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Integration of Mitogen-Activated Protein Kinase Kinase Activation in Vascular 5-Hydroxytryptamine$_{2A}$ Receptor Signal Transduction[1]

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ABSTRACT

Vascular 5-Hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) receptor signaling and contraction has been associated with the activation of L-type calcium channels, phospholipase C (PLC) and, as we previously demonstrated, tyrosine kinase activation. We hypothesize the 5-HT$_{2A}$ receptor activates all three pathways independently to elicit contraction and that one of the tyrosine kinases activated by 5-HT is mitogen-activated protein kinase kinase (MEK). Endothelium-denuded rat thoracic aorta was mounted into isolated tissue baths for measurement of isometric contractile force. 5-HT, α-methyl-5-HT and 2,5-dimethoxy-4-iodoamphetamine all contracted the rat aorta, whereas the 5-HT$_{2A}$ receptor antagonist ketanserin (30 nM) blocked contraction to 5-HT. The tyrosine kinase inhibitor genistein (5 μM) shifted contraction to 5-HT, α-methyl-5-HT and DOI ~10-fold to the right, whereas daidzein (5 μM), the inactive isomer of genistein, was unable to shift 5-HT-induced contraction. PD098059 (10 μM), an inhibitor of MEK, shifted contraction to 5-HT ~7-fold to the right. We next examined the integration of tyrosine kinase activation in 5-HT$_{2A}$ receptor signaling. 5-HT-induced contraction was reduced individually by the PLC inhibitor 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC; 100 μM) or the Ca^{++} channel inhibitor nifedipine (500 nM); the remaining response to 5-HT was reduced by further addition of either genistein or PD098059. When nifedipine and NCDC were used in combination, a part of the contraction to 5-HT remained; this contraction was further reduced by genistein or PD098059. In cultured aortic smooth muscle cells, 5-HT (0.01–100 μM) stimulated tyrosyl-phosphorylation of 42- and 44-kDa proteins identified as Erk MAPKs; this phosphorylation was reduced by PD098059 (10 μM). Neither nifedipine nor NCDC reduced 5-HT (1 μM)-stimulated Erk MAPK tyrosyl-phosphorylation, but the combination of nifedipine, NCDC and PD098059 abolished 5-HT (1 μM)-stimulated Erk MAPK tyrosyl-phosphorylation. Taken together, these studies indicate that stimulation of a vascular 5-HT$_{2A}$ receptor activates Ca^{++} channels and PLC as well as MEK to cause rat aortic contraction and that MEK activation is at least partially independent of the two pathways classically associated with 5-HT$_{2A}$ receptors.

Previously, the signaling pathway for smooth muscle contraction was considered largely separate from pathways leading to cell growth. Growth involved growth factor-induced activation of receptor and nonreceptor tyrosine kinases and activation of the MAPK pathway (Jin *et al*., 1996), whereas smooth muscle contraction was largely associated with agonist-stimulated G protein activation of the myosin light chain kinase pathway. Recent reports have suggested that signaling pathways for growth and contraction can interact to modulate vascular function. For example, growth factors like platelet-derived growth factor and epidermal growth factor induce vascular contraction (Berk *et al*., 1985, 1986), whereas hormones like angiotensin II and serotonin (5-HT), both agonists of G protein-coupled receptors, stimulate protein tyrosyl-phosphorylation (a measure of tyrosine kinase activity) and vascular smooth muscle cell growth (Lee *et al*., 1994b; Molloy *et al*., 1993; Nemecek *et al*., 1986).

Interest in the ability of 5-HT to activate a tyrosine kinase-dependent pathway stems from the fact that 5-HT produces hyperplasia and hypertrophy of smooth muscle cells (Lee *et al*., 1994b; Nemecek *et al*., 1986). 5-HT can also potentiate the mitogenic effects of other mitogenic compounds (Lee *et al*., 1991, 1994a; Nemecek *et al*., 1986) as well as augment contractile responses to other agonists (Van Nueten *et al*., 1981). 5-HT causes vasoconstriction of rat aorta through activation of 5-HT$_2$ receptors (Cohen *et al*., 1981). The

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ABBREVIATIONS: DMEM, Dulbecco's modified Eagle's medium; DOI, 2,5-dimethoxy-4-iodoamphetamine; Erk, extracellular signal-regulated kinase; ANOVA, analysis of variance; GAP, GTPase-activating factor; GRF, GTP-releasing factor; 5-HT, 5-hydroxytryptamine; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NCDC, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate; PE, phenylephrine; PH, pleckstrin homology; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; PLC, phospholipase C; SDS, sodium dodecyl sulfate; SH, src homology; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline + Tween.

5-HT_{2A} receptor is G protein linked to PLC and L-type voltage-gated Ca^{++} channels (Nakaki *et al*., 1985). Recently, we demonstrated that 5-HT-induced rat arterial contraction can be inhibited by the tyrosine kinase inhibitor genistein, indicating that 5-HT-induced contraction is also dependent on the activation of tyrosine kinases (Watts *et al*., 1996; Watts, 1996). Initial studies using Western blot analysis suggested that 5-HT can activate tyrosine kinases because 5-HT has been shown to stimulate tyrosyl-phosphorylation of rasGAP (Semenchuk and DiSalvo, 1995) and of the MAP kinases in smooth muscle (Kelleher *et al*., 1995; Watts *et al*., 1996; Watts, 1996).

The best characterized members of the MAP kinase superfamily of protein kinases are the extracellular signal-regulated protein kinases (Erk/MAP kinases, or Erks; p42 and p44 kDa) (Cobb and Goldsmith, 1995). The Erk/MAP kinases are involved in cell growth and can be activated by many different stimuli; these proteins are activated when phosphorylated on both tyrosine and threonine residues. Activation of the MAP kinase pathway ultimately results in the phosphorylation of transcription factors required for cell growth (Blenis, 1993). The protein directly responsible for Erk/MAP kinase phosphorylation is MAPK kinase/Erk kinase, or MEK (Zheng and Guan, 1993). Recently, a tyrosine kinase inhibitor was developed that is specific for MEK. PD098059 (Dudley *et al*., 1995) is able to reduce 5-HT-induced contraction in the rat aorta, indicating that the MAP kinase pathway, and specifically MEK, plays an active role in 5-HT-induced contraction (Watts, 1996).

Although it is clear that 5-HT stimulates PLC, Ca^{++} channels and tyrosine kinases (MEK) to result in contraction, it is presently unclear how these signaling mechanisms interact to cause contraction. We hypothesize that tyrosine kinase activation is somewhat independent of the signaling pathways classically associated with 5-HT-induced contraction in the rat aorta. We use both contractile and Western techniques to explore the ability of the 5-HT_{2A} receptor to activate a tyrosine kinase-dependent pathway and the manner in which these three important signaling elements—PLC, Ca^{++} channels and tyrosine kinase(s)/MEK—interact to cause 5-HT-induced vascular contraction.

Methods

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

Isolated Tissue Bath Protocol

Rats were killed (80 mg/kg pentobarbital i.p.), and thoracic aorta was 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 buffer for measurement of isometric contractile force using standard bath procedures. Physiological salt solution contained (in mM) NaCl 130, KCl 4.7, KH_2PO_4 1.18, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2 \cdot 2H_2O$ 1.6, $NaHCO_3$ 14.9, dextrose 5.5 and $CaNa_2EDTA$ 0.03. One end of the preparation was attached to a stainless steel rod, the other was attached to a force transducer (FT03; Grass Instruments, Quincy, MA), and the strip was placed under optimum resting tension (1500 mg, as determined previously). Muscle baths were filled with warmed (37°C) and aerated (95% O_2/5% CO_2) physiological salt solution. Changes in isometric force were recorded on a Grass polygraph (Grass Instruments, Quincy, MA). After 1-hr equilibration, arteries were challenged with phenylephrine (10 μM). Tissues were washed, and the status of the endothelium was examined by observing arterial relaxation to the endothelium-dependent agonist acetylcholine (1 μM) in tissues contracted by a half-maximal concentration of the *alpha*-1 adrenergic receptor agonist phenylephrine (~10 nM). The endothelium was functionally disrupted; ≤8% relaxation of the phenylephrine contraction was observed in any tissue. Tissues were then washed multiple times, and one of the following experimental protocols was followed.

