0026-895X/96/030522-07$3.00/0

MOLECULAR PHARMACOLOGY, 50:522–528 (1996).

Angiotensin II Type 1 Receptor Signals through Raf-1 by a Protein Kinase C-Dependent, Ras-Independent Mechanism

HIDENORI ARAI[1] and JAIME A. ESCOBEDO[2]

Daiichi Research Center, Cardiovascular Research Institute, University of California San Francisco, California 94143-0130

Received February 8, 1996; Accepted May 23, 1996

SUMMARY

To understand the molecular mechanism by which the angiotensin II (AII) type 1 receptor (AT_1 receptor) transduces its biological signal, we examined the role of various signaling molecules involved in AT_1 receptor signaling in Chinese hamster ovary cells stably transfected with the AT_1 receptor. AT_1 receptor-transfected cells responded to AII treatment by inhibiting adenylyl cyclase, increasing the intracellular Ca^{2+} concentration, and activating protein kinase C (PKC)α and PKCϵ. AII also activated the c-*fos* gene and mitogen-activated protein (MAP) kinases. The activation of PKC, the c-*fos* gene, and MAP kinases was blocked by inhibition of PKC induced by pretreatment with 12-*O*-tetradecanoylphorbol-13-acetate but not by pretreatment with pertussis toxin, suggesting that PKC couples to the activation of the the c-*fos* gene and MAP kinases. In addition, AII activated Raf-1 and MAP kinase kinase in a PKC-dependent manner. A dominant negative mutant of Ras had no effect on AII-induced MAP kinase or c-*fos* gene activation. Thus, the AT_1 receptor signals through Raf-1 and its downstream signaling molecules by a PKC-dependent mechanism that does not involve Ras activation.

AII, a biologically active product of the renin-angiotensin system, is a major regulator of smooth muscle cell proliferation, blood pressure, and electrolyte balance (1). These diverse actions are believed to be mediated through the AT_1 receptor, which belongs to the seven-transmembrane G protein-coupled receptor family (2–4). In the signal transduction triggered by AII, binding of AII to the AT_1 receptor stimulates a number of signaling molecules (5), including PLCβ, which is activated through the G_q family, to generate inositol-1,4,5-trisphosphate and diacylglycerol. Inositol-1,4,5-trisphosphate then releases calcium from intracellular stores, and diacylglycerol activates PKC (6). The AT_1 receptor has also been shown to couple to PTX-sensitive G_i/G_o, resulting in the inhibition of adenylyl cyclase (7). Further along this signal transduction system, AII activates Raf-1 and MAP kinases (8, 9), as previously found for tyrosine kinase receptors such as epidermal growth factor receptors (10). MAP kinases transmit mitogenic signals to effector molecules such as the proto-oncogenes c-*fos*, c-*myc*, and c-*jun*, which are involved in AT_1 receptor signaling (11–13). Among those proto-oncogenes, the c-*fos* gene is one of the best characterized examples of these immediate/early-response genes, and its induction correlates with AII-induced proliferation of smooth muscle cells (13).

Three mechanisms have been proposed to link G protein-coupled receptors to MAP kinases and further-downstream effector molecules. One possible mechanism is direct activation of Raf-1 by PKC. PKCα can activate Raf-1 by phosphorylating a serine residue at position 499 (14, 15). A second possible mechanism is Ras-dependent activation through the $\beta\gamma$ subunit of G proteins, leading to sequential activation of a group of serine/threonine kinases, such as Raf, MEK, and MAP kinases. This effect of Ras in receptor signaling has been shown in G_i-coupled acetylcholine muscarinic m2 receptors (16), lysophosphatidic acid receptors (17), α_2-adrenergic receptors (18), and thrombin receptors (19). A third possible mechanism is the recently discovered MEKK-mediated activation of MAP kinases (20), although recent data suggest that MEKK preferentially activates c-Jun amino-terminal kinase (21). Because previous reports have shown that c-*fos* gene activation by AII in cardiac myoblasts is partially PKC dependent (22) and Raf-1 activation by AII is PKC dependent

This work was supported by Daiichi Pharmaceutical Co., Ltd. H.A. was supported by grants from Japan Heart Foundation, Mochida Momorial Foundation, and the International Atherosclerosis Society.

