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, $\alpha$-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 mM) shifted contraction to the right, whereas daidzein (5 mM) was unable to shift 5-HT-induced contraction. The tyrosine kinase inhibitor genistein (5 mM) shifted contraction to 5-HT, $\alpha$-methyl-5-HT and DOI $\sim$10-fold to the right, whereas daidzein (5 mM), the inactive isomer of genistein, was unable to shift 5-HT-induced contraction. PD098059 (10 mM), an inhibitor of MEK, shifted contraction to 5-HT $\sim$7-fold to the right. We next examined the integration of tyrosine kinase activation in 5-HT$_{2A}$ receptor signaling.

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 tyrosine 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$_{2A}$ 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; MAPK, mitogen-activated protein; PLC, phospholipase C; PH, pleckstrin homology; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; PD098059, an inhibitor of MEK; NCDC, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate; PE, phenylephrine; 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$^{2+}$ 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$^{2+}$ 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$^{2+}$ 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, K$_2$HPO$_4$ 1.18, MgSO$_4$·7H$_2$O 1.17, CaCl$_2$·2H$_2$O 1.6, NaHCO$_3$ 14.9, dextrose 5.5 and CaNa$_2$EDTA 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 phenyl-ephrine (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; ≤5% 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, 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 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 genistein, 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) and 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 confluence. 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 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/ml 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 × 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 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), rebloked 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 EC50 values were calculated using a nonlinear regression analysis using the algorithm (effect = maximum response/l + (EC50/agonist concentration)). Apparent antagonist dissociation constants (K_B) values were determined according to the following equation:

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

where B is the antagonist concentration, and the dose ratio is the EC50 value of 5-HT in the presence of antagonist divided by the control EC50 of 5-HT (Arunlakshana and Schild, 1959). K_B values are reported as the mean of the negative logarithm (pK_B) of individual

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Validation of 5-HT2A Receptor Mediation of 5-HT-Induced Contraction in Aorta

Two pharmacological approaches were used to validate the receptor in the aorta as a 5-HT2A receptor. First, 5-HT, the 5-HT2 receptor full agonist α-methyl-5-HT and the 5-HT2 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 EC50 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-HT2A receptor mediates 5-HT-induced contraction in the rat aorta. The 5-HT2A

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Written by Wayne Rashband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NITIS, 5285 Port Royal Road, Springfield, VA 22161, part number PB95-504888.
receptor antagonist ketanserin (30 nM) acted as a competitive antagonist against 5-HT with an apparent antagonist dissociation constant (-log K_D = 8.70 ± 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_2 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.

**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
Effect of increasing concentrations of the MEK inhibitor PD098059 on 5-HT-induced contraction in endothelium-denuded rat aorta Data are presented as mean ± S.E.M. for the number of animals in parentheses. The EC50 value was defined as the concentration of 5-HT necessary to produce a half-maximal response (described as determined in Methods). Maximum contraction is reported as a percentage of contraction in response to phenylephrine (10−6 M).

<table>
<thead>
<tr>
<th>PD098059 concentration</th>
<th>−log EC50</th>
<th>Maximum contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (4)</td>
<td>6.17 ± 0.09</td>
<td>108.15 ± 6.27</td>
</tr>
<tr>
<td>1 (3)</td>
<td>6.40 ± 0.13</td>
<td>100.83 ± 6.82</td>
</tr>
<tr>
<td>3 (6)</td>
<td>5.78 ± 0.06a</td>
<td>85.63 ± 2.91a</td>
</tr>
<tr>
<td>10 (4)</td>
<td>5.56 ± 0.14a</td>
<td>77.86 ± 6.81a</td>
</tr>
<tr>
<td>30 (4)</td>
<td>5.33 ± 0.05a</td>
<td>67.35 ± 11.77a</td>
</tr>
<tr>
<td>50 (4)</td>
<td>5.35 ± 0.05a</td>
<td>86.65 ± 6.55a</td>
</tr>
<tr>
<td>100 (4)</td>
<td>5.53 ± 0.04a</td>
<td>89.00 ± 8.44a</td>
</tr>
</tbody>
</table>

*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-HT2A 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-dibutryrate 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 classical 5-HT2A 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). The genistein-induced shift was greater in tissues incubated with nifedipine compared with NCDC.

Integration of Transduction Pathways for 5-HT2A 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-HT2A 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...
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-HT₂A 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 DiSalvo, 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-HT₂A receptor (PLC and L-type calcium channel activation), tyrosine kinase activation should be considered a functional pathway for the vascular 5-HT₂A receptor.
In the present study, we linked tyrosine kinase activation to the vascular 5-HT\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 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\textsubscript{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\textsubscript{2A} receptor stimulates vascular contraction in part by the activation of voltage-dependent Ca\textsuperscript{++} 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\textsuperscript{++} and (2) production of diacylglycerol activates PKC. This finding...
should not take away from the role of Ca\textsuperscript{2+} 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\textsuperscript{2+} 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 \(\mu\)M), a PLC inhibitor, completely inhibited 5-HT-(100 \(\mu\)M)-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 \(\mu\)M 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 \(\mu\)M) 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. We then tested 50 nM and 1 \(\mu\)M nifedipine against 5-HT-induced contractions and found the two concentrations equally inhibited 5-HT-induced contraction. The mechanism by which tyrosine kinases stimulate contraction is uncertain, although several theories have been examined. Calcenon is an actin binding protein that inhibits smooth and skeletal muscle actin-activated myosin ATPase (Katsuyama et al., 1992). Phosphorylation of calcenon...
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/MAPK proteins (activated MAPK) that was dra-

References


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