Identification of an Essential Signaling Cascade for Mitogen-activated Protein Kinase Activation by Angiotensin II in Cultured Rat Vascular Smooth Muscle Cells

POSSIBLE REQUIREMENT OF Gq-MEDIATED p21\(^{ras}\) ACTIVATION COUPLED TO A Ca\(^{2+}\)/CALMODULIN-SENSITIVE TYROSINE KINASE\(^*\)

(Received for publication, January 18, 1996, and in revised form, March 19, 1996)

Satoru Eguchi, Takeshi Matsumoto, Evangeline D. Motley\(^\dagger\), Hiroshi Utsunomiya, and Tadashi Inagami\(^\S\)

From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 and the Department of Physiology, Meharry Medical College, Nashville, Tennessee 37208

In cultured rat vascular smooth muscle cells, angiotensin II (Ang II) induced a rapid increase in mitogen-activated protein kinase (MAPK) activity through the Ang II type 1 receptor, which was insensitive to pertussis toxin but was abolished by the phospholipase C inhibitor, U73122. The Ang II-induced MAPK activation was not affected by the protein kinase C inhibitor, GF109203X, and was only partially impaired by pretreatment with a phorbol ester, whereas both treatments completely prevented MAPK activation by the phorbol ester. Intracellular Ca\(^{2+}\) chelation by TMB-8, but not extracellular Ca\(^{2+}\) chelation or inhibition of Ca\(^{2+}\) influx, abolished Ang II-induced MAPK activation. The calmodulin inhibitor, calmidazolium, and the tyrosine kinase inhibitor, genistein, completely blocked MAPK activation by Ang II as well as by the Ca\(^{2+}\) ionophore A23187. Ang II caused a rapid increase in the binding of GTP to p21\(^{ras}\), and this was inhibited by genistein, TMB-8, and calmidazolium but not by pertussis toxin or GF109203X. These data suggest that Ang II-induced MAPK activation through the Ang II type 1 receptor could be mediated by p21\(^{ras}\) activation through a currently unidentified tyrosine kinase that lies downstream of G\(_{\alpha}\)-coupled Ca\(^{2+}\)/calmodulin signals.

The peptide hormone angiotensin II (Ang II)\(^1\) evokes diverse physiological responses, including arterial vasoconstriction, stimulation of aldosterone secretion, and renal sodium reabsorption (1). In addition, it is a growth-promoting factor for vascular smooth muscle cells (VSMC) (2–4), renal mesangial cells (5), cardiomyocytes (6), and cardiac fibroblasts (7). Since Ang II is believed to play a pivotal pathogenic role in the development of cardiovascular diseases such as hypertension and atherosclerosis (8, 9), there has been considerable interest in defining its signaling pathways that mediate the growth response of VSMC.

Pharmacological evidence has defined at least two subtypes of Ang II receptors, designated AT\(_1\) and AT\(_2\) (1). Recent molecular cloning has revealed that both receptor subtypes belong to the superfamily of G protein-coupled receptors with seven transmembrane helices (10–13). In cultured rat VSMC, AT\(_1\) activation by Ang II is initiated by stimulation of a phosphatidylinositol-specific phospholipase C (PI-PLC), leading to the generation of inositol trisphosphate (IP\(_3\)) and diacylglycerol (14), which are involved in intracellular Ca\(^{2+}\) mobilization (15) and protein kinase C (PKC) activation (16), respectively. In VSMC, Ang II also induces a rapid increase in expression of the growth-associated nuclear proto-oncogenes, c-fos, c-jun, and c-myc (17–19) and stimulates tyrosine phosphorylation of multiple substrates (20), including mitogen-activated protein kinases (MAPKs) (20–22).