Protocol 1: Determination of optimum nifedipine concentration. To determine the concentration of nifedipine to use in investigation of 5-HT_{2A} receptor signaling, multiple concentrations (10, 30, 50, 100, 300 and 1000 nM; 1-hr incubation in the dark) of nifedipine were tested against a cumulative concentration response curve to KCl (6–100 mM). KCl was used as a stimulus for L-type voltage-gated calcium channels, and only one concentration of nifedipine was examined for each tissue.

Protocol 2: Determination of optimum NCDC concentration. The concentration of NCDC used (100 μM) in these experiments was determined by the facts that this concentration (1) produced the maximal inhibition on 5-HT-induced aortic contraction and (2) completely inhibited 5-HT (100 μM)-stimulated inositol monophosphate accumulation in the rat aorta (Turla and Webb, 1990).

Protocol 3: Identification of L-type calcium channel-, PLC- and tyrosine kinase/MEK-dependent portion of 5-HT-induced aortic contraction. In fresh tissue, either vehicle, nifedipine (50 nM and 1 μM), NCDC (100 μM), genistein (5 μM) or PD098059 (10 μM) was incubated with tissues for 1 hr before conduction of a cumulative 5-HT concentration-response curve. All experiments with PD098059 or nifedipine were performed in the dark.

Protocol 4: Integration of L-type calcium channels, PLC and tyrosine kinases in 5-HT receptor signaling. In fresh tissue, either a combination of appropriate vehicles, nifedipine plus genistein, NCDC plus genisein, nifedipine plus NCDC or nifedipine plus NCDC plus genistein/PD098059 were incubated with tissues for 1 hr before conduction of a cumulative 5-HT concentration-response curve.

Culturing of Aortic Smooth Muscle Cells

Vascular smooth muscle cells were derived from the aorta of male Sprague-Dawley rats. Aortae were excised in an aseptic manner, cleaned of debris and endothelium and cut into small (2 × 2 mm) squares. These pieces of tissue were placed lumen side down in a P-60 Corning Culture dishes (Corning, NY) and layered with just enough serum-enriched media to keep the tissues moist [medium consisted of DMEM with D-glucose (4500 mg/liter), L-glutamine (1%) and HEPES buffer (25 mM) (GIBCO Life Technologies, Gaithersburg, MD) containing fetal bovine serum (40% v/v; Hyclone Laboratories, Logan, UT) and streptomycin (100 mg/ml)/penicillin (100 units/ml) (GIBCO Life Technologies)]. Plates were placed in a 5% CO_2 warming incubator. Once the tissues had attached to the plate (~18 hr), additional medium was added to the dish. After ~1 week, a sufficient number of cells had migrated from the tissue to obtain confluency. Cells were trypsinized, seeded into T75 flasks and fed with normal serum (10%) DMEM. 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, St. Louis, MO); cultured rat fibroblasts did not stain with this antibody.

Vascular Smooth Muscle Cell Experiments

Cells (P-100 plates) were switched to modified physiological salt solution (see above) for 1 hr before the addition of agonist (final volume, 4 ml). At this same time, antagonists or vehicle was added and equilibrated with tissues for 1 hr. Examination of cells after 1 hr indicated that cells do not lift off and are not destroyed in the presence of either vehicle or test compounds (data not shown). Each

dish was incubated with one agonist concentration. A 5-min incubation was used for 5-HT because we established previously this as the necessary time for maximal stimulation of Erk MAPK protein tyrosyl-phosphorylation (Watts *et al.*, 1996). After this incubation, plates were placed on ice, and the incubation buffer was aspirated. Cells were washed three times (4 ml/wash) with phosphate-buffered saline containing sodium vanadate as a tyrosine phosphatase inhibitor (10 mM sodium phosphate, 150 mM NaCl and 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 PMSF, 10 μg/μl aprotinin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate) was added to each dish, and cells were lysed with a rubber policeman. Lysate was transferred into 1.5-ml centrifuge tubes and centrifuged at 14,000 $\times$ *g* for 10 min at 4°C. The supernatant was aspirated from the pellet of cellular debris.

Western Blot Analyses

Lysate (4:1 in denaturing loading buffer, boiled 5 min) was loaded and separated on a 10% denaturing SDS-polyacrylamide gel (20-cm gels; constant current at 11 mA for ~16 hr at 4°C). Proteins were electrically transferred to prepared Immobilon-P membrane (100 V, constant voltage for 1 hr, 4°C). Transfer of rainbow molecular weight standards separated along with the lysate samples indicated the success of transfer. After transfer of proteins, gels were stained with Coomassie Brilliant Blue to allow us to view the consistency of protein loading between lanes. Immobilon-P membranes were then blocked for 3 to 4 hr in TBS-T (0.1%) containing 4% chick egg ovalbumin and 0.025% sodium azide. Mouse phosphotyrosine antibody (1:7500, clone 4G10; Upstate Biotechnology, Lake Placid, NY), mouse Erk MAPK antibody (1:5000; Zymed Laboratories, San Francisco, CA) or rabbit activated Erk MAPK antibody (1:3000; New England Biolabs, Beverly, MA) incubated with blots overnight (4°C). Blots were washed three times with TBS-T (30, 5 and 5 min) and once with TBS (5 min). Anti-mouse (1:7500; Amersham Laboratories, Arlington Heights, IL) or anti-rabbit (1:3000; Zymed Laboratories, San Francisco, CA) antibody linked to horseradish peroxidase was added for 1 hr and incubated with blots at 4°C. Blots were washed using the same protocol as after the first antibody incubation. Enhanced chemiluminescence using Amersham reagents was performed on the blots to visualize antibody-labeled bands. In some experiments, blots were stripped for reprobing with another antibody. Blots were immersed in a solution of 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris · HCl, pH 6.7, at 50°C for 30 min with agitation. Blots were washed (twice for 10 min in TBS-T), reblocked and probed as described above.

Data Analysis

Contractility data are presented as mean ± S.E.M. as a percentage of the PE (10^{-5} M) contraction for the number of animals indicated in parentheses. Unpaired or paired Student's *t* tests were used where appropriate in comparing the responses of two groups, and analysis of variance followed by a Tukey *post hoc* test was used when comparing the responses of three or more groups ($P < .05$ 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)]. Apparent antagonist dissociation constants (K_B values) were determined according to the following equation:

$$K_B = \frac{[B]}{[\text{Dose ratio} - 1]}$$

where B is the antagonist concentration, and the dose ratio is the EC_{50} value of 5-HT in the presence of antagonist divided by the control EC_{50} of 5-HT (Arunlakshana and Schild, 1959). K_B values are reported as the mean of the negative logarithm (pK_B) of individual pK_B values. Quantitation of band density was performed on a PowerMac 8100 computer using the public domain NIH Image program.[2]

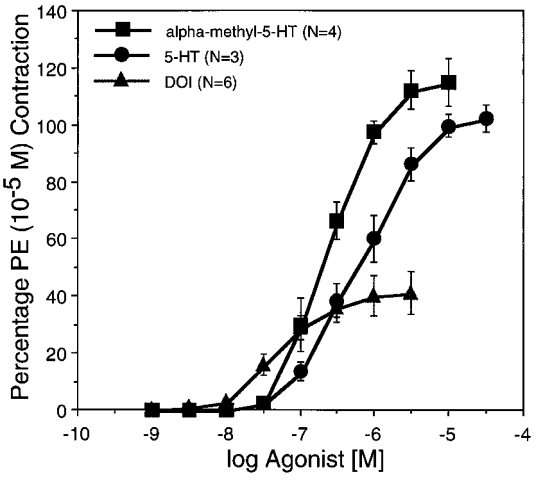

Fig. 1. Concentration-dependent contraction of endothelium-denuded rat aorta to 5-HT and the 5-HT_2 receptor agonists α-methyl-5-HT and DOI. Points represent mean ± S.E.M. for the number of animals (in parentheses).

