Dissociation of Angiotensin II-Stimulated Activation of Mitogen-Activated Protein Kinase Kinase from Vascular Contraction

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ABSTRACT

Angiotensin II (Ang II) is a potent pressor hormone, a stimulus for vascular smooth muscle hypertrophy and an activator of multiple tyrosine kinases. The physiological effects of Ang II are mediated through activation of AT1 and AT2 receptors, receptors that have been coupled to tyrosine kinase(s) and tyrosine phosphatases, respectively. Agonists of G protein-coupled receptors, of which Ang II is one, have recently been shown to stimulate smooth muscle contraction in part via activation tyrosine kinases. We tested the hypothesis that Ang II-induced contraction in the rat aorta was dependent on activation of tyrosine kinase(s) and specifically investigated the role of the tyrosine kinase mitogen-activated protein kinase kinase (MEK). Ang II, a kinase important to the mitogen activated protein kinase (MAPK) pathway. Rat thoracic aortic strips denuded of endothelium and cultured aortic smooth muscle cells were used in isolated tissue baths for measurement of isometric contractile force and Western analyses of protein tyrosyl-phosphorylation. Ang II (0.1–100 nM)–induced contraction in the aorta was completely blocked by the AT2 receptor antagonist losartan (1 μM) but unaffected by the AT1 receptor antagonist PD123319 (100 nM) or tyrosine phosphatase inhibitor sodium orthovanadate (1 μM), indicating an AT1 receptor-mediated aortic contraction to Ang II. Neither the tyrosine kinase inhibitor genistein (5 μM), inactive tyrosine kinase inhibitor daidzein (5 μM) nor MEK inhibitor PD098059 (10 μM) reduced Ang II–induced contraction; the concentrations of inhibitors used maximally reduced contraction stimulated by the combination of PD098059 and PD123319, indicating that it is unlikely AT2 receptor stimulation masks activation of the MAPK pathway through AT1 receptor activation. The nonflavone tyrosine kinase inhibitor tyrphostin B42 (30 μM) reduced Ang II–induced maximal contraction (to 11.2% control) but, unlike the other tyrosine kinase inhibitors, also reduced KCl–induced contraction (to 55.2% control), indicating a probable nonselectivity of tyrphostin B42. Ang II–induced maximal contraction was reduced by the L-type voltage gated calcium channel antagonist nifedipine (50 nM), consistent with the activation of calcium channels by Ang II. In cultured rat aortic smooth muscle cells, Ang II (0.1–1000 nM) stimulated concentration-dependent tyrosyl-phosphorylation of the extracellular signal regulated kinase (Erk) mitogen-activated protein kinase proteins (maximal stimulation, fold basal: Erk-1 = 17-fold, Erk-2 = 3-fold), indicating that Ang II can activate MEK. Losartan (1 μM) abolished Ang II (10 nM)–induced Erk tyrosyl-phosphorylation and PD098059 (10 μM), which did not diminish Ang II–induced aortic contraction, reduced Ang II (10 nM)–stimulated phosphorylation of Erk-2 by 72%. Finally, Ang II (1 μM) increased tyrosyl-phosphorylation of the Erks proteins in isolated aorta exposed to Ang II for 5 min. Thus, while Ang II can stimulate both MEK activation and vascular contraction via interaction with AT1 receptors, stimulation of MEK does not appear to be important for Ang II–induced contraction. These findings dissociate the process of Ang II–stimulated Erk protein tyrosyl-phosphorylation from Ang II–induced contraction in the rat aorta.
(Griendling et al., 1996). Several groups have done important work in demonstrating the ability of Ang II to activate multiple tyrosine kinase dependent pathways, and it is activation of these pathways to which the mitogenic actions of Ang II have been attributed (Griendling et al., 1997, Berk and Corson, 1997). AT2 receptors, while less intensively examined, also appear to be seven α-helical transmembrane domain proteins in structure (Mukoyama et al., 1993), but their linkage to G proteins has not been definitively established. AT2 receptors have been reported as receptors of antiproliferation in that they can mediate reduction in neointimal formation after balloon catheterization in carotid arteries transsected with the AT2 gene (Nakajima et al., 1995). AT2 receptors have the ability to activate a tyrosine phosphatase and, as such, have the potential to ameliorate signaling through AT1 receptors, receptors that in part depend on tyrosine kinase activation (Nakajima et al., 1995; Sabri et al., 1997).