MAPKs, also known as extracellular signal-regulated kinases, are a family of protein-serine/threonine kinases that are believed to function as integrators for mitogenic signals originating from several distinct classes of cell surface receptors, such as receptor tyrosine kinases and G protein-coupled receptors (23, 24). In their activated forms, p44\(^\text{ERK1}\) and p42\(^\text{ERK2}\) transmit extracellular stimuli by phosphorylating a variety of substrates including transcriptional factors and kinases (25). MAPKs are activated by phosphorylation of both threonine and tyrosine residues (26) catalyzed by an MAPK kinase (27) also known as MEK (28). MEK is in turn regulated by serine phosphorylation by several MAPK kinase kinases, including Raf-1 (29, 30). Recently, the cascade from growth factor receptor tyrosine kinases to MAPK has been elucidated. The adapter protein Grb2 links the tyrosine-phosphorylated receptor to Sos, which acts as a guanine nucleotide exchange factor for p21\(^{ras}\) (31), and the active GTP-bound p21\(^{ras}\) stimulates Raf-1 kinase activity toward MEK (32, 33). However, the pathway originating from G protein-coupled receptors in the activation of MAPK is not clearly defined.

Earlier reports proposed a dominant role of PKC in the mechanism of Ang II-mediated MAPK activation in VSMC (20, 21), whereas more recent studies have indicated that calcium signals rather than PKC are critical for MAPK activation by Ang II in cardiac cells (34, 35). To define the signal transduction cascades leading to MAPK activation by the AT\(_1\) receptor, we examined the roles of various signaling molecules activated by Ang II through AT\(_1\) in cultured rat aortic VSMC. We found that AT\(_1\) signals to p21\(^{ras}\) and subsequently to MAPK, possibly...
through Ca\(^{2+}\)/calmodulin-sensitive activation of a protein tyrosine kinase by a G\(_{13}\)-coupled pathway.

**EXPERIMENTAL PROCEDURES**

**Materials—** Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, penicillin, and streptomycin were obtained from Life Technologies, Inc. Ang II was purchased from Peninsula Laboratories, U73122, GF109203X, BAPTA-AM, TMB-8, nifedipine, W-7, calmidazolium chloride, genistein, and ST638 were purchased from Calbiochem. The AT\(_1\) antagonist DUP753 was a generous gift of DuPont Merck Pharmaceutical Co., and the AT\(_2\) antagonist PD123319 was purchased from Research Biochemicals, Inc. Pertussis toxin (PTX), phorbol 12-myristate 13-acetate (PMA), lysophosphatidic acid (LPA), and EGTA were obtained from Sigma.

Cell Culture—VSMC were prepared from the thoracic aorta of 12-week-old Sprague-Dawley rats (Charles River Laboratories) by the explant method and cultured in DMEM containing 10% fetal calf serum, penicillin, and streptomycin as described previously (36). Subcultured VSMC from passages 3–15, used in the experiments, showed >99% positive immunostaining of smooth muscle \(\alpha\)-actin antibody (Sigma) and were negative for mycoplasma infection by the polymerase chain reaction kit (Stratagene). The expressions of AT\(_1\) receptors and endothelin type A receptors (36) were confirmed on the basis of binding studies with specific receptor antagonists. For the experiments, cells at ~80% confluence in culture wells were made quiescent by incubation with serum-free DMEM for 3 days, unless otherwise stated.

**MAPK Activity—** VSMC grown on a 24-well plate were stimulated with agonists at 37°C in serum-free DMEM for specified durations. The reaction was terminated by the replacement of medium with the ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 20 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na\(_3\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml leupeptin, and 10 \(\mu\)g/ml aprotinin). After brief sonication (10 s), the samples were centrifuged for 5 min at 14,000 \(\times\) g, and the supernatant was assayed for MAPK activity with an assay kit (Amersham Corp.) that measures the incorporation of [\(\gamma\)\(^{32}\)P]ATP into a synthetic peptide (KRELVEPLTPAGEAPNQALLR) as a specific MAPK substrate. The reaction was carried out with the cell lysate (~1 \(\mu\)g of protein) in 75 mM HEPES buffer, pH 7.4, containing 1.2 mM MgCl\(_2\), 2 mM substrate peptide, and 1.2 mM ATP, 1 \(\mu\)Ci of [\(\gamma\)\(^{32}\)P]ATP for 30 min at 30°C. The resultant solution was applied to a phosphocellulose membrane and extensively washed in 1% acetic acid and then in \(\mathrm{H}_2\mathrm{O}\). The radioactivity trapped on the membrane was measured by liquid scintillation counting.