Chemicals

Compounds made with deionized water (unless indicated otherwise in parentheses) included acetylcholine chloride, aprotinin, β-mercaptoethanol, chick egg ovalbumin, EGTA, 5-HT hydrochloride, leupeptin, nifedipine, NCDC, phenylephrine hydrochloride, sodium azide, sodium dodecyl sulfate, sodium orthovanadate, Tris base, Tris · HCl, Triton X-100, Tween-20 (Sigma Chemical, St. Louis, MO); α-methyl-5-HT, daidzein (dimethylsulfoxide), DOI, genistein (dimethylsulfoxide), ketanserin tartrate (dimethylsulfoxide) (Research Biochemicals, Natick, MA) and Coomassie Brilliant Blue (Bio-Rad, Hercules, CA). PD098059 was a kind gift from Dr. David Dudley at Parke Davis (Ann Arbor, MI).

Results

In all experiments, aortic strips were denuded of endothelium; this was documented by observing a lack of relaxation to acetylcholine in phenylephrine (EC_{50})-contracted tissues. Strips that had no more than an 8% relaxation to acetylcholine were used in the following experiments.

Validation of 5-HT_{2A} Receptor Mediation of 5-HT-Induced Contraction in Aorta

Two pharmacological approaches were used to validate the receptor in the aorta as a 5-HT_{2A} receptor. First, 5-HT, the 5-HT_2 receptor full agonist α-methyl-5-HT and the 5-HT_2 receptor partial agonist DOI were examined for contractile activity in the aorta. Figure 1 demonstrates that 5-HT and α-methyl-5-HT were similarly efficacious, with α-methyl-5-HT being slightly more potent. Control $-\log EC_{50}$ values were 6.19 ± 0.07 M for 5-HT and 6.56 ± 0.11 M for α-methyl-5-HT. DOI was a partial agonist with a statistically greater potency (7.25 ± 0.07 M) than that for both 5-HT and α-methyl-5-HT. These findings suggest that a 5-HT_2 receptor mediates 5-HT-induced contraction in the rat aorta. The 5-HT_{2A}

[2] Written by Wayne Rasband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Road, Springfield, VA 22161, part number PB93-504868.

receptor antagonist ketanserin (30 nM) acted as a competitive antagonist against 5-HT with an apparent antagonist dissociation constant ($-\log K_B = 8.70 \pm 0.01$ M), which is consistent with interaction with the 5-HT$_{2A}$ receptor subtype in the aorta (data not shown).

In some tissues, 5-HT has been observed to stimulate *alpha*-1 adrenergic receptors. To ensure that the responses we later studied were distinctly addressing serotonergic receptor and not adrenergic receptor signaling, we examined the effects of the *alpha*-1 adrenergic receptor antagonist prazosin against 5-HT in the rat aorta. Prazosin (100 nM), in a concentration that completely eliminated aortic contraction to the *alpha*-1 adrenergic agonist phenylephrine, did not shift or reduce 5-HT-induced contraction (data not shown), indicating that 5-HT does not interact with *alpha*-1 adrenergic receptors to cause aortic contraction. Thus, our conclusions can be addressed specifically to the 5-HT$_{2A}$ receptor.

Effect of Tyrosine Kinase Inhibitors on 5-HT-Induced Contraction in Aorta

Having established that a 5-HT$_{2A}$ receptor mediates contraction to 5-HT in the aorta, we next examined the ability of the general tyrosine kinase inhibitor genistein (5 μM) to reduce 5-HT-stimulated contraction. If 5-HT$_{2A}$ receptors are linked to tyrosine kinase activation, then genistein should reduce not only 5-HT-induced vascular contraction but also contraction stimulated by the 5-HT$_2$ receptor agonists α-methyl-5-HT and DOI. Figure 2 depicts the results of these experiments. Genistein (5 μM) shifted 5-HT-induced contraction to all three agonists, ~10-fold rightward, providing evidence that the 5-HT$_{2A}$ receptor is coupled to a tyrosine kinase. The presence of genistein significantly affected the potency of these three agonists with calculated $-\log EC_{50}$ values determined to be 6.05 ± 0.14 M (DOI), 5.61 ± 0.05 M (α-methyl-5-HT) and 5.49 ± 0.09 M (5-HT). One of the tyrosine kinases stimulated by activation of the 5-HT$_{2A}$ receptor is MEK; the MEK inhibitor PD098059 (10 μM) shifted and reduced aortic contraction to 5-HT (fig. 3). It should be noted that 10 μM PD098059 maximally inhibited 5-HT-induced contraction; further increases in the concentration of PD098059 do not further reduce contraction to 5-HT (table 1). Thus, these experiments link tyrosine kinase activation, in particular that of MEK, to 5-HT$_{2A}$ receptor signaling.

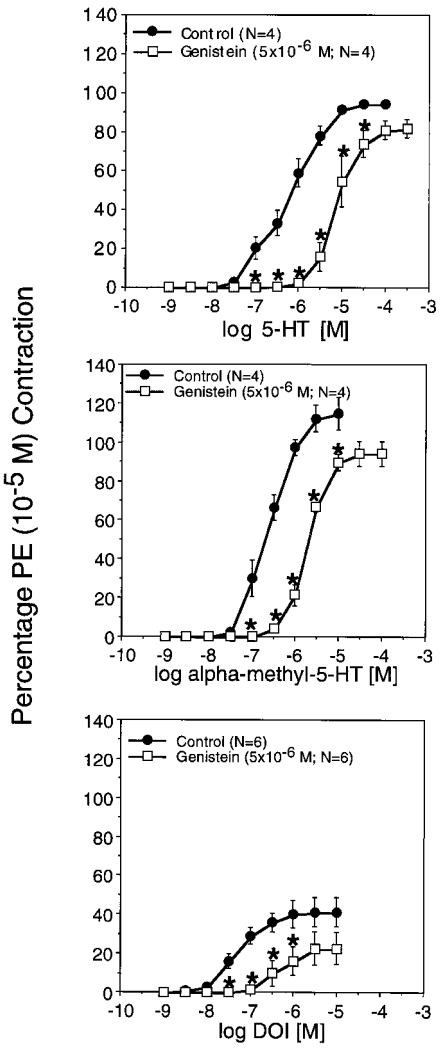

Fig. 2. Effect of tyrosine kinase inhibitor genistein (5×10^{-6} M) on endothelium-denuded rat aortic contraction induced by 5-HT (top), α-methyl-5-HT (middle) and DOI (bottom). Points represent mean ± S.E.M. for the number of animals (in parentheses). *Statistically significant ($P < .05$) between responses of control and genistein-incubated tissues.

Stimulation of Protein Tyrosyl-Phosphorylation by 5-HT in Rat Aortic Vascular Smooth Muscle Cells

The contractile experiments above demonstrate that 5-HT activates tyrosine kinase(s), including MEK, to result in vascular contraction. We next used a more direct measure of tyrosine kinase activation by investigating changes in levels of protein tyrosyl-phosphorylation caused by 5-HT. These experiments were performed in smooth muscle cells cultured from the thoracic aorta, the same vessel used in the contractile experiments.