Ang II-stimulated protein synthesis and vascular cell growth are sensitive to the effects of tyrosine kinase inhibitors (Servant et al., 1996) and the ability of Ang II to stimulate tyrosyl-phosphorylation of multiple signaling proteins is argument that Ang II activates tyrosine kinases. Tyrosine kinases have been, until the last decade, classically involved in growth or cytokine signaling. It has become clear, however, that tyrosine kinase(s) also play a role in the dynamic process of smooth muscle contraction. Hollenberg and co-workers were among the first to provide evidence that smooth muscle contraction (nonvascular) elicited by agonists of G protein-coupled receptors could be reduced by inhibitors of tyrosine kinases (Yang et al., 1992, 1993; Hollenberg, 1994; Laniyonu et al., 1994). The first agonist investigated in the initial contractile experiments by Hollenberg was Ang II. Other groups have since demonstrated that vascular smooth muscle contraction caused by norepinephrine (DiSalvo et al., 1993) and 5-HT (Watts 1996, Watts et al., 1996) could be diminished by tyrosine kinase inhibitors including PD098059, a selective inhibitor of the tyrosine kinase MEK.

Western assays using a phosphotyrosine antibody made it possible to investigate the ability of receptor agonists that couple to G proteins to increase protein tyrosyl-phosphorylation, a measure of tyrosine kinase activity. Ang II can stimulate phosphorylation and activation of a host of different proteins in vascular smooth muscle, including tyrosyl-phosphorylation of the Erk MAPKs (Erk-1 and Erk-2) in rat aortic smooth muscle cells derived from aorta of male Sprague-Dawley rats (250–300 g) using the explant method (described previously, Florian and Watts, 1998) and used in 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). Cells (P-100 culture dish) were switched to physiological salt solution (see above) for 1 hr before addition of agonist (final volume = 4 ml). Antagonists or vehicle were added at this time. Each dish was incubated with one agonist concentration. A 5-min incubation was used for Ang II as we have previously established this as the necessary time for maximal stimulation of Erk1/2 tyrosyl-phosphorylation (data not shown). Incubation buffer was aspirated and 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, 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/µl aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate) were added to each dish, and cells were lysed. Lysate was transferred into 1.5-ml centrifuge tubes and centrifuged at 14,000 × g for 10 min at 4°C. The supernatant, as the sample, was aspirated from the pellet of cellular debris.

**Methods**

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

**Contractile studies.** Male Sprague-Dawley rats (250–300 g, Charles River) were euthanized (80 mg kg⁻¹ 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 buffer for measurement of isometric contractile force using standard bath procedures. Physiological salt solution contained (in mM) NaCl, 130; KCl, 4.7; KH2PO4, 1.18; MgSO4·7H2O, 1.17; CaCl2·2H2O, 1.6; NaHCO3, 14.9; dextrose, 5.5; and CaNa2EDTA, 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 placed under optimum resting tension (1500 mg). This tension had been determined in previous experiments to be that at which aortic strips respond with a maximal force to a maximal concentration of KCl (100 mM). 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. After 1 hr equilibration, arteries were challenged with phenylephrine (10⁻⁵ M). 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 alpha-1 adrenergic receptor agonist phenylephrine (1 × 10⁻⁴ M). The endothelium was functionally disrupted as we saw no greater than an 8% relaxation of the phenylephrine contraction in any tissue. Tissues were then washed multiple times, and one of the following experimental protocols was used.

**Vascular smooth muscle cells experiments.** Vascular smooth muscle cells were derived from aorta of male Sprague-Dawley rats (250–300 g) using the explant method (described previously, Florian and Watts, 1998) and used in 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). Cells (P-100 culture dish) were switched to physiological salt solution (see above) for 1 hr before addition of agonist (final volume = 4 ml). Antagonists or vehicle were added at this time. Each dish was incubated with one agonist concentration. A 5-min incubation was used for Ang II as we have previously established this as the necessary time for maximal stimulation of Erk1/2 tyrosyl-phosphorylation (data not shown). Incubation buffer was aspirated and 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, 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/µl aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate) were added to each dish, and cells were lysed. Lysate was transferred into 1.5-ml centrifuge tubes and centrifuged at 14,000 × g for 10 min at 4°C. The supernatant, as the sample, was aspirated from the pellet of cellular debris.

