Mechanisms of 5-Hydroxytryptamine2A Receptor Activation of the Mitogen-Activated Protein Kinase Pathway in Vascular Smooth Muscle

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ABSTRACT
5-Hydroxytryptamine (5-HT) activates the extracellular signal-regulated kinase (Erk) mitogen-activated protein kinases (MAPKs) in the vasculature, resulting in contraction. The mechanisms by which this occurs are unclear. G protein-coupled receptors can activate Erk MAPK pathways through a variety of mechanisms, including stimulation of Src, phosphoinositide-3 kinase (PI-3-K), protein kinase C (PKC), or the epidermal growth factor (EGF) receptor tyrosine kinase. We hypothesize that 5-HT uses one or more of these pathways. In isolated strips of rat aorta, the MAPK/Erk kinase inhibitor U0126 (50 μM), Src inhibitor PP1 (0.5 μM), PKC inhibitors calphostin C (1 μM) and chelerythrine (10 μM), and the PI-3-K inhibitor LY294002 (1–20 μM) reduced 5-HT-induced contraction. The EGF receptor tyrosine kinase inhibitor AG1478 (0.25–1 μM) was without effect. Thus, 5-HT activates PKC, Src, and possibly PI-3-K to result in contraction. In rat aortic myocytes, 5-HT (1 μM) activated Erk MAPK proteins 2- to 3-fold over basal values; activation was reduced by U0126, PP1, and LY294002 and unaffected by calphostin C or chelerythrine, wortmannin, or AG1478. The lack of effect of EGF receptor tyrosine kinase and PI-3-K inhibitors was confirmed in that the EGF receptor immunoprecipitated from 5-HT-exposed cells did not display an increase in autophosphorylation, nor did 5-HT significantly increase activation of Akt/protein kinase B, a downstream substrate for PI-3-K. These data suggest that the rat aortic 5-HT2A receptor uses Src but not PKC, PI-3-K, or the EGF receptor tyrosine kinase in stimulating Erk MAPK activation.

5-Hydroxytryptamine (5-HT, serotonin) is an autacoid with a myriad of actions in the cardiovascular system. In large arteries isolated from the rat, 5-HT stimulates a 5-HT2A receptor-dependent contraction that is partially mediated by activation of mitogen-activated protein kinases (MAPKs), specifically the extracellular signal-regulated kinases (Erk MAPKs; Watts, 1996; Watts et al., 1996; Florian and Watts, 1998). Signaling pathways for the 5-HT2A receptor have classically included activation of phospholipase C (PLC) and plasma membrane calcium channels that are sensitive to inhibition by dihydropyridines. Studies in rat aortic smooth muscle cells indicate that as expected, it is also the 5-HT2A receptor that mediates 5-HT-stimulated phosphorylation and activation of the Erk MAPKs. Data from other laboratories support the ability of 5-HT to activate the Erk MAPK pathway in smooth muscle (Kelleher et al., 1995; Semenchuk and DiSalvo, 1995; Chopra et al., 1997). Activation of the Erk MAPK pathway by 5-HT in aortic tissue is independent of PLC and plasma membrane calcium channel activation (Florian and Watts, 1998). What is unknown is the mechanisms by which 5-HT can stimulate the Erk MAPK pathway.

We examined several of the mechanisms that have been described for activation of the Erk MAPK pathway via the stimulation of G protein-coupled receptors (Gutkind, 1998). These are not the only mechanisms that could be activated by G protein-coupled receptors but represent the most likely candidates because of what is understood about classic 5-HT2A receptor signaling and Erk MAPK activation by agonists of G protein-coupled receptors in smooth muscle. First, protein kinase C (PKC) can associate with the ras-raf-1 complex, an element of the Erk MAPK pathway (Marais et al., 1998; Schonwasser et al., 1998). Because 5-HT-induced contraction is clearly dependent on PKC, this is a reasonable pathway to investigate. Second, G protein-coupled receptors may activate phosphoinositide-3 kinase (PI-3-K) through pleckstrin homology associations via the βγ subunit of the G