[1] Current affiliation: Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, CA 94141-9100.

[2] Current affiliation: Chiron Corporation, Emeryville, CA 94608.

ABBREVIATIONS: AII, angiotensin II; AT_1, type 1 angiotensin II; PLC, phospholipase; PKC, protein kinase C; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CHO, Chinese hamster ovary; β-gal, β-galactosidase; PTX, pertussis toxin; GST, glutathione-*S*-transferase; PBS, phosphate-buffered saline; FCS, fetal calf serum; MBP, myelin basic protein(s); TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDGF, platelet-derived growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

in smooth muscle cells (8), we investigated which signaling events were induced by AII and whether these pathways were PKC dependent in CHO cell lines stably expressing the human AT$_1$ receptor. We also explored the role of Ras in c-*fos* activation by using a dominant negative mutant of Ras.

Experimental Procedures

Materials and chemicals. AII was obtained from Peninsula Laboratories (Belmont, CA). Lipofectamine, OPTI-MEM I, and anti-PKC isoform-specific antibodies were obtained from Life Technologies (Grand Island, NY). Luciferase assay system and Galacto-Light for β-gal assay were obtained from Promega (Madison, WI) and Tropix (Bedford, MA), respectively. PTX was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Anti-ERK2 antibody and anti-Raf-1 antibody were purchased from Santa Cruz Biochemicals (Santa Cruz, CA), and anti-MEK1&2 antibody was from Transduction Laboratories (Lexington, KY). Anti-HA antibody (12CA5) was kindly provided by Qianjin Hu (University of California, San Francisco, CA). *Escherichia coli* producing GST fused to MEK (GST-MEK) and GST fused to a kinase negative mutant of ERK1 (GST-KNERK1) were kindly provided by R. L. Erikson (Harvard University, Cambridge, MA). GST-MEK and GST-KNERK were purified as described previously (23), and GST-MEK was digested by thrombin to remove GST.

Plasmids. The human AT$_1$ receptor cDNA was cloned by polymerase chain reaction using human aortic smooth muscle cDNA (InVitrogen, San Diego, CA) as a template and cloned into expression vector pBJ-1 using *Not*I and *Eco*RV sites. p2FTL, a luciferase reporter plasmid that has the serum response element of c-*fos*, was kindly provided by G. Rosenfeld (University of California, San Diego, CA). pSV-β-gal control vector was obtained from Promega. A dominant negative mutant of c-*Ras* (N^{17}Ras) was kindly provided by Akira Kikuchi (University of California, San Francisco). pcDNAI-ERK2 was provided by Henry Bourne (University of California, San Francisco).

Cell culture and establishment of stable cell lines. CHO-K1 cells were grown at 37° in 5% CO_2 in Ham's F-12 medium containing 10% FCS (Intergen, Purchase, NY), streptomycin (100 μg/ml), and penicillin (100 IU/ml). AT$_1$ receptor stable transfectants were obtained by cotransfection of pBJ-1-AT$_1$ and a vector containing the neomycin-resistant gene. CHO cells were incubated for 24 hr with 1 μg of pSV-7Dneo plus 5 μg of pBJ-1-AT$_1$ in the presence of 30 μl of lipofectamine in 5 ml of OPTI-MEM. On the next day, the cells were switched to full growth medium containing 400 μg/ml G-418, an analog to neomycin (GIBCO BRL, Baltimore, MD). The selection of individual neomycin-resistant cell clones was carried out ~1 week later. Neomycin-resistant clones were tested for expression of the recombinant form of the AT$_1$ receptor by a binding experiment.