**Western analyses; cultured cells.** The supernatant of the lysate (4:1 in denaturing loading buffer, boiled 5 min) was loaded and separated on a 10% denaturing SDS-polyacrylamide gel. Proteins were transferred electrochemically to prepared Immobilon-P membrane and membranes were then blocked for 3 to 4 hr in Tris buffer saline and 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, Lake Placid, NY) or mouse Erk1/2 antibody (1:5000, Zymed Laboratories, San Francisco, CA) were incubated with blots overnight (4°C) on a rocker. Blots were
washed 3 times with TBS-T (30 min, 5 min, 5 min) and once with TBS (5 min). Anti-mouse antibody (1:7500, Amersham Laboratories, IL) linked to horseradish peroxidase was added for 1 hr and incubated with blots at 4°C on a rocker. 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, 2% SDS in 62.5 mM Tris-HCl, pH 6.7, at 60°C for 30 min with agitation. Blots were washed (2 × 10 min in TBS-T), reblocked and probed as described above.

Western analyses; whole arteries. Aortic strips denuded of endothelium were mounted in isolated tissue baths, and after a 1-hr incubation and initial challenge to phenylephrine, tissues were exposed to one concentration of Ang II (1 μM) or vehicle (5 min incubation). Strips were immediately frozen with liquid nitrogen-cooled tongs and pulverized in ice-cold homogenization buffer (255 mM sucrose, 10 mM Tris-HCl, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/μl aprotinin, 10 μg/μl leupeptin and 1 mM sodium orthovanadate). The homogenate was briefly centrifuged to pellet debris and aliquots of the supernatant were taken for protein content using a BioRad protein assay. Supernatant proteins were then solubilized in Laemmli’s sample buffer (4:1, boiled for 5 min) and loaded and separated on a 10% SDS-polyacrylamide gel and then probed as described above.

Data analysis. Contractile data are presented as mean ± S.E.M. as a percentage of the phenylephrine (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 two groups responses (P < .05 considered statistically significant). Agonist EC50 values were calculated using a nonlinear regression analysis using the algorithm [effect = [maximum response/1 + (EC50/ agonist concentration)ln]. Quantification of band density was performed on a PowerMac 8100 computer using the public domain NIH Image program (written by Wayne Rasband at the U. S. 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 Rd., Springfield, VA 22161, part number PB93–504868). Band density is reported in arbitrary densitometry units.

Materials. Drug solutions were made in deionized water unless indicated otherwise in parentheses: acetylicholine chloride, angiotensin II, aprotinin, β-mercaptoethanol, chick egg ovalbumin, EGTA, leupeptin, nifedipine (ethanol), phenelphrine hydrochloride, sodium azide, sodium dodecyl sulfate, sodium orthovanadate, Tris base, Tris-HCl, Triton X-100, Tween-20 (Sigma Chemical); daidzein (dimethylsulfoxide), genistein (dimethylsulfoxide) and PD123319 (dimethylsulfoxide) (Research Biochemicals International, Natick, MA); Cooamassie brilliant blue (BioRad, Hercules, CA); tyrophostin B42 (dimethylsulfoxide; Calbiochem-Novabiochem International, San Diego, CA). PD098059 (dimethylsulfoxide) was a kind gift from Dr. David Dudley at Parkes Davis (Ann Arbor, MI).

Results

The first experiments performed were to determine the classification of the angiotensin receptor mediating contraction in the rat thoracic aorta. The AT1 receptor antagonist losartan (1 μM), at a concentration that minimally interacts with the AT2 receptor (Griendling et al., 1996), abolished contraction to Ang II (fig. 1). Moreover, neither the AT2 receptor antagonist PD123319 (100 nM) nor the tyrosine phosphatase inhibitor sodium orthovanadate (1 μM) altered Ang II-induced aortic contraction (fig. 1). These findings confirm that of multiple other investigators in that they suggest an AT1 receptor is solely responsible for mediating Ang II-induced aortic contraction.