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; EGF, epidermal growth factor; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; PI-3-K, phosphoinositide-3-kinase; PKB, protein kinase B; PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate; PLC, phospholipase C; PP1, 4-amino-5-(4-methylphenyl)-7-(1,3-dioxo-2-propenyl)[3,4-d]pyrimidine; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline and 0.1% Tween-20; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene.
protein and thereby activate the Erk MAPK pathway (Koch et al., 1994; Thomason et al., 1994; Lopez-Illasaca et al., 1997). Third, stimulation of G protein-coupled receptors has been associated with the transactivation of the epidermal growth factor (EGF) receptor tyrosine kinase, an event that leads to Erk MAPK activation (Schlessinger, 1993; Daub et al., 1996). Such transactivation has been described for muscarinic (Keely et al., 1998), angiostatin AT1 (Eguchi et al., 1998), endothelin (Daub et al., 1996; Iwasaki et al., 1998), purinergic (Soltoff, 1998), lysophosphatidic acid (Daub et al., 1996), thrombin (Daub et al., 1996), and arachidonic acid receptors (Dulin et al., 1998). Of particular note is the fact that transactivation of the EGF receptor tyrosine kinase through AT1 (Eguchi et al., 1998) and endothelin (Iwasaki et al., 1998) receptors occurs in rat aortic vascular smooth muscle cells. Finally, we investigated the role of Src in 5-HT-induced activation of Erk MAPKs because Src is necessary for angiostatin II activation of Erk MAPK proteins in aortic smooth muscle cells (Ishida et al., 1995, 1998).

We tested the hypothesis that one or more of these pathways mediates 5-HT-induced activation of the Erk MAPK activation. We used a pharmacological approach in parallel experiments in the isolated tissue bath and Western analyses to investigate the role of these pathways in 5-HT-induced activation of the Erk MAPK pathway in the rat aorta. Our previous findings in contractile experiments (whole aorta) corresponds well with those observed in Western analyses (aortic smooth muscle cells), so we use these two approaches together (Watts, 1996, Watts et al., 1996; Florian and Watts, 1998). We used pharmacological inhibitors of each of these pathways to modulate contraction and protein phosphorylation. U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) is a recently described inhibitor of mitogen-activated protein kinase kinase (MAPKK; Favata et al., 1998), and the Src family of tyrosine kinases can be inhibited by PP1 (Hanke et al., 1996; Schindler et al., 1999). Chelerythrine (Herbert et al., 1990) and calphostin C (Brus et al., 1991) are structurally distinct inhibitors of PKC inhibitor, whereas both LY294002 (Vlahos et al., 1994) and wortmannin (Arcaro and Wyman, 1993; Uii et al., 1995) are inhibitors of PI-3-K isoenzymes. Finally, AG1478 (Daub et al., 1996) and 4,5-dianilinophthalalimide (Buchdunger et al., 1994) are used as specific inhibitors of the EGF receptor tyrosine kinase. The findings of this pharmacological approach support the idea that the 5-HT2A receptor uses Src but not the other pathways to activate Erk MAPK proteins in aortic smooth muscle.

**Materials and Methods**

All animal procedures were in accordance with the institutional guidelines of Michigan State University.