Adenylyl cyclase assays. CHO-AT$_1$ cells were grown until confluent in 12-well dishes and labeled overnight with 2 μCi/ml [^{3}H]adenine (25–30 Ci/mmol) in Ham's F-12 with 10% FCS in the presence or absence of 100 ng/ml PTX. On the next day, the cells were washed with serum-free media supplemented with 1 mg/ml bovine serum albumin and 10 mM HEPES. After removal of the washing media, the cells were stimulated by the addition of fresh media containing 50 μM forskolin and 100 nM AII in the presence of 1 mM 3-isobutyl-1-methylxanthine for 20 min at room temperature. The incubation was terminated by replacement of the media with 1 ml of ice-cold 5% trichloroacetic acid containing 1 mM cAMP and 1 mM ATP. After incubation at 4° for 30 min, the labeled [^{3}H]ATP and [^{3}H]cAMP pools were separated and quantified by chromatography on Dowex 50W and neutral alumina columns, as described previously (24). The 1-ml acid supernatant was loaded onto 1-ml Dowex columns, and the ATP pools eluted with 3 ml of H_2O. The Dowex 50W columns were then placed over 1-ml alumina columns, and 10 ml of H_2O was added to the Dowex columns and the eluent allowed to drip directly onto the alumina. The cAMP pool was then eluted directly from the alumina with 5 ml of 0.1 M imidazole and 0.01 mM sodium azide. Adenylyl cyclase activity was expressed as a ratio by the equation cAMP/(ATP + cAMP).

Luciferase and β-gal assay. CHO cells in each well (12-well plates; Nunc, Naperville, CT) were transfected for 6–8 hr with a total of 0.4–0.6 μg of plasmid DNA in 0.4 ml of OPTI-MEM I containing 1.2 μl of lipofectamine. Then, cells were switched to full medium and incubated 18 hr. After a 24-hr incubation in Ham's F-12 medium containing 0.5% FCS, cells were stimulated for indicated times, and the cell lysate was used for measuring luciferase and β-gal activity according to the manufacturer's instructions. Transfection of pSV-β-gal was done to correct the transfection efficiency. Both activities were measured in the linear range, and endogenous β-gal activity was negligible (<1%).

MAP kinase assay. Subconfluent CHO-AT$_1$ cells were serum starved for 24 hr and stimulated with agonists for 5 min. After being washed twice with ice-cold PBS containing 1 mM sodium orthovanadate, the cells were lysed in 500 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 1% Triton X-100, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride). After centrifugation at 13,000 × *g* for 15 min, equal amounts of cell lysates were incubated with anti-ERK2 antibody for 2 hr at 4° and collected with Protein A-Sepharose beads. Samples were then washed three times with lysis buffer and twice with washing buffer (25 mM Tris, pH 7.4, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate) and then incubated in 50 μl of kinase buffer (25 mM HEPES, pH 7.4, 10 mM magnesium acetate, 2 mM dithiothreitol, 2 mM EGTA, 200 μM sodium orthovanadate, 50 μM ATP, 250 μg/ml MBP, 2 μM protein kinase A inhibitor, and 2 μCi of [γ-^{32}P]ATP) for 30 min at 25°C. MAP kinase activity was assayed in two ways: autoradiography and ^{32}P-incorporation into p81 phosphocellulose paper. After incubation, the reaction was stopped by the addition of Laemmli loading buffer (26), the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the phosphoproteins were visualized by autoradiography. Alternately, after incubation, 25-μl aliquots were spotted