We next examined the ability of various tyrosine kinase inhibitors to influence Ang II-induced contraction in the rat aorta. Genistein, a general tyrosine kinase inhibitor, was used at a concentration (5 × 10−6 M) that does not influence KCl-induced contraction or phorbol dibutyrate-induced contraction in the vasculature (Watts et al., 1996). Thus, we believe this concentration of genistein is relatively selective. Moreover, this concentration of genistein reduces aortic contraction to 5-HT, an agonist known to activate the MAPK pathway in the aorta and vascular smooth muscle cells (Watts, 1996; Watts et al., 1996). Genistein did not shift or reduce Ang II-induced contraction in the rat aorta (fig. 2, top). As expected, the inactive isomer of genistein, daidzein, also did not alter Ang II-induced contraction (fig. 2, bottom). In some experiments, after incubation with genistein (5 μM), tissues were challenged with just one concentration of angiotensin II (10 nM). This protocol minimizes the issue of desensitization, which may occur during a cumulative concentration-response curve to Ang II. Even in these experiments, genistein did not reduce Ang II-induced contraction. We then tested a more specific tyrosine kinase inhibitor, PD098059. PD098059 is an inhibitor of MEK, a dually active tyrosine and threonine kinase vital to the MAPK pathway (Dudley et al., 1995). PD098059 (10 μM) did not reduce or shift Ang II-induced rat aortic contraction (fig. 3, top). This finding confirms the general observations made with genistein: Ang II-induced contraction in the endothelium-denuded rat aorta does not appear to require tyrosine kinase activation. The same experiment was done in the presence of PD123319 with the idea that if AT2 receptors were activated, they may stimulate tyrosine phosphatases and mask the effect of tyrosine kinase activation mediated by AT1 receptors. This does not, however, appear to be the case as the combination of the MEK inhibitor PD098059 and AT2 receptor antagonist PD123319 did not alter Ang II-induced aortic contraction (fig. 3, bottom).

Tyrophostins are a group of tyrosine kinase inhibitors chem-
ically distinct from the flavone-derived genistein. Figure 4 (top) demonstrates that at 30 μM, tyrphostin B42, Ang II-induced contraction was significantly reduced but so to was aortic contraction activated by high potassium (fig. 4, bottom). In this instance, KCl-induced contraction was used as an indirect measure of L-type voltage-gated calcium channel activation. These findings suggest that while tyrphostin B42 may reduce Ang II-induced contraction, this reduction is likely because of inhibition of calcium channels. Maximal aortic contraction to Ang II was reduced by the L-type voltage-gated calcium channel blocker nifedipine (50 nM; fig. 5). This concentration of nifedipine reduced KCl-induced aortic contraction (fig. 5), and in past studies we have determined this to be the minimal concentration of nifedipine necessary to produce a maximal inhibition of KCl-induced aortic contraction (Florian and Watts, 1998). These data indicate that Ang II does utilize calcium channels for contraction and this is consistent with the inhibition observed with tyrphostin B42.

To assess more directly the ability of Ang II to activate tyrosine kinase(s), we used Western analyses to measure changes in protein tyrosyl-phosphorylation. Rat aortic vascular smooth muscle cells were incubated with one concentration of Ang II for 5 min, lysed and then taken through the Western protocol described in the methods. Figure 6 depicts the results of one experiment (experiment performed in 3 separate explants at least once in each explant where each explant is from a different rat); shown is a portion of the gel. Ang II potently (EC₅₀ between 0.1 and 1 nM) and steeply activated tyrosyl-phosphorylation of bands at 42 and 44 kDa (top gel strip). When the blot was reprobed with an antibody for the Erk MAPKs, two bands with a molecular weight consistent with that of Erk-1 (44 kDa) and Erk-2 (42 kDa) comigrated exactly with those identified by the phosphotyrosine antibody (fig. 6, bottom gel strip). These findings suggest that, as found by multiple other groups, Ang II can activate the MAPK pathway and the tyrosine kinase important to the MAPK pathway, MEK. These findings begin to dissociate the activation of the MAPK pathway from functioning in contraction as stimulated by Ang II.