**Isolated Tissue Bath Protocol.** Rats were euthanized (80 mg/kg pentobarbital i.p.), and thoracic aortae were removed. Arteries were dissected into helical strips (0.25 × 1 cm), and the endothelial cell layer was removed by rubbing the luminal side of the vessel with a moistened cotton swab. Tissues were placed in physiological salt solution for measurement of the isometric contractile force using standard bath procedures. The physiological salt solution contained 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH2PO4, 1.17 mM MgSO4 · 7H2O, 1.6 mM CaCl2 · 2H2O, 14.9 mM NaHCO3, 5.5 mM dextrose, and 0.05 mM CaNa2EDTA. One end of the preparation was attached to a stainless steel rod, and the other was attached to a force transducer (FT03; Grass Instruments, Quincy, MA) and placed under optimum resting tension (1500 mg, determined previously). Muscle baths were filled with warmed (37°C), aerated (95% O2/5% CO2) physiological salt solution. Changes in isometric force were recorded on a Grass polygraph (Grass Instruments). After a 1-h equilibration, arteries were challenged with phenylephrine (10–5 M). This contraction to phenylephrine within each experimental group was not different, and thus this response to phenylephrine was used to normalize contractile data. Tissues were washed, and the status of the endothelium was examined by observing arterial relaxation to the endothelium-dependent agonist acetylcholine (1 × 10–6 M) in tissues contracted by a half-maximal concentration of the α1-adrenergic receptor agonist phenylephrine (1 × 10–6 M). Tissues were then washed multiple times, and one of the following inhibitors was added for a 1-h incubation: vehicle (0.1–0.5% dimethyl sulfoxide), the MAPKK inhibitor U0126 (20–50 μM), Src inhibitor PP1 (0.5 μM), PKC inhibitor chelerythrine (10 μM) or calphostin C (1 μM), PI-3-K inhibitor wortmannin (0.05–1 μM) or LY294002 (1–20 μM), EGF receptor tyrosine kinase inhibitor 4,5-dianilinophthalalimide (100 nM to 10 μM), or the EGF receptor tyrosine kinase inhibitor AG1478 (250 nM to 1 μM). In some experiments, U0126 (50 μM) and nifedipine (50 nM) were both added to the bath. Experiments using wortmannin or nifedipine were performed in the dark because both compounds are light sensitive. Calphostin C was exposed to daylight for 45 min before use because the activation of calphostin C is light dependent (Brus et al., 1991). After this incubation, aortic strips were exposed to cumulative concentrations of 5-HT (10–9 to 10–5 M), KCl (6–100 mM), or phorbol-12,13-dibutyrate (PDBu; 10–9 to 10–5 M).

**Aortic Smooth Muscle Cell Experiments.** Vascular smooth muscle cells were derived from aorta of male Sprague-Dawley rats in an explant method previously described (Florian and Watts, 1998). Cells were plated onto P-100 plates and used when confluent between passages 2 and 9. With each new isolation, the cells were positively stained for smooth muscle α-actin (Sigma Chemical Co., St. Louis, MO; rat fibroblasts do not stain with this antibody.

**Aortic Smooth Muscle Cell Experiments.** Cells (P-100 plates) were switched to physiological salt solution (4 ml; see above) for 1 h before the addition of agonist. At this same time, inhibitors (as listed above and including okadaic acid) or vehicle (0.1–0.5% dimethyl sulfoxide) were added and equilibrated with tissues for 1 h. Each dish was incubated with one agonist concentration. A 5-min incubation was used for agonist incubation. Plates were placed on ice, and incubation buffer was aspirated. Plates were washed three times (4 ml/wash) with ice-cold PBS containing sodium orthovanadate as a tyrosine phosphatase inhibitor (10 mM sodium phosphate, 150 mM NaCl, 1 mM sodium orthovanadate, pH 7.0). Five hundred microliters of supplemented RIPA lysis buffer (50 mM Tris · HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate) were added to each dish, and cells were harvested with a rubber policeman. Lysate was centrifuged at 14,000g for 10 min at 4°C. Supernatant total protein was measured (Bio-Rad, Hercules, CA), and the supernatant was taken through either an immunoblotting or an immunoprecipitation protocol.

**Immunoblotting Protocol.** Supernatant (4:1 in denaturing loading buffer, boiled 5 min) was loaded, separated on 10% denaturing SDS-polyacrylamide gels, and transferred to Immobilon-P membranes. Membranes were blocked for 3 to 4 h in Tris-buffered saline plus Tween-20 (0.1%); TBS-T containing 4% chick egg ovalbumin and 0.025% sodium azide. Mouse phosphotyrosine antibody (1:7500, clone 4G10; Upstate Biotechnology Inc., Lake Placid, NY), rabbit Akt/protein kinase B (PKB), or rabbit phosphospecific Akt/PKB (1:1000; New England Biolabs, Beverly, MA) was incubated with blots overnight (4°C). After the washes, the appropriate secondary antibody linked to horseradish peroxidase (anti-mouse (1:7500, Amer sham Laboratories, Arlington Heights, IL) or anti-rabbit (1:2000, Zymed Laboratories, S. San Francisco, CA)) was added for 1 h and
incubated with blots at 4°C. Enhanced chemiluminescence was performed (Amersham Laboratories, Arlington Heights, IL). In some experiments, blots were stripped for re-probing with another antibody by incubating blots in Re-blot (Chemicon Co., Temecula, CA).