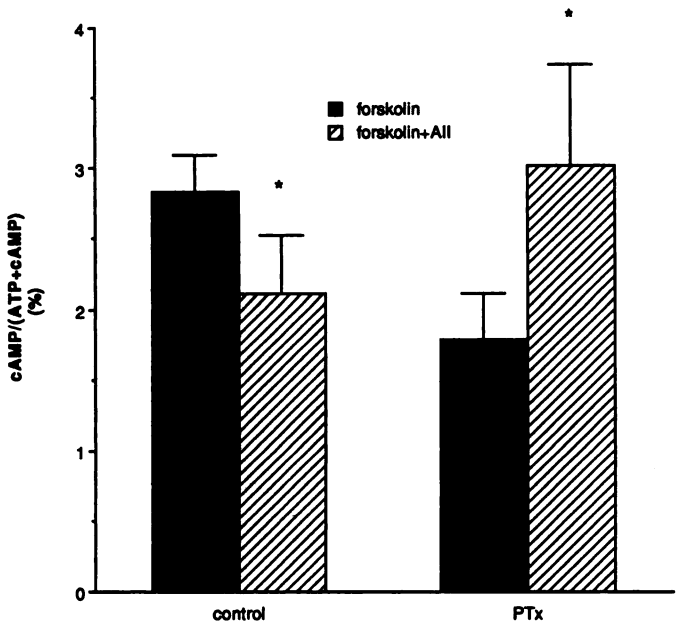

Fig. 1. AT$_1$ receptor mediates inhibition of adenylyl cyclase. CHO-AT$_1$ cells were labeled with [^{3}H]adenine in the presence or absence of 100 ng/ml PTX (*PTx*) and stimulated with 50 μM forskolin in the presence or absence of 100 nM AII. The adenylyl cyclase activity was measured as described in Materials and methods. Data were expressed as mean ± standard deviation from two independent experiments with triplicate determinations. *, $p < 0.05$ versus forskolin.

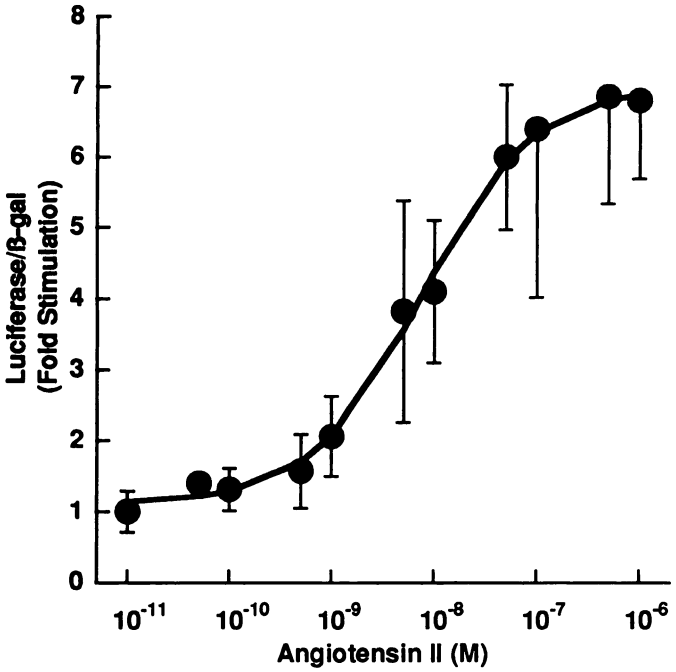

Fig. 2. Dose-dependency of c-*fos* gene activation induced by AII in CHO-AT_1 cells. After CHO-AT_1 cells were transiently transfected with the reporter plasmid p2FTL and pSV-β-gal as an internal control (0.2 μg/well each) by lipofectamine and were serum starved for 24 hr, they were stimulated with various concentrations of AII for 6 hr. Data are expressed as mean ± standard deviation of fold increase over basal from three independent experiments with triplicate determinations.

onto p81 phosphocellulose paper and extensively washed with 150 mM phosphoric acid. The paper was dried, and ^{32}P-incorporation into MBP was measured with a scintillation counter (27) .

Ca^{2+} measurement. After collection of the cells by incubation in 2 mM EDTA-PBS for 10 min at 37°, AII-induced increases in cytoplasmic Ca^{2+} were measured using an Hitachi F-2000 fluorometer