The next experiments also supported this conclusion. Rat aortic smooth muscle cells were incubated with Ang II (10 nM) and either vehicle, PD098059 or losartan. The concentration of these two inhibitors used in cell experiments was identical to that used in the contractile studies (losartan 1 μM, PD098059 10 μM). The hypothesis tested in this experimental series was that if MEK can be activated but is not involved in contraction, then PD098059 should reduce Ang II-induced tyrosyl-phosphorylation of MAPK but should not, as we found, be able to reduce Ang II-induced contraction. Figure 7 shows the results of one of these experiments. Tyrosyl-phosphorylation activated by Ang II (10 nM) was com-
pletely abolished by losartan. Moreover, PD098059 reduced Ang II-stimulated phosphorylation of Erk-1 and Erk-2 by 70%, indicating that (1) the proteins were MAPKs as MAPKs are the substrate for MEK; and (2) Ang II is clearly capable of activating MEK. Taken together, these experiments demonstrate that while Ang II-induced Erk1/2 tyrosyl-phosphorylation and vascular contraction are similarly dependent on stimulation of the AT1 receptor, vascular contraction is not dependent on activation of tyrosine kinase(s), in particular MEK.

Figure 8 provides a connection between the cellular experiments and contractile experiments. This fig. shows the results from experiments in which aortic strips (denuded of endothelium) were contracted to Ang II and, 5 min into the contraction, were frozen with liquid-nitrogen cooled tongs, pulverized with liquid nitrogen, homogenized and loaded onto polyacrylamide gels for ultimate visualization of the tyrosyl-phosphorylation status of the Erk MAPK proteins. Ang II is capable of stimulating tyrosyl-phosphorylation of Erk1/2 during contraction and the inability of an inhibitor of the kinase responsible for phosphorylation of Erk1/2 (PD098059) to block Ang II-induced contraction suggests that this phosphorylation event and thus activation of the MAPK pathway is not important for contraction.

Discussion

The ability of Ang II to activate tyrosine kinases in vascular smooth muscle cells is an indisputable fact. Berk and Corson (1997) and Griendling et al. (1997) have done an outstanding job in detailing the proteins tyrosyl-phosphorylated upon Ang II-stimulation in the cardiovascular system. Included in this list are the extracellular signal-regulated kinases (Erk) mitogen-activated protein kinases (MAPKs) and other proteins known to participate in the MAPK cas-
ical event with activation of the MAPK pathway (Servant et al., 1997). This pathway is important in vascular smooth muscle growth as inhibitors of tyrosine kinase(s), specifically MEK, can reduce protein synthesis in vascular smooth muscle cells. Antagonists incubated with cells 1 hr prior to the addition of Ang II for 5 min. Representative of four experiments performed on cells derived from four separate explants, each from a different rat.

This is shown by the lack of ability of tyrosine kinase inhibitors to block Ang II-induced contraction. Specifically, neither the general tyrosine kinase inhibitor genistein nor an inhibitor of MEK, PD098059, shifted or reduced Ang II-induced contraction. It is possible that we have used submaximal concentrations of inhibitors, but several points lead us to believe this is not the case. First, these concentrations of inhibitors were chosen based on previous experiments with serotonin (Watts et al., 1996; Watts, 1996). These compounds were able to reduce contraction to 5-HT in the rat aorta and reduce 5-HT-stimulated tyrosyl-phosphorylation of the Erk1/2 proteins in rat aortic vascular smooth muscle. The concentration of genistein used presently is below that which can significantly influence L-type voltage-gated calcium channels (Wijetunge et al., 1992) and that of PD098059 nearly abolished the activity of glutathione-S-transferase MEK (Dudley et al., 1995) and caused maximal reduction of 5-HT-stimulated rat aortic contraction and tyrosyl-phosphorylation (Florian and Watts, 1998; Watts et al., 1996, Watts 1996). The concentration of tyrophostin B42 is in the range consistent with interaction with multiple tyrosine kinase(s), but useful concentrations of the tyrophostins seem to parallel those at which tyrophostins also block L-type voltage-gated calcium channel current in vascular smooth muscle cells (Wijetunge et al., 1992, Watts et al., 1996). For example, we have gone down in tyrophostin B42 concentration to 10 μM and find that while it does not inhibit Ang II-induced aortic contraction, tyrophostin B42 also does not reduce KCl-induced contraction (unpublished data). Our findings are in agreement with the interaction of tyrophostin B42 with calcium channels as the drug blocked KCl-induced contraction, a measure of activation of L-type voltage-gated calcium channels. Ang II-induced contraction was blocked by this concentration of tyrophostin B42, but it likely this effect was at least partially nonselective as Ang II-induced aortic contraction was reduced by nifedipine, supporting the dependence of Ang II on calcium channel activation for contraction. However, all of the inhibitory effects of tyrophostin B42 cannot be solely ascribed to inhibition of calcium channels because tyrophostin B42 reduced Ang II-induced maximal contraction to a greater extent than did a maximal concentration of nifedipine. These data suggest that tyrophostin B42 may inhibit the activity of other proteins important to contraction. Nonetheless, our data are consistent with idea that a substantial portion of the reduction of Ang II-induced aortic by tyrophostin B42 is through inhibition of calcium channel activation.