**Immunoprecipitation Protocol.** The EGF receptor was immunoprecipitated from lysate supernatant using standard techniques (1 μg/ml goat anti-epidermal growth factor receptor, protein A/G Sepharose beads; Santa Cruz Biotechnologies, Santa Cruz, CA). Beads were boiled in sample buffer (120 μl) for 5 min and centrifuged. The supernatant was loaded onto SDS-polyacrylamide gels and run as described above except that gels were 5% and transfer buffer contained 5% methanol. Blots were probed with a mouse phosphotyrosine primary antibody (1:7500; Upstate Biotechnology, Inc.) or the EGF receptor antibody (1 μg/ml; and the appropriate secondary was used (1:3000 for anti-goat secondary antibody for EGF receptor antibody; Zymed Laboratories, S. San Francisco, CA) and then processed using enhanced chemiluminescent techniques.

**Data Analysis.** Data from isolated tissue bath experiments are presented as mean ± S.E. as a percentage of the phenylephrine (10^{-5} M) contraction for the number of animals indicated in parentheses. Cell experiments were performed three or four times, with each repetition of the experiment being performed in cells in which ex- plants were derived from different animals. Thus, experiments are representative of responses of three or four different animals. Un- paired or paired Student’s *t* tests were used where appropriate in comparing two group responses, and ANOVA followed by a Tukey post hoc test was used when comparing responses of three or more groups (*p* < 0.05 was considered statistically significant). Agonist EC_{50} values were calculated using a nonlinear regression analysis using the algorithm [effect = maximum response/1 + (EC_{50}/agonist concentration)] and the computer program Prism (GraphPAD, San Diego, CA). Quantitation of band density was performed using the public domain NIH Image.

**Chemicals.** Solutions of compounds were prepared in deionized water unless indicated otherwise. Acetylcholine chloride, angioten- sin II, aprotinin, β-mercaptoethanol, bovine serum albumin, chick egg ovalbumin, EGTA, 5-hydroxytryptamine hydrochloride, leupep- tin, phenylephrine hydrochloride, sodium azide, sodium dodecyl sul- fate, sodium orthovanadate, Tris base, Tris · HCl, Triton X-100, and Tween-20, wortmannin (dimethyl sulfoxide) were obtained from Sigma Chemical Co. EGF (10 mM acetic acid plus 1 mg/ml bovine serum albumin) was obtained from Upstate Biotechnology Inc. So- lutions of all of the following compounds were made in dimethyl sulfoxide: AG1478, calphostin C, LY294002, PDBu, and PP1 (all obtained from BIOMOL, Plymouth Meeting, PA); chelerythrine chlori- de and 4,5-dianilinophthalimide (both obtained from Research Bio- chemicals Inc., Natick, MA); and U0126 (obtained from Promega, Madison, WI).

**Results**

**Validation of MAPKK Activation.** Figure 1 (top) displays the effects of the MAPKK inhibitor U0126 on 5-HT-induced contraction in the endothelium-denuded rat thoracic aorta. U0126 (50 μM) shifted and significantly reduced 5-HT-induced contraction. In cultured rat aortic smooth muscle cells, U0126 also virtually abolished 5-HT-stimulated ty- rosyl-phosphorylation of Erk-1 (44 kDa) and Erk-2 (42 kDa; Fig. 1, bottom). The concentration of 5-HT used in cell exper- iments (1 μM) is a maximal but not supramaximal concentra- tion for causing Erk MAPK tyrosyl-phosphorylation. Inter- estingly, this same concentration of U0126 reduced KCl-induced contraction (Fig. 1; middle), where KCl was used as an indirect activator of L-type voltage-gated calcium channels. However, U0126 was still capable of inhibiting 5-HT-induced contraction in the presence of L-type calcium chan-nel antagonist nifedipine (Fig. 1; 50 nM). This concentration of nifedipine was the minimal concentration that maximally inhibited KCl- and 5-HT-induced contraction (Florian and Watts, 1998).

