]ATP into the GST-J un substrate as described under “Materials and Methods.” Each point is the mean of two replicates. B, time course of J NK activation in endothelin-treated ASM cells. J NK activity was measured as described for A. The kinetics of thrombin activation of J NK were similar to endothelin. In both cases maximal activation was at 15 min. C, activation of J NK by seven-transmembrane receptors and inhibition by forskolin. Serum-starved quiescent ASM cells were treated with or without 10 μM forskolin for 15 min prior to stimulation with TNF-α (10 ng/ml), endothelin (200 nM), or thrombin (40 nM) for 15 min. J NK enzymatic activity was measured by phosphorylation of the GST-J un fusion protein as described under “Materials and Methods.” An autoradiogram of the phosphorylated Jun fusion protein is shown. TNF-α, endothelin, and thrombin activate the J NKs; pretreatment with forskolin blocks agonist-induced activation of the J NKs. The intensities of the GST-J un bands are expressed relative to the unstimulated control. Lane 1, 1; lane 2, 2.1; lane 3, 2.4; lane 4, 3.6; lane 5, 0.6; lane 6, 1.6; lane 7, 0.8; lane 8, 0.8.

Activation of PKA with forskolin (Fig. 2C). Activation of PKA prior to agonist treatment significantly decreased the ability of TNF-α, endothelin, and thrombin to activate J NK1. To confirm that the observed kinase activation was due to J NKs, J NK1 was immunoprecipitated with a J NK1 specific antibody (24), and its catalytic activity was measured by immune complex kinase assay using GST-J un (1–79) as a substrate. The catalytic activity of J NK1 was increased significantly by TNF-α, endothelin, and thrombin, as measured by the ability of J NK1 to phosphorylate a GST-J un (1–79) fusion protein (Fig. 3).

Seven-transmembrane receptors bind to and activate heterotrimeric GTP-binding proteins, or G proteins, which subsequently interact with downstream effector molecules. Activation of G proteins can be mimicked pharmacologically using aluminum fluoride (AlF₄⁻). Fluoroaluminate is thought to interact with GDP in the nucleotide-binding site of the G protein and mimic the γ phosphate of GTP, thereby activating the G protein. AlF₄⁻ has been used frequently in characterizing signaling pathways that employ G proteins (33). To determine if J NK activation may occur as a result of G protein activation, ASM cells were treated with 0.1, 1, and 5 mM AlF₄⁻ for 15 min prior to assaying the catalytic activity of the J NKs. Treatment of ASM cells with AlF₄⁻ activated the J NK pathway as indicated by increased J NK catalytic activity (Fig. 4).

Weak Activation of Raf-1 by Thrombin and Endothelin—The Raf-1 kinase is thought to be the primary mechanism for activating MEK by phosphorylation of Ser-218 and Ser-222 (14–16). Phosphorylated MEK in turn activates the MAP kinases (9, 10). To determine if the activation of ERK2 by thrombin and endothelin was mediated by the Raf-1 kinase, Raf-1 MEK kinase activity was examined by immune complex kinase assay using a kinase-inactive mutant of MEK as an in vitro Raf-1 substrate. Treatment of airway smooth muscle cells with PDGF or PMA stimulated Raf-1 kinase activity. Thrombin treatment was less effective at stimulating Raf-1 activity, and endothelin did not appreciably stimulate Raf-1 activity (Fig. 5).

Activation of PKA by increasing cAMP has been reported to inhibit the ability of epidermal growth factor to activate Raf-1 and the ERKs (34). The effects of PKA activation in ASM cells were examined by activation of PKA prior to agonist treatment. Activation of PKA resulted in a decrease in the ability of PDGF and PMA to activate Raf-1, although the ability of PDGF to activate ERK2 was unaffected by PKA activation (Fig. 5).