**Immuno blotting—** VSMC grown on a 6-well plate were stimulated with agonists at 37°C in serum-free DMEM for specified durations. The reaction was terminated by the replacement of medium with 100 \(\mu\)l of SDS-polyacrylamide gel electrophoresis buffer, pH 6.8, containing 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue. After brief sonication (5 s), the samples were boiled for 5 min at 95°C and centrifuged (14,000 \(\times\) g, 5 min) at 4°C. The resultant solution was applied to a phosphocellulose membrane and extensively washed in 1% acetic acid and then in \(\mathrm{H}_2\mathrm{O}\). The radioactivity trapped on the membrane was measured by liquid scintillation counting.

**Immunoblotting**—VSMC grown on a 6-well plate were stimulated with agonists at 37°C in serum-free DMEM for specified durations. The reaction was terminated by the replacement of medium with 100 \(\mu\)l of SDS-polyacrylamide gel electrophoresis buffer, pH 6.8, containing 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue. After brief sonication (5 s), the samples were boiled for 5 min at 95°C and centrifuged (14,000 \(\times\) g, 5 min) at 4°C, and the supernatant (25 \(\mu\)l) was subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gel were transferred to a polyvinylidene difluoride membrane (Schleicher & Schuell) by electroblotting. The membrane was treated with rabbit polyclonal phospho-specific MAPK antibodies (New England Biolabs Inc.) that detect p42\(^{mitogen}\) and p44\(^{mitogen}\) only when catalytically activated by phosphorylation at Tyr-204. After incubation with secondary anti-rabbit antibodies, immunoreactive proteins were detected by the CDP-Star chemiluminescent system (New England Biolabs Inc.).

**Analysis of GTP-bound Ras—** Detection of guanine nucleotides bound to p21\(^{ras}\) was performed essentially as described previously (37). VSMC grown on a 6-well plate were prelabeled with 0.1 \(\mu\)Ci/ml carrier-free \(\gamma\)\(^{32}\)P-orthophosphate for 18 h in phosphate-free DMEM. Cells were stimulated with agonists at 37°C for specified durations. The reaction was terminated by aspirating the media, and cells were solubilized in 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 20 mM MgCl\(_2\), 1 mM Na\(_3\)VO\(_4\), 0.4 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml leupeptin, and 10 \(\mu\)g/ml aprotinin. After centrifugation for 5 min at 2000 \(\times\) g, the supernatant was immunoprecipitated with an anti-Ha-Ras-agarose conjugate (Santa Cruz Biotechnology, Inc.) for 90 min at 4°C. The immune complexes were washed 4 times with solubilization buffer, and p21\(^{ras}\)-associated guanine nucleotides were eluted in 2 \(\mu\l\) EDTA, pH 8.0, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP for 20 min at 65°C. Eluted GTP and GDP were separated on a polyethyleneimine cellulose plate by thin layer chromatography using 1.2\% ammonium formate, 0.8\% hydrochloric acid as the solvent. Labeled nucleotides were visualized and analyzed by PhosphorImager (Molecular Dynamics).

**RESULTS**

**Ang II Type I Receptor Stimulation Leads to MAPK Activation—** In cultured rat VSMC, the Ang II-induced (100 nM) stimulation of MAPK activity peaked at 5 min and declined in 10–30 min (Fig. 1A). The MAPK activation was dependent on the concentration of Ang II (Fig. 1B); increased activity was initially detectable at 0.01 nM, half-maximal at approximately 0.2 nM, and maximal at 10–100 nM. Treatment with 100 nM Ang II for 5 min also resulted in marked tyrosine phosphorylation of p44\(^{mitogen}\) and p42\(^{mitogen}\) only when catalytically activated by phosphorylation at Tyr-204. After incubation with secondary anti-rabbit antibodies, immunoreactive proteins were detected by the CDP-Star chemiluminescent system (New England Biolabs Inc.).