**Involvement of PKC.** Calphostin C (1 μM), a PKC inhibitor used at a concentration documented to reduce PDBu- induced contraction (Fig. 2, top right), and Erk MAPK ty- rosyl-phosphorylation (Fig. 2, bottom right) significantly reduced 5-HT-induced contraction (Fig. 2, top left). However, the same concentration of calphostin C was unable to reduce 5-HT-stimulated tyrosyl-phosphorylation of Erk-2 (Fig 2, bottom); similar results were seen with another PKC inhibitor, chelerythrine (10 μM; data not shown). These data sug- gest that although activation of PKC is important for 5-HT- induced contraction, it is not important for activation of the Erk MAPK pathway.
Involvement of PI-3-K. The PI-3-kinase inhibitor LY294002 caused a modest concentration-dependent inhibition of 5-HT-induced contraction (Fig. 3). Similar contractile experiments with the structurally distinct PI-3-K inhibitor wortmannin (Arcaro and Wyman, 1993; Ui et al., 1995) were also performed, but because of the ability of wortmannin (0.25–1 μM) to irreversibly inhibit myosin light chain kinase and thereby abolish 5-HT-induced contraction, these data are difficult to interpret and are not shown. In cell experiments, only the higher concentration of LY294002 significantly inhibited 5-HT-stimulated tyrosyl-phosphorylation of Erk-1 (44 kDa) and Erk-2 (42 kDa). Numbers above each lane indicate the arbitrary densitometry units for the 42-kDa protein. Blots represent one of four such experiments.

Figure 2. Top, effect of PKC inhibitor calphostin C (1 μM) on 5-HT- (left) and PDBu- (right) induced contraction in the endothelium-denuded rat thoracic aorta. Points represent mean values and vertical bars represent the S.E. for the number of animals indicated in parentheses. PE, phenylephrine. * statistical difference from response in vehicle-incubated tissues. Bottom, blot of the effect of the same concentration of calphostin C (1 μM) on 5-HT- (1 μM; left) and PDBu- (100 nM; right) stimulated tyrosyl-phosphorylation of Erk-1 (44 kDa) and Erk-2 (42 kDa). Numbers above each lane indicate the arbitrary densitometry units for the 42-kDa protein. Blots represent one of four such experiments.

Figure 3. Top, effect of LY294002 (1–20 μM), a reversible PI-3-K inhibitor, on 5-HT-induced contraction in the endothelium-denuded rat thoracic aorta. Points represent mean values and vertical bars represent the S.E. for the number of animals indicated in parentheses. PE, phenylephrine. * statistical difference from response in vehicle-incubated tissues. Blot of the effect of LY294002 (middle; 1, 20 μM) or wortmannin (bottom; 0.25–1 μM) on 5-HT (1 μM)-stimulated tyrosyl-phosphorylation of Erk-1 (44 kDa) and Erk-2 (42 kDa). Numbers above each lane indicate the arbitrary densitometry units for the 42-kDa protein. ND, not detectable. Blots represent one of four such experiments.

PKB phosphorylation. All concentrations of wortmannin tested (0.050–1 μM) abolished EGF-stimulated Akt/PKB phosphorylation. 5-HT (10^{-9}–10^{-5} M) did not appreciably activate Akt/PKB (Fig. 5); only a slightly retarded mobility of Akt/PKB in cells exposed to a high (10 μM) concentration of 5-HT was observed. The phosphospecific antibody was specific for phosphorylated Akt/PKB as this antibody did not recognize a nonphosphorylated Akt/PKB protein (Fig. 5, top blot). To be sure that phosphorylation of Akt/PKB was a modifiable event in these cells, we tested the ability of EGF and okadaic acid to enhance Akt/PKB phosphorylation. EGF caused a significant increase in band density and retarded mobility of Akt/PKB (Fig. 5, bottom blot). Comparably, 5-HT (10^{-9}–10^{-5} M) had a negligible effect on phosphorylation, even in the presence of the phosphatase 1 and 2A inhibitor okadaic acid (Fig. 5, top blot). Okadaic acid itself significantly
increased phosphorylation and activation of Akt/PKB. Collectively, these data suggest that 5-HT does not appreciably activate PI-3-K.