Cultured cells were incubated with various concentrations of 5-HT for 5 min, and the lysate of cells was separated and transferred to Immobilon-P membrane. This membrane was probed with an antibody against phosphotyrosine and visualized with a horseradish peroxidase-linked secondary antibody. Figure 4 (left gel) shows a picture of the resultant blots from one such experiment. 5-HT caused a concentration-dependent increase in tyrosyl-phosphorylation (top left) of proteins that comigrated with 42- and 44-kDa proteins identified by an antibody against Erk MAPKs (bottom left). We next used an antibody against activated MAPK, in which (middle left) it was shown that 5-HT caused a concentration-dependent increase in the amount of activated MAPK protein. As another control measure, phosphorylated (activated) and unphosphorylated MAPK proteins were added to the last two lanes to ensure we were in fact examining the effect of 5-HT on MAPK phosphorylation. The tyrosine kinase responsible for tyrosyl-phosphorylation of the Erk MAPKs is MEK; thus, in agreement with the contractile data given above, these Western data suggest that 5-HT activates MEK. This finding is further supported by the fact that the increase in 5-HT-stimulated tyrosyl-phosphorylation of Erk MAPK (compare lanes 1 and 2) can be significantly reduced by the MEK inhibitor PD098059 (compare lanes 3 and 4, 10 μM, fig. 4, right) used at the same concentration that inhibited 5-HT-induced contraction. In addition, basal phosphorylation of

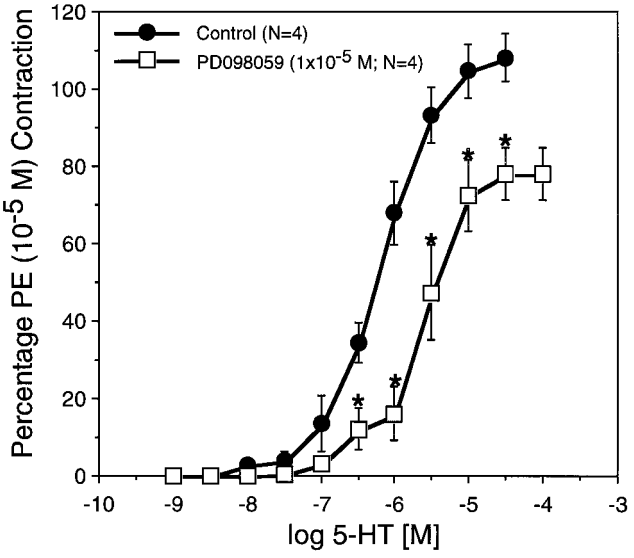

Fig. 3. Effect of MEK inhibitor PD098059 (1×10^{-5} M) on 5-HT-induced contraction in endothelium-denuded rat aorta. Points represent mean ± S.E.M. for the number of animals (in parentheses). *Statistically significant ($P < .05$) between responses of control and PD098059-incubated tissues.

TABLE 1

Effect of increasing concentrations of the MEK inhibitor PD098059 on the potency and maximal effect of 5-HT in the endothelium denuded rat aorta

Data are presented as mean ± S.E.M. for the number of animals in parentheses. The EC_{50} value was defined as the concentration of 5-HT necessary to produce a half-maximal response (determined as described in Methods). Maximum contraction is reported as a percentage of contraction in response to phenylephrine (10^{-5} M).

PD098059 concentration	Potency $-\log EC_{50}$	Maximum contraction
μM	M	% PE contraction
Vehicle (4)	6.17 ± 0.09	108.15 ± 6.27
1 (3)	6.40 ± 0.13	100.83 ± 6.82
3 (6)	5.78 ± 0.06[a]	85.63 ± 2.91[a]
10 (4)	5.56 ± 0.14[a]	77.86 ± 6.81[a]
30 (4)	5.33 ± 0.05[a]	67.35 ± 11.77[a]
50 (4)	5.35 ± 0.05[a]	86.65 ± 6.55[a]
100 (4)	5.53 ± 0.04[a]	89.00 ± 8.44[a]

[a] Statistically significant differences between vehicle (greatest percentage of DMSO, 0.2%) and PD098059 responses ($P < .05$, ANOVA followed by Tukey's test).

the Erk MAPK proteins was reduced by the addition of PD098059 alone. Collectively, these experiments provide significant support for the hypothesis that 5-HT can activate tyrosine kinase(s), including MEK, in vascular smooth muscle. The next series of experiments investigate how this signaling pathway integrates into the known and established pathways for the 5-HT_{2A} receptor. To do so, we first needed to determine the appropriate concentrations of inhibitors of each pathway to be used.

Identification of Optimal Inhibitor Concentrations

L-type voltage-gated calcium channels. Increasing concentrations of nifedipine were examined against KCl-induced contraction; the depolarization and resultant contraction caused by KCl is due to opening of L-type calcium channels. Nifedipine reduced contraction, and 50 nM nifedipine was the lowest concentration of inhibitor that caused a maximal inhibition of KCl-induced contraction (fig. 5, ANOVA followed by Tukey's *post hoc*). This was the concentration of nifedipine used in later experiments.

PLC. The NCDC concentration used (100 μM) caused maximal inhibition of 5-HT-induced contraction (data not shown) but, more importantly, has been previously determined as the concentration that completely abolished phosphoinositide hydrolysis stimulated by maximal 5-HT (100 μM) in the rat aorta (Turla and Webb, 1990).

Tyrosine kinase(s). We previously used and demonstrated that 5 μM genistein (as shown in fig. 2) is a concentration that does not inhibit contraction stimulated by the PKC agonist phorbol-12, 13-dibutryate or KCl (Watts *et al*., 1996). To determine the optimal concentration of PD098059, multiple concentrations of PD098059 (1–100 μM) were tested against 5-HT-induced contraction in the aorta; 10 μM was the lowest concentration of PD098059 that caused maximal inhibition of 5-HT-induced contraction (table 1). These findings provide the appropriate concentration of tyrosine kinase inhibitors to use in our experiments investigating the integration of a tyrosine kinase dependent pathway into classic 5-HT_{2A} receptor signaling. Moreover, these results suggest that tyrosine kinases do mediate at least a portion of 5-HT stimulated contraction in the aorta but only a portion; contraction could not be blocked completely by PD098059.

Effect of Inhibitors on 5-HT-Induced Contraction in Rat Aorta

Figure 6 depicts the effects of our determined concentrations of nifedipine (top left) and NCDC (top right) on 5-HT-induced contraction in the endothelium-denuded rat aorta. Contraction to 5-HT was equally inhibited by 50 nM and 1 μM nifedipine. As expected, both compounds independently inhibited 5-HT-induced contraction. Note that a portion of contraction to 5-HT remains in the presence of either nifedipine or NCDC; the contraction that was remaining after nifedipine or NCDC treatment could be further reduced with the addition of genistein (fig. 6, bottom left and right). This genistein-induced shift was greater in tissues incubated with nifedipine compared with NCDC.

Integration of Transduction Pathways for 5-HT_{2A} Receptor

In these experiments, the effects were determined of a combination of nifedipine and NCDC in the absence or presence of genistein or PD098059 on 5-HT-induced contraction. When the tyrosine kinase inhibitor genistein (fig. 7, top) was added to the combination of nifedipine and NCDC, the curve was shifted 5-fold rightward and reduced maximal contraction to 5-HT. PD098059 (fig. 7, bottom) did not shift but did reduce maximal 5-HT-induced contraction. These data suggest that for the aortic 5-HT_{2A} receptor, (1) there are signal transduction pathways available in addition to L-type calcium channel and PLC activation, (2) one of those pathways is tyrosine kinase dependent and (3) the tyrosine kinase important to the MAPK pathway, MEK, is one of those tyrosine kinases.

Effect of Inhibitors on 5-HT-Stimulated Protein Tyrosyl-Phosphorylation in Rat Aortic Vascular Smooth Muscle Cells. These experiments were conducted to parallel the contractile data for the effects of nifedipine, NCDC, PD098059 and the combination of these three inhibitors on