**Analysis of GTP-bound Ras—** Detection of guanine nucleotides bound to p21\(^{ras}\) was performed essentially as described previously (37). VSMC grown on a 6-well plate were prelabeled with 0.1 \(\mu\)Ci/ml carrier-free \(\gamma\)\(^{32}\)P-orthophosphate for 18 h in phosphate-free DMEM. Cells were stimulated with agonists at 37°C for specified durations. The reaction was terminated by aspirating the media, and cells were solubilized in 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 20 mM MgCl\(_2\), 1 mM Na\(_3\)VO\(_4\), 0.4 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml leupeptin, and 10 \(\mu\)g/ml aprotinin. After centrifugation for 5 min at 2000 \(\times\) g, the supernatant was immunoprecipitated with an anti-Ha-Ras-agarose conjugate (Santa Cruz Biotechnology, Inc.) for 90 min at 4°C. The immune complexes were washed 4 times with solubilization buffer, and p21\(^{ras}\)-associated guanine nucleotides were eluted in 2 \(\mu\l\) EDTA, pH 8.0, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP for 20 min at 65°C. Eluted GTP and GDP were separated on a polyethyleneimine cellulose plate by thin layer chromatography using 1.2\% ammonium formate, 0.8\% hydrochloric acid as the solvent. Labeled nucleotides were visualized and analyzed by PhosphorImager (Molecular Dynamics).

**RESULTS**

**Ang II Type I Receptor Stimulation Leads to MAPK Activation—** In cultured rat VSMC, the Ang II-induced (100 nM) stimulation of MAPK activity peaked at 5 min and declined in 10–30 min (Fig. 1A). The MAPK activation was dependent on the concentration of Ang II (Fig. 1B); increased activity was initially detectable at 0.01 nM, half-maximal at approximately 0.2 nM, and maximal at 10–100 nM. Treatment with 100 nM Ang II for 5 min also resulted in marked tyrosine phosphorylation of p44\(^{mitogen}\) and p42\(^{mitogen}\) in VSMC, whereas no significant phosphorylation was observed without the stimulation (for examples see Figs. 2, 3, 5, and 7). Therefore, subsequent MAPK experiments were performed with 100 nM Ang II stimulation for 5 min. To determine which Ang II receptor subtype mediates MAPK activation, VSMC were pretreated with either the AT\(_1\) antagonist DUP753 or the AT\(_2\) antagonist PD123319. MAPK activation by Ang II was markedly inhibited by 10 \(\mu\)M DUP753 but not by 10 \(\mu\)M PD123319 (Fig. 1C), indicating that Ang II-induced MAPK activation is mainly mediated by the AT\(_1\) receptor in VSMC.
AT1 receptors have been reported to be coupled to either Gq or Gi, which activates PI-PLC or inhibits adenylate cyclase, respectively (1, 38). To determine which G protein-mediated signaling is involved in the MAPK activation, the effects of PTX and the specific PI-PLC inhibitor U73122 (39) on Ang II-induced MAPK activation in VSMC. Treatment with PTX (1 μg/ml) for 24 h did not affect Ang II-induced MAPK activation, whereas it inhibited MAPK activation induced by LPA that had been reported to use Gi for MAPK activation (40, 41) (Fig. 2A). In contrast, U73122 completely suppressed Ang II-induced MAPK activation in a dose-dependent manner without affecting basal MAPK activity (Fig. 2B). The half-maximal inhibition (2.5 μM) was comparable with that for PI-PLC (39). U73122 (10 μM) also inhibited Ang II-induced tyrosine phosphorylation of p44<sup>mapk</sup> and p42<sup>mapk</sup> in VSMC (Fig. 2C). These data suggest that PI-PLC activation through a PTX-insensitive G protein (probably G<sub>i</sub>) plays a critical role in Ang II-induced MAPK activation in VSMC.