Involvement of EGF Receptor. Two approaches were taken to examine the role of the EGF receptor tyrosine kinase in 5-HT-induced stimulation of the Erk MAPK pathway.

First, in concentrations at and above those documented to reduce EGF receptor tyrosine kinase activity in vascular smooth muscle cells, the EGF receptor tyrosine kinase inhibitor AG1478 did not shift or reduce 5-HT-induced aortic con-
tachment (Fig. 6, top). Similar results were seen with another EGF receptor tyrosine kinase inhibitor, 4,5-dianilinophthal-alamide (data not shown). AG1478 (1, 10 μM) appeared to alter the absolute maximal tyrosyl-phosphorylation of the Erk MAPKs stimulated by 5-HT (1 μM; Fig. 6, bottom), but with compared to basal levels, the fold stimulation of tyrosyl-phosphorylation caused by 5-HT was not inhibited by AG1478.

Second, we immunoprecipitated the EGF receptor from aortic smooth muscle cells treated with 5-HT and determined whether 5-HT can increase the tyrosyl-phosphorylation of the EGF receptor. EGF (10 nM) stimulated tyrosyl-phosphorylation of the EGF receptor (Fig. 7, second to last lane, top blot), whereas 5-HT (10^{-8}–10^{-5} M) did not stimulate detectable tyrosyl-phosphorylation of the EGF receptor. Lack of tyrosyl-phosphorylation did not occur because the EGF receptor was not appropriately immunoprecipitated in these samples; the bottom blot of Fig. 7 shows the top blot stripped and reprobed with an EGF receptor antibody. Lysate from HER cells that overexpress the EGF receptor was used as a positive control for EGF receptor identification. The reverse experiment (immunoprecipitate with phosphotyrosine and probe for EGF receptor) was performed and similar results were observed; 5-HT does not stimulate tyrosyl-phosphorylation of the EGF receptor in rat aortic smooth muscle cells. Thus, transactivation of the EGF receptor tyrosine kinase by 5-HT cannot be included as a mechanism for 5-HT-induced stimulation of the Erk MAPK pathway.

Involvement of Src. PP1 (0.5 μM) was used as an inhibitor of Src/Src-like tyrosine kinases (Hanke et al., 1996; Schindler et al., 1999). PP1 did not alter the KCl (6–100 mM)-induced aortic contraction (n = 6) but rightward shifted contraction to 5-HT (Fig. 8, top). PP1 also significantly reduced 5-HT-stimulated Erk MAPK tyrosyl-phosphorylation (Fig. 8, bottom). Not shown are gels demonstrating the ability of PP1 to abolish angiotensin II (100 nM)-stimulated tyrosyl-phosphorylation of Erk MAPKs, an event established as Src dependent (Ishida et al., 1996, 1998). Although indirect proof, we show in Fig. 8 (top right) data that are consistent with the ability of 5-HT to activate Src. In aortic smooth muscle cells, 5-HT stimulated an increase in tyrosyl-phosphorylation of a protein or proteins approximately 60 kDa in mass. This band set exactly comigrates with that activated by angiotensin II (100 nM; Fig. 8, top right), a known activator of Src in vascular cells (Ishida et al., 1996, 1998). Thus, although indirect, this experiment provides preliminary evidence that 5-HT can activate Src with an ultimate increase in Erk MAPK activity.