Endothelin- or Thrombin-induced DNA Synthesis Is Inhibited by Forskolin—The effects of endothelin and thrombin on airway smooth muscle cell proliferation were determined by measuring DNA synthesis. Both endothelin and thrombin treatment of airway smooth muscle cells resulted in increased DNA synthesis as measured by [³H]thymidine incorporation (Fig. 6). Pretreatment of ASM cells with 10 μM forskolin (to activate PKA) inhibited the ability of endothelin and thrombin...
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Fig. 5. Raf-1 activity in agonist-stimulated ASM cells. ASM cells were pretreated with 10 μM forskolin and subsequently stimulated with PDGF (10 ng/ml), thrombin (40 nM), endothelin (200 nM), and PMA (1 μM). The Raf-1 kinase was immunoprecipitated, and MEK kinase activity was determined by immune complex kinase assay using kinase-inactive recombinant (KR) MEK as an in vitro substrate. PDGF-, PMA-, and thrombin-stimulated cells showed increased Raf-1 activity as measured by MEK phosphorylation. Endothelin resulted in little appreciable Raf-1 activation. The intensities of the GST-Jun bands are expressed relative to the unstimulated control. Lane 1, 1; lane 2, 16.8; lane 3, 10.5; lane 4, 8.0; lane 5, 17.1; lane 6, 3.0; lane 7, 11.1; lane 8, 0.8; lane 9, 0.3; lane 10, 17.3.

Fig. 6. Inhibition of endothelin- or thrombin-induced DNA synthesis by forskolin. DNA synthesis in agonist-stimulated ASM cells was measured by [3H]thymidine incorporation. Pretreatment with forskolin inhibited the ability of endothelin or thrombin to stimulate DNA synthesis. Each condition is an average of four replicates. The data are representative of three experiments. **, indicates statistical significance, p < 0.01.

DISCUSSION

In the present studies, we have determined that stimulation of the endothelin or thrombin receptors in airway smooth muscle cells results in activation of both the ERKs and JNKs. Taken together the data suggest that activation of the seven-transmembrane endothelin and thrombin receptors mediates their proliferative effects via coupling to the ERK and JNK pathways. Whether both of these kinase pathways contribute positive input to cell growth has yet to be determined.

The upstream kinases that couple the endothelin or thrombin receptors to activation of the ERK and JNK pathways are not defined at this point. Endothelin and thrombin receptor activation stimulates phospholipase activity and results in increased diacylglycerol levels (37, 38) as well as activation of PKC (4, 39). PKC is reported to phosphorylate and activate Raf-1 (40), suggesting a possible PKC-dependent mechanism of ERK activation. However, the ability of PKC to activate Raf-1 is controversial since PKC phosphorylation of Raf-1 appears to preferentially stimulate its autokinase activity rather than its Raf-1 kinase activity (41). Our results in airway smooth muscle cells indicate that endothelin is a very poor activator of Raf-1 catalytic activity (but a strong activator of the ERKs and JNKs), suggesting that these agonists employ Raf-1-independent mechanisms of activating the ERKs. Furthermore, our experiments indicate that in ASM cells the ERK and JNK activator(s) stimulated by endothelin or thrombin appears to be inhibited by PKA.

Dissecting the effects of cellular cAMP on cell growth has been enigmatic since cAMP appears to be growth stimulatory in some cells, such as 3T3, and growth inhibitory in other cell types, such as T cells and Src-transformed cell lines (42, 43). Several reports indicate that the PKA-induced inhibition of the ERKs is mediated at least in part by phosphorylation of Raf-1. For instance, agonists that activate PKA, such as forskolin and dibutyryl cAMP, inhibit growth factor-induced activation of Raf-1 and the MAP kinases (34, 44). PKA phosphorylates Raf-1 on Ser-43, and this phosphorylation inhibits the Raf-1 autokinase activity, suggesting that these agonists employ Raf-1-independent mechanisms of activating the ERKs. Interestingly, activation of ERK2 in response to PDGF does not appear to be inhibited by activation of PKA (Fig. 2), suggesting that seven-transmembrane receptors and tyrosine kinase growth factor receptors couple to the MAP kinase pathway via distinct mechanisms in airway smooth muscle cells. The inhibition of the ERKs and JNKs by forskolin correlates with an inhibition in endothelin- or thrombin-induced mitogenesis, suggesting that these kinases may regulate the mitogenic effects of these agonists. Although forskolin has striking effects on MAP kinase activation, PKA activation has pleiotropic effects on cell growth that are poorly understood, making it difficult to establish causality between inhibition of the ERKs or JNKs and inhibition of mitogenesis.

While the mechanism of inhibition of the ERKs and JNKs by PKA in ASM cells is currently unknown, there are interesting parallels with another seven-transmembrane receptor, the β-adrenergic receptor, which is desensitized by phosphorylation (47). Perhaps PKA-induced receptor phosphorylation inhibits seven-transmembrane coupling to the MAP kinase path-
way. The mechanism of seven-transmembrane receptor activation of these MAP kinases is an area of intense study.

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