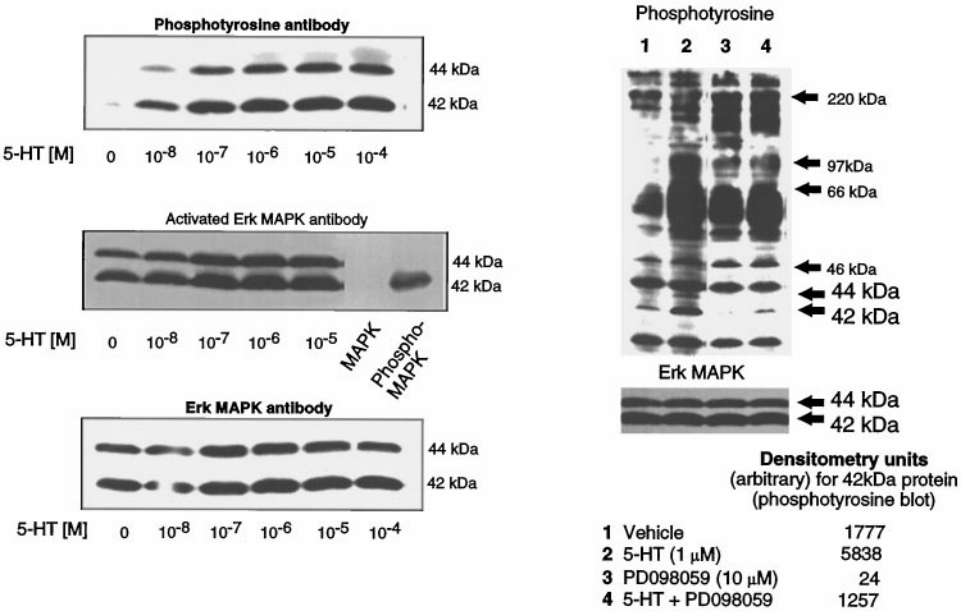

Fig. 4. Effect of 5-HT on protein tyrosyl-phosphorylation in cultured rat aortic smooth muscle cells. Left, visualization of blots probed with phosphotyrosine (top), Erk MAPK (bottom) or activated Erk MAPK antibody (middle); control unphosphorylated and phosphorylated MAPK (10 μg) were used in the last two lanes. Concentration marked between strips indicates the concentration of 5-HT that was incubated with each individual plate. Right, visualization of the effects of the MEK inhibitor PD098059 on basal and 5-HT-stimulated increases in tyrosyl-phosphorylation of Erk MAPKs. Bottom, equal loading of Erk MAPK proteins to lanes. Representative of four experiments on cells derived from four separate explants.

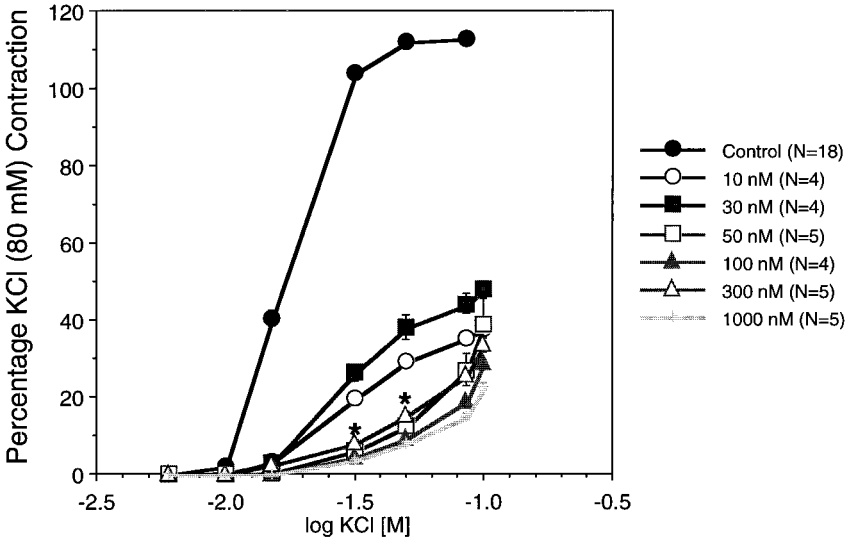

Fig. 5. Concentration-dependent inhibition of endothelium-denuded rat aortic contraction to depolarizing KCl by the L-type voltage-gated calcium channel inhibitor nifedipine. Points represent mean ± S.E.M. for the number of animals (in parentheses). *Statistically significant ($P < .05$) between responses of 10- and 30-nM incubations and those with ≥50 nM nifedipine. All responses above 10 mM KCl were significantly different from control.

tyrosyl-phosphorylation by 5-HT. Cultured cells were exposed to inhibitors for 1 hr before a 5-min incubation with 5-HT. The lysate of cells was separated and transferred to Immobilon-P membrane, probed with an antibody against phosphotyrosine and then visualized with a horseradish peroxidase-linked secondary antibody. Figure 8 shows the resultant blots from one such experiment. Neither nifedipine nor NCDC reduced tyrosyl-phosphorylation by 5-HT; in fact, nifedipine appeared to potentiate 5-HT-stimulated Erk MAPK tyrosyl-phosphorylation. PD098059 (10 μM) reduced 5-HT-stimulated tyrosyl-phosphorylation of the Erk MAPK proteins; however, the combination of nifedipine, NCDC and PD098059 abolished 5-HT-stimulated tyrosyl-phosphorylation. These experiments demonstrate that neither nifedipine nor NCDC inhibits tyrosyl-phosphorylation of the Erk MAPK proteins. In addition, these experiments indicate that the tyrosine kinase (MEK)-dependent pathway is a valuable pathway for the 5-HT$_{2A}$ receptor and that this pathway is activated independently of the other two pathways classically associated with this receptor.

Discussion

The cardiovascular actions elicited by 5-HT have been studied extensively. 5-HT stimulates vasoconstriction of multiple vascular beds (Hamel *et al*., 1993; Herve *et al*., 1990) and is a mitogen with the ability to induce smooth muscle cell growth as well as potentiate the mitogenic activity of other growth factors (Kavanaugh *et al*., 1988; Lee *et al*., 1994a; Nemecek *et al*., 1986). Recent studies have demonstrated that 5-HT stimulates tyrosyl-phosphorylation of proteins in smooth muscle cells (Kelleher *et al*., 1995; Semenchuk and Di Salvo, 1995; Watts *et al*., 1996). Moreover, rat vascular contraction induced by 5-HT can be reduced by general tyrosine kinase inhibitors as well as by a specific MEK inhibitor, PD098059 (Watts, 1996; Watts *et al*., 1996). Taken together, these initial studies suggest that in addition to classic signaling mechanisms associated with the 5-HT$_{2A}$ receptor (PLC and L-type calcium channel activation), tyrosine kinase activation should be considered a functional pathway for the vascular 5-HT$_2$ receptor.