Roles of Protein Kinase C in Ang II-induced MAPK Activation—In cultured VSMC, PI-PLC activation by Ang II leads to production of two second messengers, IP<sub>3</sub> and diacylglycerol (14), that induce the release of Ca<sup>2+</sup> from intracellular stores (15) and PKC activation (16). Since PKC activation by a phorbol ester has been reported to stimulate MAPK (42), we examined whether phorbol ester-sensitive PKC is essential for Ang II-induced MAPK activation in VSMC. Although depletion of PKC by a 24-h pretreatment with PMA (10–1000 nM) moderately increased basal MAPK activity, it completely inhibited Ang II-induced MAPK activation in VSMC (44, 45). Therefore, we further examined the effect of the specific PKC inhibitor GF109203X on Ang II-induced MAPK activation in VSMC. Pretreatment with 2 μM GF109203X completely inhibited MAPK activation in response to 100 nM PMA without affecting basal MAPK activity, whereas no significant inhibition was observed in Ang II-induced MAPK activation (Fig. 3B). GF109203X (2 μM) also inhibited tyrosine phosphorylation of p44<sup>mapk</sup> and p42<sup>mapk</sup> induced by PMA but not by Ang II in VSMC (Fig. 3C and D). These data indicate that Ang II-induced MAPK activation was at least independent of GF109203X-sensitive PKC in VSMC.

Calcium and Calmodulin-dependent MAPK Activation by Ang II—In cultured VSMC, Ang II has been shown to cause a
rapid and transient elevation of cytosolic Ca\(^{2+}\) released from the IP\(_3\)-sensitive intracellular stores by the activation of PI-PLC. This is followed by a sustained elevation of cytosolic Ca\(^{2+}\) through its influx mediated by an L-type Ca\(^{2+}\) channel (46, 47). Since intracellular Ca\(^{2+}\) elevation has been reported to be a sufficient stimulus for MAPK activation (48), we sought to determine whether the MAPK activation by Ang II was Ca\(^{2+}\)-dependent. Pretreatment with BAPTA-AM (10 \(\mu\)M) or TMB-8 (100 \(\mu\)M), drugs commonly used as intracellular Ca\(^{2+}\) chelators, resulted in complete loss of MAPK activation induced by Ang II (Fig. 4, A and B). In contrast, extracellular Ca\(^{2+}\) chelation by EGTA or blockade of L-type Ca\(^{2+}\) channels with nifedipine failed to inhibit Ang II-induced MAPK activation (Fig. 4, C and D) even though these treatments abolished the Ang II-induced sustained phase of Ca\(^{2+}\) elevation. Parallel inhibitory patterns in Ang II-induced tyrosine phosphorylation of p44\(^{\text{mapk}}\) and p42\(^{\text{mapk}}\) were observed with BAPTA-AM and TMB-8, whereas nifedipine did not affect the tyrosine phosphorylation of MAPKs (Fig. 5). These data indicate that the release of Ca\(^{2+}\) from IP\(_3\)-sensitive stores, rather than Ca\(^{2+}\) influx, may play a major role in Ang II-induced MAPK activation in VSMC.

Elevation of cytosolic Ca\(^{2+}\) is known to activate a variety of enzymes through its interaction with calmodulin (49). To examine whether calmodulin mediates MAPK activation in response to Ang II, VSMC were preincubated with well characterized calmodulin inhibitors, W-7 or calmidazolium. Although pretreatment with high concentrations of W-7 decreased basal MAPK activity, it dose dependently and completely inhibited Ang II-induced MAPK activation (Fig. 6A). Half-maximal inhibition was observed at ~25 \(\mu\)M, which was comparable with that for Ca\(^{2+}\)/calmodulin-dependent phosphodiesterase (50). Pretreatment with 10 \(\mu\)M calmidazolium also completely inhibited both activation of MAPK (Fig. 6B) and tyrosine phosphorylation of p44\(^{\text{mapk}}\) and p42\(^{\text{mapk}}\) (data not shown) induced by Ang II in VSMC. Treatment with a Ca\(^{2+}\) ionophore, A23187 (10 \(\mu\)M), resulted in marked stimulation of MAPK activity that was also inhibited by calmidazolium but not by GF109203X (Fig. 6C). These data suggest that Ang II stimulates MAPK activity through a Ca\(^{2+}\)/calmodulin-dependent mechanism.