Discussion

These experiments were undertaken to determine whether activation of Src, PKC, PI-3-K, or the EGF receptor tyrosine kinase was a mechanism by which 5-HT activates the Erk MAPK pathway in aortic smooth muscle. Activation of this biochemical pathway was confirmed by using a newly available MAPKK-specific inhibitor, U0126. U0126 is a butadiene derivative specific for MEK1/MEK2 with little activity against PKC, Erk MAPKs, or JNK MAPKs (Favata et al., 1998). At this time, little is known about the effects of U0126 on elements important to smooth muscle contractility. U0126 (50 μM) reduced 5-HT-stimulated tyrosyl-phosphorylation and aortic contraction, agreeing with previous findings using an inhibitor of MAPKK activation, PD98059 (Dudley et al., 1995; Watts, 1996). It is unlikely that inhibition of 5-HT-induced contraction by U0126 can be attributed to calcium channel blockade in that U0126 reduced 5-HT-induced contraction in the presence of nifedipine; higher concentrations of nifedipine do not further reduce 5-HT-induced aortic contraction (Florian and Watts, 1998). The Erk MAPK pathway appears to participate in smooth muscle contraction by inhibiting the activation of caldesmon (Adam et al., 1989), thereby disinhibiting actin-myosin ATPase, or possibly by Erk MAPK proteins through direct phosphorylation of myosin light chain (Jin et al., 1996).

The 5-HT_{2A} receptor has the structure of a G protein-coupled receptor and is coupled to pertussis toxin-insensitive G_{αi} (Roth et al., 1998). We have not determined whether this is the actual G protein used for signal transduction in the rat aortic smooth muscle cells because tools to selectively block the interaction of the 5-HT_{2A} receptor and G protein are not readily available. Nevertheless, it is a reasonable assumption that the 5-HT receptor in the rat aorta is coupled to a G protein. We first examined the ability of PKC, as activated by 5-HT, to stimulate the Erk MAPK pathway. PKC has been reported to activate the c-rat-ras complex (Marais et al., 1998; Schönwasser et al., 1998), a kinase complex that precedes MAPKK in the Erk MAPK pathway. PKC is clearly important to 5-HT-induced contraction (Fig. 2), and this is consistent with the coupling of the 5-HT_{2A} receptor with

![Fig. 7. Inability of 5-HT to stimulate tyrosyl-phosphorylation of the EGF receptor in cultured aortic smooth muscle cells. Top, immunoprecipitation performed using an antibody against the EGF receptor; blot probed with phosphotyrosine antibody. Numbers above blot are arbitrary densitometry units for the EGF receptor band, ND, not detectable. Bottom, same blot as above but stripped and reprobed with EGF receptor antibody to validate that similar amounts of EGF receptor were immunoprecipitated in each lane. Blots are representative of three such experiments.](image-url)
phospholipase C, one metabolic product of which (diacylglycerol) can activate PKC. It does not appear that PKC activation is important for Erk-MAPK phosphorylation because the PKC inhibitors calphostin C and chelerythrine were ineffective in reducing 5-HT-stimulated tyrosyl-phosphorylation of the Erk MAPKs. This agrees with previous results from our laboratory in which the inhibition of PLC, activation of which can precede PKC stimulation, reduced contraction to 5-HT but did not reduce 5-HT-stimulated Erk MAPK tyrosyl-phosphorylation (Florian and Watts, 1998). Moreover, the general tyrosine kinase inhibitor genistein and PD098059, an inhibitor of MAPKK activation, both significantly shifted and reduced 5-HT-induced aortic contraction in the presence of PLC inhibition (Florian and Watts, 1998). These experiments underscore that the Erk MAPK pathway is not sequential with a PLC-dependent pathway and that PKC, at least isozymes sensitive to blockade by calphostin C and chelerythrine, is not a mediator of 5-HT-stimulated activation of the Erk MAPK pathway.