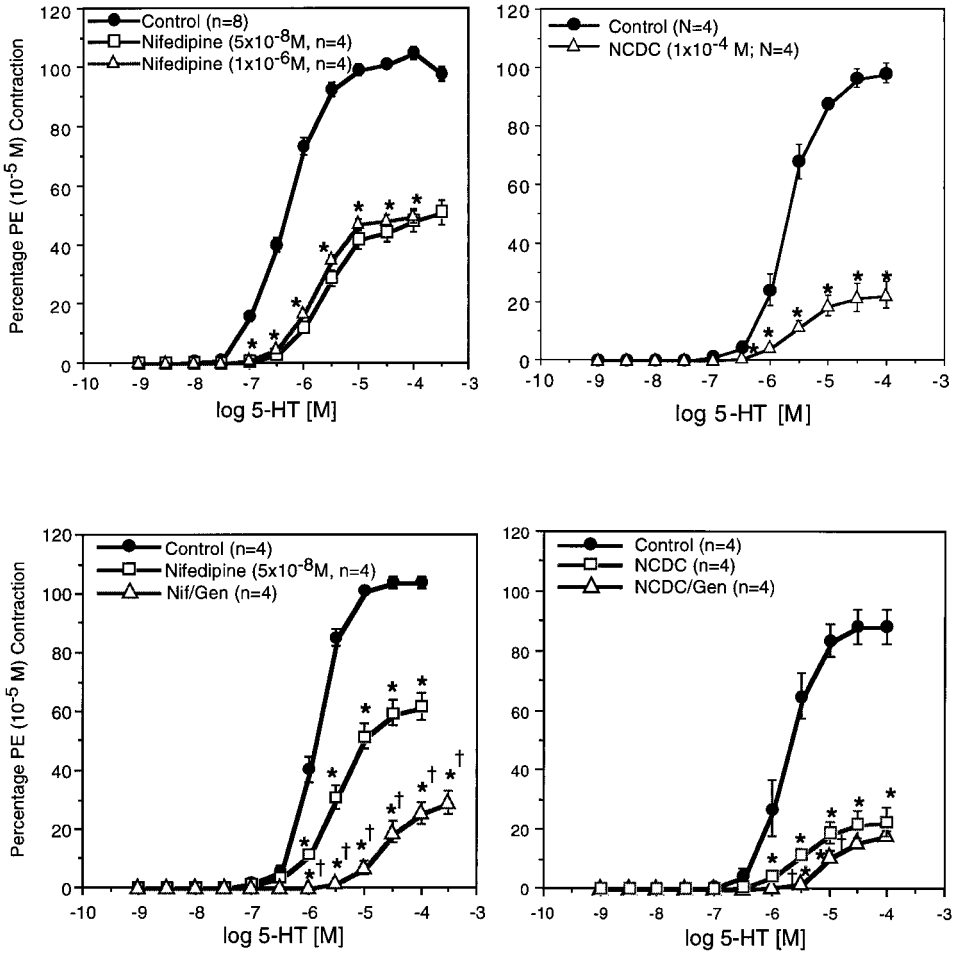

Fig. 6. Inhibition of 5-HT-induced contraction in endothelium-denuded rat aorta by maximally effective concentrations of the L-type voltage-gated calcium channel inhibitor nifedipine (top left) and PLC inhibitor NCDC (top right), nifedipine and genistein (bottom left) and NCDC and genistein (bottom right). Points represent mean ± S.E.M. for the number of animals (in parentheses). *Statistically significant ($P < .05$) between responses of control and inhibitor-incubated tissues. †Statistically significant ($P < .05$) between responses of nifedipine and nifedipine plus genistein or NCDC and NCDC plus genistein.

In the present study, we linked tyrosine kinase activation to the vascular 5-HT_{2A} receptor. The specific MEK inhibitor PD098059 inhibited 5-HT-induced aortic contraction, indicating that MEK is at least one of the tyrosine kinases activated in response to signaling by the 5-HT_{2A} receptor. It is not obvious how G protein-linked receptors can activate a tyrosine kinase such as MEK, although several theories have been examined. PLC is activated in response to 5-HT_{2A} receptor signaling. One product of PLC activity is 1,2-diacylglycerol, an activator of PKC (Takai *et al*., 1979). Kolch *et al*. (1993) found that several isoforms of PKC directly phosphorylate and activate Raf-1; activated Raf-1 can then directly phosphorylate and activate MEK (Dent *et al*., 1992). These findings represent one mechanism by which the 5-HT_{2A} receptor could recruit the MAP kinase pathway in cell signaling and one way in which the classic pathways for this receptor could interact with tyrosine kinase-dependent signaling pathways. Alternatively, the *beta-gamma* subunits of some G proteins have been shown to bind and interact with the pleckstrin homology (PH) domains (Koch *et al*., 1994, van Biesen *et al*., 1995) of several proteins, including PLC-γ (Inglese *et al*., 1995), Ras (Koch *et al*., 1994), Ras-GRF and Ras-GAP (Touhara *et al*., 1994), all of which play a role in the MAP kinase pathway. Recent studies have investigated the role of PI3K in G protein-coupled receptor activation of tyrosine kinases. PI3K catalyzes the phosphorylation of inositol phospholipids and appears to be involved in both growth factor and G protein receptor signaling (Cantley *et al*., 1991). PI3K contains SH2 domains that enable it to interact with tyrosyl-phosphorylated tyrosine kinases; in addition, PI3K is activated by the *beta-gamma* subunits of G proteins (Thomason *et al*., 1994). Lopez-llasaca *et al*. (1997) demonstrated that stimulation of a G protein-coupled receptor induced MAPK activation and that this activation could be inhibited by wortmannin, an inhibitor of PI3K. The finding that the *beta-gamma* subunits of G proteins can activate tyrosine kinases provides support for the idea that at least two separate but parallel pathways—(1) classic G protein effector activation of enzymes like PLC and adenylate cyclase and (2) MAPK pathway—would be available to signal changes in cell function elicited by activation of receptors coupled to G proteins. Clearly, this dual activation is available to vascular 5-HT_{2A} receptors; it is unknown whether activation of parallel signaling mechanisms occurs upon stimulation of other 5-HT receptors.

In addition to the activation of tyrosine kinases like MEK, the 5-HT_{2A} receptor stimulates vascular contraction in part by the activation of voltage-dependent Ca^{++} channels and PLC (Nakaki *et al*., 1985). Our studies confirmed these data. NCDC exhibited a greater ability to inhibit 5-HT-induced contraction than either nifedipine or the tyrosine kinase inhibitors. One explanation for this greater effect of NCDC may be because PLC is able to activate two second-messenger pathways to elicit contraction: (1) production of inositol triphosphate signals the release of intracellular Ca^{++} and (2) production of diacylglycerol activates PKC. This finding

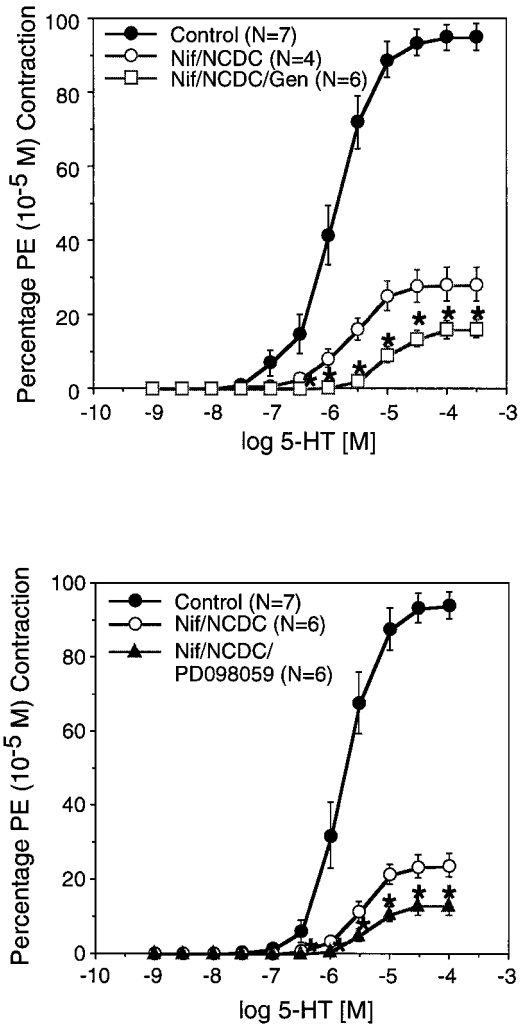

Fig. 7. Reduction of nifedipine and NCDC-insensitive portion of the 5-HT concentration response curve by the general tyrosine kinase inhibitor genistein (top; 5×10^{-6} M) and the MEK inhibitor PD098059 (bottom; 1×10^{-5} M). Points represent mean ± S.E.M. for the number of animals (in parentheses). *Statistically significant ($P < .05$) between responses of nifedipine/NCDC/kinase inhibitors and nifedipine/NCDC-incubated tissues.

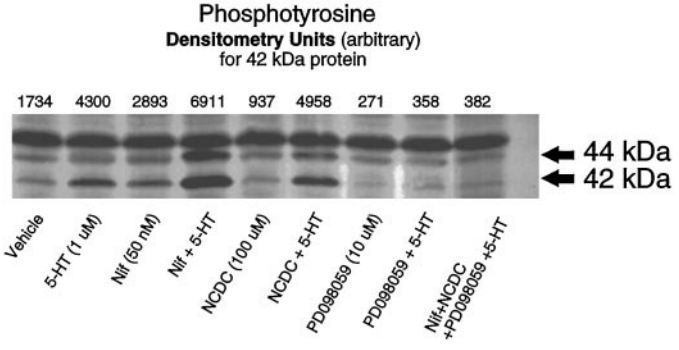

Fig. 8. Effect of nifedipine, NCDC, PD098059 and the combination of all three together on basal and 5-HT-stimulated Erk MAPK protein tyrosyl-phosphorylation in cultured rat aortic smooth muscle cells. Visualization of blots probed with phosphotyrosine antibody. Inhibitors were incubated for 1 hr before the 5-HT (1 μM) incubation for each individual plate. Arbitrary densitometry units for the 42-kDa protein are reported above each lane. Results are representative of four experiments on cells derived from four separate explants.

should not take away from the role of Ca^{++} channels and tyrosine kinases in 5-HT-induced contraction, so we conducted a series of experiments to examine how the two classic pathways were integrated with tyrosine kinase activation. The addition of genistein to strips incubated with nifedipine or NCDC was able to further reduce the contractile response to 5-HT, although, again, contraction was not completely inhibited. The combination of genistein and nifedipine caused a greater shift in 5-HT-induced contraction than that of genistein and NCDC, indicating that Ca^{++} channels are more closely coupled to tyrosine kinases; this finding is supported by the Western blot showing treatment with nifedipine actually potentiated 5-HT-stimulated Erk MAPK tyrosyl-phosphorylation. Similarly, the combination of nifedipine plus NCDC significantly reduced 5-HT-induced contraction; however, the remaining contraction could be further reduced with the addition of genistein or PD098059. The use of all three inhibitors was unable to completely block 5-HT-induced contraction. Several possibilities exist as to why a contraction remains after exposure to the three inhibitors.