Our findings with respect to the involvement of tyrosine kinase(s) in Ang II-induced smooth muscle signaling of contraction differs from those of other groups in some respects. Ang II (1 μM)-induced contraction in guinea pig longitudinal and circular gastric smooth muscle was blocked by losartan and could be reduced by both genistein (7.4 μM) and tyrophostin (RG50864; 20 μM) (Yang et al., 1993). In the isolated guinea pig smooth muscle, Ang II (1 μM) also stimulated tyrosyl-phosphorylation of a protein in the 42–44 kDa range, suggesting the ability of Ang II to activate tyrosyl-phosphorylation of proteins (possibly the Erks). Thus, the AT1 receptor in guinea pig smooth muscle is different from that in the rat aorta in terms of its ability to link into the Erk MAPK pathway to influence contractility. In the vasculature, tyrophostin A42 (1 μM) but genistein (1 μM) was able to reduce Ang II (1 nM)-stimulated increases in intracellular calcium in primary cultures of Wistar-Kyoto mesenteric re-
sistance vessels (Touyz and Schiffrin, 1996), suggesting a possible interaction of tyrophostins with calcium channels.

One study in particular conflicts with our findings. The AT\textsubscript{1} receptor antagonist losartan, general tyrosine kinase inhibitor tyrphostin (AGS2; 40 \textmu M) and genistein (7.5 \textmu M) were shown to reduce Ang II (30 nM)-induced contraction in porcine coronary artery; in the same study, tyrphostin and genistein reduced Ang II (20 nM)-induced rat aortic contraction (Laniyonu et al., 1994). The general ability of tyrosine kinase inhibitors to reduce Ang II-induced contraction is different from what we observed. There are several possible reasons for these differences. First, it is possible that there is tissue specific ability of Ang II to activate the Erk1/2 in contraction: the rat aorta AT\textsubscript{1} receptor may not, for some reason, be similar to the porcine AT\textsubscript{1} receptor in its signal transduction. Second, while Laniyonu et al. (1994) demonstrated inhibition of Ang II-induced contraction by tyrosine kinase inhibitors in the aorta and stated that this was independent of inhibiting calcium channel activity (as measured by KCl-induced contraction), they make a point in their manuscript that deserves repeating. They state that at concentrations of genistein above 10 \textmu M, genistein cannot be used selectively as “there was no longer a clearcut selectivity in blocking agonist-mediated contraction” (Laniyonu et al., 1994). Genistein has been demonstrated to block L-type voltage-gated calcium channel current in vascular smooth muscle cells (Wijetunge et al., 1992) and we also have found that concentrations of genistein at 15 \textmu M causes the effects of daidzein, genistein’s inactive isomer, to cause similar reduction of 5-HT-induced contraction in the rat aorta (unpublished data). Moreover, we have demonstrated that tyrphostins can also reduced KCl-induced arterial contraction. These findings suggest that, if inappropriate concentrations of tyrosine kinase inhibitors are used, one can reduce arterial contraction with tyrosine kinases and possible lead to the false conclusion of tyrosine kinase involvement. In our experiments, we used 5 \textmu M genistein, a concentration that does not reduce KCl-induced contraction. Most importantly, we know that the concentrations of PD098059 which exert a strong and significant inhibition of Ang II-stimulated Erk1/2 tyrosyl-phosphorylation do not influence contraction stimulated by Ang II, nor do they inhibit KCl-induced arterial contraction (Watts, 1996). Thus, we believe the appropriate concentrations of inhibitors have been used in the present experiments.

The third difference between our studies and those of Laniyonu et al. (1994) is that the aortic strips in their study had intact endothelium while those in ours did not. Thus, our collective findings are not necessarily in disagreement but suggest that Ang II stimulates/inhibits production of a substance from the endothelium which activates/inhibits a tyrosine-kinase dependent pathway in smooth muscle, respectively. An example of such a substance is endothelin-1 and we are currently pursuing this avenue of research.