The next mechanism examined, transactivation of the EGF receptor tyrosine kinase, also is one that does not appear to be stimulated by 5-HT to activate the Erk MAPK pathway. The EGF receptor possesses an intrinsic tyrosine kinase that, once activated, uses adaptor proteins to activate the Erk MAPK pathway (Schlessinger, 1993). In vascular smooth muscle cells, transactivation of the Erk MAPK pathway via the EGF receptor tyrosine kinase has been reported for angiotensin AT_1 (Eguchi et al., 1998) and endothelin ET_A (Iwasaki et al., 1998) receptors. Similar to the 5-HT_2A receptor, the AT_1 and ET_A receptor have been associated with G_q proteins. Thus, transactivation of the EGF receptor in vascular smooth muscle cells by 5-HT is a mechanism that is reasonable to investigate. Only supramaximal concentrations of AG1478 reduced 5-HT-stimulated tyrosyl-phosphorylation. These findings, in combination with the observation that EGF but not 5-HT induced activation of the EGF receptor tyrosine kinases in smooth muscle cells, suggest that 5-HT does not activate the EGF receptor to stimulate the Erk MAPK pathway.

Another mechanism investigated was the involvement of the PI-3-K pathway in 5-HT-stimulated activation of the Erk MAPK pathway. PI-3-K is activated in smooth muscle by...
both growth factors and G protein-coupled receptors (Walker et al., 1998), and Akt/PKB phosphorylation and activation has been described as generally being induced universally on PI-3-K activation (Cooker et al., 1998). Two inhibitors of PI-3-K, LY294002 and wortmannin, inhibited 5-HT-induced contraction but to different magnitudes. The qualitatively different findings with these inhibitors can be attributed to several factors. First, there may be isoform specificity with respect to inhibition of PI-3-K. Three major PI-3-K classes exist (I–III; Fruman et al., 1998). The PI-3-K isoform preferentially activated by βγ subunits of heterotrimeric G proteins is the γ form; this protein has been reported as sensitive to inhibition by the concentrations of LY294002 and wortmannin tested here (Fruman et al., 1998). The dramatic inhibitory effects of wortmannin can be attributed to its irreversible inhibition of myosin light chain kinase (Nakanishi et al., 1992). Reduction in 5-HT-induced contraction and Erk MAPK tyrosyl phosphorylation by the same concentration of LY294002 is less readily explained, especially in light of the fact that 5-HT did not appreciably increase tyrosyl-phosphorylation of Akt/PKB, a downstream substrate of PI-3-K. This was true even when phosphatases 1 and 2A were inhibited by okadaic acid. One possible explanation for the inhibition of contraction by LY294002 is that it may be a 5-HT2A receptor antagonist in that the structural nucleus of both LY294002 and the classic 5-HT2A receptor antagonist ketanserin are similar. To our knowledge, however, there have been no reports to this effect, and it is important to note such a potential nonspecificity. Nevertheless, the lack of an observable increase in Akt/PKB activation by 5-HT in an optimized system suggests that 5-HT does not activate PI-3-K in vascular smooth muscle cells.

Recently, it has been reported that phosphorylation and internalization of the 5-HT1A receptor (transfected) were necessary for the Erk MAPK pathway to be activated (Cowen et al., 1996; Della Rocca et al., 1999). Important to this process is the association of an adaptor protein with the tyrosine kinase Src. We have found that Src, or a Src-like tyrosine kinase such as Lck and Fyn, is an important player in 5-HT2A receptor-mediated Erk MAPK activation because the Src family inhibitor PP1 (Hanke et al., 1996; Schindler et al., 1999) shifted aortic contraction to 5-HT and reduced 5-HT-stimulated Erk MAPK tyrosyl-phosphorylation. It will be interesting to discover whether the 5-HT2A receptor, like the 5-HT1A receptor, requires internalization and association with Src for activation of the Erk MAPK pathway and whether activation of Src is the only mechanism necessary for Erk MAPK activation. Because 5-HT is a vascular mitogen, it is important to understand the mechanisms by which the Erk MAPK pathway is activated because this may provide insight into mechanisms that underlie the abnormal vascular smooth muscle growth and contractility commonly observed in cardiovascular diseases such as hypertension and atherosclerosis.

In summary, these studies combining a functional and cellular approach indicate that in rat aortic vascular smooth muscle, the 5-HT2A receptor activates the Erk MAPK pathway through at least Src but does not do so through stimulation of PKC, the EGF receptor tyrosine kinase, or PI-3-K.

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