First, there is the possibility that we have used inappropriate concentrations of the inhibitors, so the three pathways are not completely inhibited. This must remain a possibility, but we believe this is not the case. Previous studies have shown that NCDC (100 μM), a PLC inhibitor, completely inhibited 5-HT-(100 μM)-induced phosphoinositide hydrolysis in rat aorta (Turla and Webb, 1990) and maximally reduced 5-HT-stimulated aortic contraction (Nakaki *et al.*, 1985). We believe that the PLC pathway is appropriately inhibited in these experiments. Second, nifedipine was first tested against KCl-induced contraction as an index of calcium channel activation, and it was determined that increases in the concentration of nifedipine above 50 nM did not further reduce KCl-induced contraction. We then tested 50 nM and 1 μM nifedipine against 5-HT-induced contraction and found the two concentrations equally inhibited 5-HT-induced contraction. Thus, we concluded that L-type calcium channels are likely completely inhibited. Similarly, we demonstrated that the concentration of PD098059 used in these experiments (10 μM) completely inhibited the portion of the 5-HT-induced contraction that was due to MEK activation because increasing concentrations of PD098059 did not further reduce 5-HT-induced contraction (Watts, 1996). Moreover, this concentration of PD098059 significantly reduced 5-HT-stimulated tyrosyl-phosphorylation of Erk/MAPK (p42 and p44) proteins. Taken together, we believe that we have used concentrations of inhibitors that maximally affect their intended targets with minimal nonselectivity. More likely is the idea that there are yet other mechanisms, such as the activation of transient calcium channels (T-type), that are responsible for the contraction remaining after combined treatment. Based on these results, we propose that all three pathways are activated somewhat independently of one another to result in vascular contraction. The potential for interaction between these pathways arises, for example, with the knowledge that PLC can act as a substrate for Erk.

The mechanism by which tyrosine kinases stimulate contraction is uncertain, although several theories have been examined. Caldesmon is an actin binding protein that inhibits smooth and skeletal muscle actin-activated myosin ATPase (Katsuyama *et al.*, 1992). Phosphorylation of caldesmon

reverses its inhibitory activity, allowing smooth muscle contraction to occur (Ngai and Walsh, 1984). Several studies have shown that MAPK is capable of associating with (Khalil *et al*., 1995) and phosphorylating (Adam *et al*., 1989; Childs *et al*., 1992; Katoch and Moreland, 1995) caldesmon. This represents one mechanism by which 5-HT_{2A} receptor activation of the MAPK pathway could lead to vascular contraction. An attempt has also been made to link the activation of the MAP kinase pathway with the myosin light chain kinase pathway that is involved in smooth muscle contraction. Fetal calf serum, known to contain several growth factors that activate receptors with intrinsic tyrosine kinase activity, induced phosphorylation of myosin light chain and induced contraction in the rat aorta and pulmonary artery. Moreover, these effects could be inhibited by the tyrosine kinase inhibitor genistein (Jin *et al*., 1996). These results support the idea of MAPK activation as a valid mechanism by which vascular contraction can occur.

Vascular smooth muscle cell culture is a useful tool with which to examine the biochemical effects of 5-HT on cell signaling. Western analysis was used to more directly assess the effect of 5-HT on tyrosine kinase activation in cultured rat aortic smooth muscle cells. 5-HT stimulated a concentration-dependent increase in tyrosyl-phosphorylation of the Erk/MAP kinase proteins (activated MAPK) that was dramatically reduced by the MEK inhibitor PD098059. 5-HT-stimulated tyrosyl-phosphorylation was not inhibited by NCDC and was actually potentiated by nifedipine; however, the combination of nifedipine, NCDC and PD098059 greatly reduced this phosphorylation. The enzyme responsible for Erk/MAP kinase tyrosyl-phosphorylation is MEK (Zheng and Guan, 1993), further indicating that stimulation of a 5-HT_{2A} receptor leads to activation of MEK. Because neither nifedipine nor NCDC inhibited tyrosyl-phosphorylation of the Erk MAPK proteins by 5-HT, it appears that the tyrosine kinase (MEK)-dependent pathway is activated independently of the other two pathways activated by 5-HT. Studies have demonstrated that other contractile agonists, like angiotensin II and endothelin-1, mediate their actions through G protein-coupled receptors and stimulate tyrosyl-phosphorylation of the Erk/MAPK proteins (Koide *et al*., 1992; Lucchesi *et al*., 1996; Molloy *et al*., 1993). However, although angiotensin II and endothelin-1 stimulate tyrosine kinase activity, it does not appear that the activation of tyrosine kinases by endothelin-1 and angiotensin II mediates contraction because genistein is unable to block aortic contraction to these agonists.[3] Western studies correlate well with our contractile studies, providing further evidence that 5-HT_{2A} receptor stimulation leads to the activation of MEK to result in contraction. Thus, it appears that in the case of 5-HT_{2A} receptor signaling, MEK is specifically activated to cause aortic contraction and is an important signaling mechanism for the 5-HT_{2A} receptor.

The idea that 5-HT utilizes tyrosine kinases in cell signaling is especially compelling in disease states like hypertension and atherosclerosis. Vascular reactivity to agonists and, in particular, 5-HT is increased in hypertension (Bohr and Webb, 1989; Turla and Webb, 1989). Some forms of hypertension are also associated with structural changes in the blood vessel wall in which the vessel wall thickens. Vascular cell growth likely depends on the activity of tyrosine kinases, which when activated are capable of modulating the expression of genes required for cell growth. It can be postulated that in disease states in which there is a hypersensitivity to 5-HT, there may be enhanced activation of the MAP kinase pathway, resulting in increased smooth muscle cell growth and possibly contraction. Thus, 5-HT may regulate both smooth muscle contraction and growth in normal as well as diseased states through the activation of tyrosine kinases such as MEK.

In summary, rat aortic contraction 5-HT occurs *via* 5-HT_{2A} receptors leading to the activation of PLC, L-type Ca^{++} channels as well as tyrosine kinases, including MEK. Furthermore, these signaling pathways are activated somewhat independently of each other to stimulate aortic contraction. Taken together, these studies indicate that in addition to classic signaling pathways associated with the 5-HT_{2A} receptor, the MAPK pathway is an important signaling pathway that should be considered.

[3] S. W. Watts, unpublished data.

References

Adam LP, Haeberle JR and Hathaway DR (1989) Phosphorylation of caldesmon in arterial smooth muscle. *J Biol Chem* **264:**7698–7703.

Arunlakshana O and Schild HO (1959) Some quantitative uses of drug antagonists. *Br J Pharmacol* **14:**48–58.

Berk BC, Alexander RW, Brock TA, Gimbrone MA and Webb RC (1986) Vasoconstriction: A new activity for platelet-derived growth factor. *Science* **232:**87–89.

Berk BC, Brock TA, Webb RC, Taubman MB, Atkinson WJ, Gimbrone MA and Alexander RW (1985) Epidermal growth factor, a vascular smooth muscle mitogen, induces rat aortic contraction. *J Clin Invest* **75:**1083–1086.

Blenis J (1993) Signal transduction via the MAP kinases: Proceed at your own RSK. *Proc Natl Acad Sci USA* **90:**5889–5892.