One of the most powerful pieces of data to dissociate Ang II-induced contraction from activation of the Erk MAPK pathway are those that involve PD098059. PD098059 is a specific inhibitor of MEK (Dudley et al., 1995) and we have used this compound before to associate 5-HT-induced contraction with Erk MAPK activation (Watts, 1996). Ang II-stimulated tyrosyl-phosphorylation of the Erk MAPK and protein synthesis was dramatically reduced by PD098059 (IC\textsubscript{50} = 4.3 \textmu M; Servant et al., 1996). In agreement with this study, we demonstrated that PD098059 could reduce Ang II-stimulated tyrosyl-phosphorylation of the Erk MAPK proteins in aortic vascular smooth muscle cells. This same concentration of PD098059 did not shift or reduce Ang II-induced aortic contraction. It is possible that AT receptor signaling is different in rats with arteriovascular smooth muscle cell and isolated rat aorta, but two points make this unlikely. First, an AT\textsubscript{1} receptor mediates both contraction and protein tyrosyl-phosphorylation as the AT\textsubscript{1} receptor antagonist losartan completely abolished both phosphorylation and aortic contraction. Moreover, we have presented data in this paper demonstrating the ability of Ang II to stimulate tyrosyl-phosphorylation of the Erk1/2 in the isolated rat aorta contracted to Ang II. Thus, these data suggest that while Ang II has the capability of activating MEK, this pathway does not appear to play a role in Ang II-induced rat aortic contraction.

AT\textsubscript{2} receptors have recently been linked to modulation of tyrosine phosphatase activity (Bottari et al., 1992; Kambara-yashi et al., 1993a, 1993b). If these receptors are present in aortic vasculature and activated at the same time as the AT\textsubscript{1} receptor, the signal transduction pathways activated by AT\textsubscript{2} receptors may balance those stimulated by the AT\textsubscript{1} receptor such that tyrosine kinase inhibitors might not be effective. Our data suggest that AT\textsubscript{2} receptors do not mediate Ang II-induced contraction, nor do they mask AT\textsubscript{1} receptor mediated tyrosine kinase activation. Thus, it is the AT\textsubscript{1} receptor that is primarily responsible for Ang II-induced contraction.

It is clear that agonists of G protein-coupled receptors can modulate vascular smooth muscle contraction via classic pathways and, as recently revealed, tyrosine kinase-dependent pathways. This does not, however, appear to be true for all agonists as we have demonstrated a disassociation of Ang II-induced contraction and protein tyrosyl-phosphorylation; the reasons for this are not obvious. Our results with respect to the involvement of the MAPK and/or tyrosine kinase-dependent pathway in Ang II-induced contraction differ from those of Sauro et al. (1996) in which Ang II was administered to the conscious rat intraperitoneally and the aorta harvested later (0–30 min). Western analyses of aortic homogenates showed phosphorylation of two proteins of ~55 kDa in size; phosphorylation of Erk1/2 was not addressed. Sauro et al. (1996) also demonstrated that Ang II-induced maximal aortic contraction in vitro could be slightly reduced by tyrphostin (100 \textmu M) in endothelium-denuded rings. It is important to note that while we have shown dissociation of MEK activation from Ang II-induced contraction in the rat aorta, this pathway may very well be important in smaller vessels of the rat (Molloy and Sauro, 1996). Understanding the ability of Ang II to activate this pathway, and its pursuant actions, is important as Ang II is a mitogen (Dubey et al., 1992; Huwiler et al., 1995; Leduc et al., 1995), can potentiate the mitogenic effects of EGF (Sambhi et al., 1992) and clearly plays a role in some forms of hypertension.

In summary, we have presented evidence that Ang II-induced contraction in the endothelium-denuded rat aorta is mediated solely through activation of an AT\textsubscript{1} receptor and cannot be blocked by the general tyrosine kinase inhibitor genistein, the MEK inhibitor PD098059 or enhanced by the tyrosine phosphatase inhibitor sodium orthovanadate; contraction could be inhibited by tyrphostin B42, but this inhibition is likely due to blockade of calcium channels. Western
analyses demonstrated that Ang II is capable of activating MEK in both aortic cells and whole aorta. Thus, Ang II-induced contraction is not dependent on activation of the MAPK pathway in the rat aorta.

References

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