Roles of Protein Tyrosine Kinase in Calcium-dependent MAPK Activation—Ang II has been shown to cause a rapid increase in tyrosine phosphorylation of multiple cellular proteins prior to MAPK activation in VSMC (20). To determine whether tyrosine kinase activity is required for the Ca\(^{2+}\)-dependent MAPK activation in response to Ang II, VSMC were

![Figure 4](image-url) **Fig. 4. Effects of calcium signal inhibitors on Ang II-induced MAPK activation.** VSMC were pretreated with the intracellular Ca\(^{2+}\) chelator BAPTA-AM (10 \(\mu\)M) for 30 min (A), another intracellular Ca\(^{2+}\) chelator TMB-8 (100 \(\mu\)M) for 30 min (B), the extracellular Ca\(^{2+}\) chelator EGTA (5 mM) for 3 min (C), or the L-type Ca\(^{2+}\) channel blocker nifedipine (1 \(\mu\)M) for 3 min (D) and then stimulated with Ang II (100 nM) for 5 min. Results shown are the mean ± S.D. of at least triplicate determinations.

![Figure 5](image-url) **Fig. 5. Effects of calcium signal inhibitors on Ang II-induced tyrosine phosphorylation of MAPKs.** VSMC were pretreated with 10 \(\mu\)M BAPTA-AM for 30 min, 100 \(\mu\)M TMB-8 for 30 min, or 1 \(\mu\)M nifedipine for 3 min and then stimulated with Ang II (100 nM) for 5 min. Blots shown are representative of three separate experiments.

![Figure 6](image-url) **Fig. 6. Roles of calmodulin in Ang II-induced MAPK activation.** A, VSMC were pretreated with indicated concentrations of the calmodulin inhibitor W-7 for 30 min and stimulated with Ang II (100 nM) for 5 min. B, VSMC were pretreated with the calmodulin inhibitor calmidazolium (10 \(\mu\)M) for 30 min and stimulated with Ang II (100 nM) for 5 min. C, VSMC were pretreated with either calmidazolium (10 \(\mu\)M) or GF109203X (GFX, 2 \(\mu\)M) for 30 min and then stimulated with the Ca\(^{2+}\) ionophore A23187 (10 \(\mu\)M) for 5 min. Results shown are the mean ± S.D. of at least triplicate determinations.
pretreated with genistein, a protein kinase inhibitor with a strong preference for tyrosine-specific kinases that acts as a competitive inhibitor of ATP binding (51), and stimulated by either Ang II or A23187. Genistein (100 μM) completely abolished both Ang II- and A23187-induced MAPK activations without affecting basal MAPK activity in VSMC (Fig. 7A). A similar inhibitory effect of genistein was observed in tyrosine phosphorylation of p44\textsuperscript{MAPK} and p42\textsuperscript{MAPK} induced by Ang II (Fig. 7B). To confirm the effect of genistein, we tested another specific tyrosine kinase inhibitor (ST638) that acts as a competitive inhibitor of substrate binding (52). Pretreatment with 100 μM ST638 for 60 min significantly inhibited MAPK activation induced by Ang II or A23187 in VSMC (data not shown). These data point to the possibility that Ang II-induced MAPK activation is at least in part mediated by a currently unidentified protein tyrosine kinase that lies downstream of a Ca\textsuperscript{2+}/calmodulin-activated system in VSMC.

Ang II Increases GTP-bound Ras—To further characterize the signaling cascade leading to MAPK activation in response to Ang II, we tested whether Ang II stimulates p21\textsuperscript{Ras} activity in VSMC. Ang II (100 nM) induced a rapid accumulation of GTP-bound p21\textsuperscript{Ras} that reached a maximum in 3-4 min, when it increased approximately 2-fold (Fig. 8A and B). This response returned to the baseline level in 20 min (Fig. 8B). We also found that treatment with 10 μM A23187 increased the GTP-bound p21\textsuperscript{Ras} in VSMC to a similar extent (Fig. 8C). To investigate whether the activation of p21\textsuperscript{Ras} and MAPK by Ang II requires similar upstream signaling, the effects of several signal transduction inhibitors were tested on Ang II-induced p21\textsuperscript{Ras} activation. Pretreatment with genistein (100 μM), TMB-8 (100 μM), or calmidazolium (10 μM), but not with PTX (1 μg/ml) or GF109203X (2 μM), prevented Ang II-induced p21\textsuperscript{Ras} activation (Fig. 9). Taken together, these data suggest that an AT\textsubscript{1}-mediated Ca\textsuperscript{2+}/calmodulin signal initiated by G\textsubscript{q} may activate the sequential cascade from p21\textsuperscript{Ras} to MAPK, a pathway common to the tyrosine kinase receptor, through a tyrosine kinase-dependent mechanism in VSMC.