Bohr DF and Webb RC (1989) Vascular smooth muscle membrane in hypertension. *Annu Rev Pharmacol Toxicol* **28:**389–409.

Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R and Soltoff S (1991) Oncogenes and signal transduction. *Cell* **64:**281–302.

Childs TJ, Watson MH, Sanghera JS, Campbell DL, Pelech SL and Mak AS (1992) Phosphorylation of smooth muscle caldesmon by mitogen-activated protein (MAP) kinase and expression of MAP kinase in differentiated smooth muscle cells. *J Biol Chem* **267:**22853–22859.

Cobb MH and Goldsmith EJ (1995) How MAP kinases are regulated. *J Biol Chem* **270:**14843–14846.

Cohen ML, Fuller RW and Wiley KS (1981) Evidence for 5-HT_2 receptors mediating contraction in vascular smooth muscle. *J Pharmacol Exp Ther* **218:**421–425.

Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM and Sturgill TW (1992) Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in Vitro. *Science* **257:**1404–1407.

Dudley DT, Pang L, Decker SJ, Bridges AJ and Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA* **92:**7686–7689.

Hamel E, Gregoire L and Lau B (1993) 5-HT_1 receptors mediating contraction in bovine cerebral arteries: A model for human cerebrovascular 5-$HT_{1D\beta}$ receptors. *Eur J Pharmacol* **242:**75–82.

Herve P, Drouet L, Dosquet C, Launay J, Rain B, Simmoneau G, Caen J and Duroux P (1990) Primary pulmonary hypertension in a patient with a familial platelet storage pool disease: role of serotonin. *Am J Med* **89:**117–120.

Inglese J, Koch WJ, Touhara K and Lefkowitz RJ (1995) $G_{\beta\gamma}$ interactions with PH domains and Ras-MAPK signaling pathways. *Trends Biol Chem* **20:**151–156.

Jin N, Siddiqui RA, English D and Rhoades RA (1996) Communication between tyrosine kinase pathway and myosin light chain kinase pathway in smooth muscle. *Am J Physiol* **271:**H1348–H1355.

Katoch SS and Moreland RS (1995) Agonist and membrane depolarization induced activation of MAP kinase in swine carotid artery. *Am J Physiol* **269:**H222–H229.

Katsuyama H, Wang C-LA and Morgan KG (1992) Regulation of vascular smooth muscle tone by caldesmon. *J Biol Chem* **267:**14555–14558.

Kavanaugh WM, Tilliams LT, Ives HE and Coughlin SR (1988) Serotonin-induced deoxyribonucleic acid synthesis in vascular smooth muscle cells involves a novel, pertussis toxin-sensitive pathway. *Mol Endocrinol* **2:**599–605.

Kelleher MD, Abe MK, Chao T-SO, Jain M, Green JM, Solway J, Rosner MR and Hershenson MB (1995) Role of MAP kinase activation in bovine tracheal smooth muscle mitogenesis. *Am J Physiol* **268:**L894–L901.

Khalil RA, Menice CB, Wang C-LA and Morgan KG (1995) Phosphotyrosine-dependent targeting of mitogen-activated protein kinase in differentiated contractile vascular cells. *Circ Res* **76:**1101–1108.

Koch WJ, Hawes BE, Allen LF and Lefkowitz RJ (1994) Direct evidence that G_i-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of $p21^{ras}$. *Proc Natl Acad Sci USA* **91:**12706–12710.

Koide M, Kawahara Y, Tsuda T, Ishida Y, Shii K and Yokoyama M (1992) Endothelin-1 stimulates tyrosine phosphorylation and the activities of two mitogen-

activated protein kinases in cultured vascular smooth muscle cells. *J Hypertens* **10:**1173–1182.
Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D and Rapp UR (1993) Protein kinase Cα activates RAF-1 by direct phosphorylation. *Nature* **364:**249–252.
Lee S-L, Wang WW, Moore BJ and Fanburg BL (1991) Dual effect of serotonin on growth of bovine pulmonary artery smooth muscle cells in culture. *Circ Res* **68:**1362–1368.
Lee S-L, Wang WW, Lanzillo JJ and Fanburg BL (1994a) Serotonin produces both hyperplasia and hypertrophy of bovine pulmonary artery smooth muscle cells in culture. *Am J Physiol* **266:**L46–L52.
Lee S-L, Wang WW, Lanzillo JJ and Fanburg BL (1994b) Regulation of serotonin-induced DNA synthesis of bovine pulmonary artery smooth muscle cells. *Am J Physiol* **266:**L53–L60.
Lopez-llasaca M, Crespo P, Pellici PG, Gutkind JS and Wetzker R (1997) Linkage of G- protein-coupled receptors to the MAPK signaling pathway through PI3-kinase γ. *Science* **275:**394–397.
Lucchesi PA, Bell JM, Willis LS, Byron KL, Corson MA and Berk BC (1996) Ca^{2+}-dependent mitogen-activated protein kinase activation in spontaneously hypertensive rat vascular smooth muscle defines a hypertensive signal transduction phenotype. *Circ Res* **78:**962–970.
Molloy CJ, Taylor DS and Weber H (1993) Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. *J Biol Chem* **268:**7338–7345.
Nakaki T, Roth BL, Chuang D-M and Costa E (1985) Phasic and tonic components in 5-HT$_2$ receptor-mediated rat aorta contraction: Participation of Ca^{++} channels and phospholipase C. *J Pharmacol Exp Ther* **234:**442–446.
Nemecek GM, Coughlin SR, Handley DA and Moskowitz MA (1986) Stimulation of aortic smooth muscle cell mitogenesis by serotonin. *Proc Natl Acad Sci USA* **83:**674–678.
Ngai PK and Walsh MP (1984) Inhibition of smooth muscle actin-activated myosin Mg^{2+}-ATPase activity by caldesmon. *J Biol Chem* **259:**13656–13659.
Semenchuk LA and DiSalvo J (1995) Receptor-activated increases in intracellular calcium and protein tyrosine phosphorylation in vascular smooth muscle cells. *FEBS Lett* **370:**127–130.
Takai Y, Kishimoto A, Kikkawa U, Mori T and Nishizuka Y (1979) Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. *Biochem Biophys Res Commun* **91:**1218–1224.
Thomason PA, James SR, Casey PJ and Downes CP (1994) A G-protein $\beta\gamma$-subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol. *J Biol Chem* **269:**16525–16528.
Touhara K, Inglese J, Pitcher JA, Shaw G and Lefkowitz RJ (1994) Binding of G-protein $\beta\gamma$-subunits to pleckstrin homology domains. *J Biol Chem* **269:**10217–10220.
Turla MB and Webb RC (1989) Vascular responsiveness to 5-hydroxytryptamine in experimental hypertension, in *The Peripheral Actions of 5-hydroxytryptamine* (Fozard JR, ed) pp 327–353, Oxford University Press, Oxford.
Turla MB and Webb RC (1990) Augmented phosphoinositide metabolism in aortas from genetically hypertensive rats. *Am J Physiol* **258:**H173–H178.
van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM and Lefkowitz RJ (1995) Receptor-tyrosine-kinase- and G $\beta\gamma$-mediated MAP kinase activation by a common signalling pathway. *Nature* **376:**781–784.
Van Nueten JM, Janssen PA, van Beek J, Xhonneux R, Verbeuren TJ and Vanhoutte PM (1981) Vascular effects of ketanserin (R41468), a novel antagonist of 5-HT$_2$ serotonergic receptors. *J Pharmacol Exp Ther* **218:**217–230.
Watts SW (1996) Serotonin activates the mitogen-activated protein kinase pathway in vascular smooth muscle: Use of the mitogen-activated protein kinase kinase inhibitor PD098059. *J Pharmacol Exp Ther* **279:**1541–1550.
Watts SW, Yeum CH, Campbell G and Webb RC (1996) Serotonin stimulates protein tyrosyl phosphorylation and vascular contraction via tyrosine kinase. *J Vasc Res* **33:**288–298.
Zheng C-F and Guan K-L (1993) Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. *J Biol Chem* **268:**11435–11439.

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