**DISCUSSION**

The signaling mechanism leading to MAPK activation from heterotrimeric G protein-coupled receptors has not been clearly defined yet. In the present study, we have proposed a novel signaling pathway from AT\textsubscript{1} to the MAPK cascade involving p21\textsuperscript{Ras} activation mediated by a Ca\textsuperscript{2+}/calmodulin-dependent tyrosine kinase.

As we demonstrated by specific Ang II receptor antagonists, G protein-coupled AT\textsubscript{1} stimulation leads to MAPK activation in cultured rat VSMC. The doned AT\textsubscript{1} receptor can couple to either G\textsubscript{q} or G\textsubscript{i} (38). Recent evidence suggests that the G\textsubscript{i}-coupled LPA receptor induces PTX-sensitive MAPK activation through p21\textsuperscript{Ras} stimulation (40, 41, 53). This process is believed to be mediated by the βγ subunit of G\textsubscript{i} proteins (54–56). In the present study, control experiments using LPA as the agonist showed that PTX treatment abolished its MAPK activation in VSMC, indicating the presence of this cascade in our VSMC. However, Ang II-induced MAPK activation was PTX-insensi-
Angiotensin II Stimulates \( p21^{\text{ras}} \) and MAPK

**FIG. 9. Effects of signaling inhibitors on Ang II-induced \( p21^{\text{ras}} \) activation.** VSMC were incubated with PTX (1 \( \mu \)g/ml) for 24 h, GF109203X (GFX, 2 \( \mu \)M) for 30 min, TMB-8 (100 \( \mu \)M) for 30 min, calmidazolium (10 \( \mu \)M) for 30 min, or genistein (100 \( \mu \)M) for 45 min and then stimulated with 100 nM Ang II for 3 min. Results shown are the mean \( \pm \) S.D. of at least three independent experiments.

by guest on April 26, 2019http://www.jbc.org/Downloaded from
p21ras activation are under investigation.

It is likely that other signaling pathways capable of mediating MAPK activation via the AT1 receptor may exist. The typical G<sub>a</sub> coupled <sub>a</sub>jpressed adrenergic receptor and M1 muscarinic cholineric receptor transfected into COS-7 cells have been shown to induce PKC-dependent and p21ras-independent MAPK activation that is insensitive to genistein (59), whereas PKC was also reported to induce p21ras-dependent MAPK activation in PC12 cells (76). The anaphylatoxin C5a receptor, which can be coupled to either G<sub>i</sub> or G<sub>q</sub> (a homologue of G<sub>b</sub>), requires both a G<sub>i</sub>-mediated Gi<sub>b</sub> signal and G<sub>q</sub>-mediated PKC activation for maximal PKC activation (77). These data support the concept that the mechanism utilized by a given receptor to stimulate MAPK activation is likely to depend on the class of G proteins and downstream components available in a given cell type (59). Therefore, under physiological conditions, it is possible that additional signaling molecules such as Gi<sub>b</sub> and PKC may contribute synergistically to Ang II-induced MAPK activation in a cell- and tissue-type-dependent manner.

In summary, we have presented several lines of evidence that Ang II-induced p21ras and MAPK activation in VSMC is mainly mediated by a Ca<sup>2+</sup>/calmodulin-dependent tyrosine kinase nase through PI-PLC-mediated Ca<sup>2+</sup> release coupled to Gi<sub>b</sub>. The cascade demonstrated here will have to be considered when defining the pathophysiologic role of Ang II in the abnormal growth of VSMC observed in cardiovascular diseases.

Acknowledgments—We thank T. Fitzgerald and E. Price for excellent technical assistance and T. Stack for secretarial assistance.

REFERENCES

1. Timmermans, P. B. M. W. M., Wong, P. C., Chiu, A. T., Herblin, W. F., Benfield, P., Carini, D. J., Lee, R. J., Weiler, R. R., Saye, J. A., and Smith, R. D. (1993) Pharmacol. Rev. 45, 205–251

2. Gerstberger, S. A., Peach, M. J., and O’Connor, I. J. (1988) Circ. Res. 62, 749–755

3. Gibbons, G. H., Pratt, R. E., and Dauz, V. J. (1992) Clin. Invest. 90, 456–461

4. Weber, H., Taylor, D. S., and Molloy, C. J. (1994) J. Biol. Chem. 269, 23129–23135

5. Sasaki, K., Yamamoto, Y., Nakamura, T., Tsubouchi, H., Ishikawa, N., and Nishizuka, Y. (1993) J. Biol. Chem. 268, 14553–14556

6. Naftilan, A. J., Gilliland, G. K., Eldridge, C. S., and Kraft, A. S. (1990) J. Biol. Chem. 265, 20717–20720

7. Kambayashi, Y., Bardhan, S., Takahashi, K., Tsuzuki, S., Inui, H., Hamakubo, T., and Ogawa, H. (1993) J. Biol. Chem. 268, 14557–14562

8. Naftilan, A. J., Gilliland, G. K., Eldridge, C. S., and Kraft, A. S. (1990) J. Biol. Chem. 265, 20717–20720

9. Akiyama, T., and Ogawara, H. (1991) J. Biol. Chem. 266, 201, 362–370

10. Akiyama, T., and Ogawara, H. (1991) J. Biol. Chem. 266, 201, 362–370

11. Berridge, M. J. (1987) J. Biol. Chem. 262, 16916–16925

12. Berridge, M. J. (1987) J. Biol. Chem. 262, 16916–16925

13. Blenis, J. (1993) Science 268, 14553–14556

14. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556

15. McCormick, F. (1993) Nature 365, 15–16

16. Moodie, S. A., Wilkinson, B. M., Weber, J. M., and Wolfman, A. (1993) Science 260, 1658–1661

17. Yeh, S. H., Hsu, C. H., Chen, H. J., and Tsai, C. J. (1993) Cell 74, 205–214

18. Naftilan, A. J., Gilliland, G. K., Eldridge, C. S., and Kraft, A. S. (1990) J. Biol. Chem. 265, 20717–20720

19. Huckle, W. R., Prokop, C. A., Dy, R. C., Herman, B., and Earp, S. (1990) J. Biol. Chem. 265, 20717–20720

20. Kruse, H., Bauriedel, G., Heimerl, J., and Weber, P. C. (1994) Cardiovasc. Pharmacol. 24, 328–335

21. Peters, R. L., Gerke, J., and Gerke, J. (1993) J. Biol. Chem. 268, 19876–19883

22. Hao, J. F., and Schulman, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 91, 11267–11270

23. Bock, C. M., Alessandrini, A., and Erikson, R. L. (1992) Science 257, 205–214

24. Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., and Rapp, U. R. (1994) Science 268, 478–480

25. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556

26. Angiotensin II Stimulates p21ras and MAPK Ratios and MAPK Activation in VSMC is...
Identification of an Essential Signaling Cascade for Mitogen-activated Protein Kinase Activation by Angiotensin II in Cultured Rat Vascular Smooth Muscle Cells: POSSIBLE REQUIREMENT OF Gq-MEDIATED p21ras ACTIVATION COUPLED TO A Ca2+/CALMODULIN-SENSITIVE TYROSINE KINASE
Satoru Eguchi, Takeshi Matsumoto, Evangeline D. Motley, Hirotoshi Utsunomiya and Tadashi Inagami

J. Biol. Chem. 1996, 271:14169-14175.
doi: 10.1074/jbc.271.24.14169

Access the most updated version of this article at http://www.jbc.org/content/271/24/14169

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 77 references, 45 of which can be accessed free at http://www.jbc.org/content/271/24/14169.full.html#ref-list-1