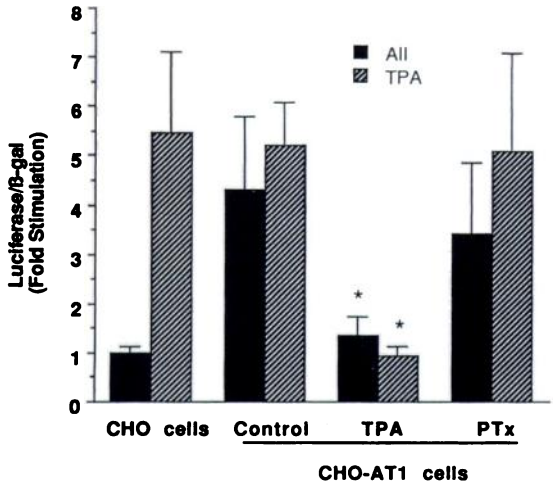

Fig. 3. Effect of pretreatment with TPA or PTX on c-*fos* gene activation by AII in CHO-AT_1 cells. After transient transfection with pF2TL and pSV-β-gal (0.2 μg/well each), CHO-AT_1 cells were serum starved in the presence of vehicle (control), 100 ng/ml TPA, or 100 ng/ml PTX (*PTx*) for 24 hr and stimulated with vehicle (basal), 100 nM AII, or 100 ng/ml TPA for 4 hr. Data are expressed as fold increase over basal luciferase/β-gal activity after stimulation with AII and MBP and are mean ± standard deviation of three independent experiments with triplicate determinations. Data for CHO cells are also shown. *, $p < 0.05$ versus control.

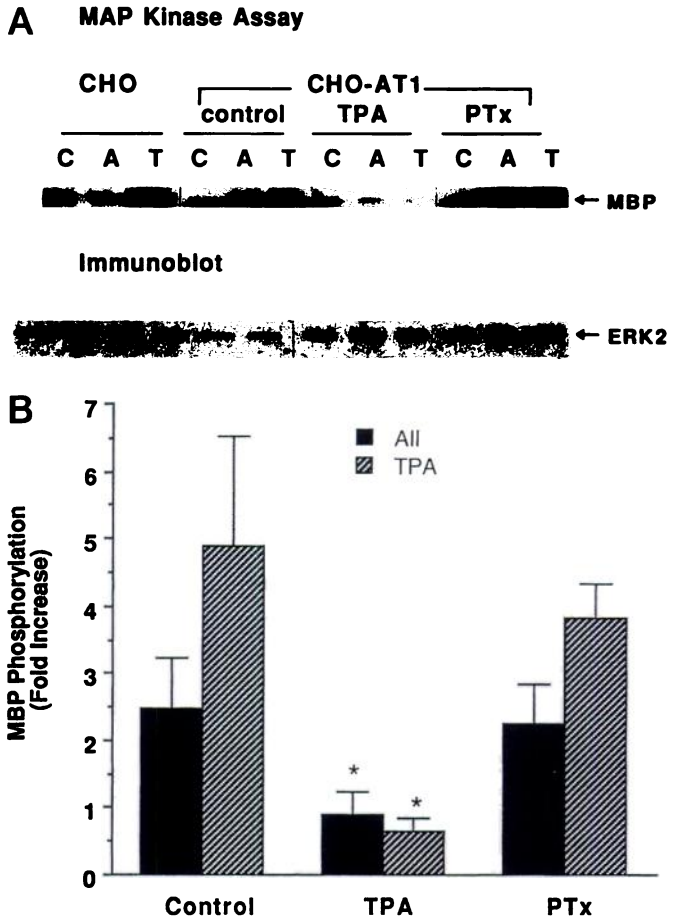

Fig. 4. Effect of pretreatment with TPA or PTX (*PTx*) on MAP kinase activation in CHO-AT_1 cells. Subconfluent CHO-AT_1 cells were serum starved for 24 hr in the presence of vehicle (control), 100 ng/ml TPA, or 100 ng/ml PTX for 24 hr and stimulated with vehicle (basal, *C*), 100 nM AII (*A*), or 100 ng/ml TPA (*T*) for 5 min. *Top*, Phosphorylated MBP were visualized by autoradiography. The amount of the immunoprecipitate was assessed by Western blotting. A representative autoradiogram from four independent experiments is shown. *Bottom*, incorporation of ^{32}P into MBP. Data are expressed as fold increase of MBP phosphorylation over basal after stimulation with AII and TPA and are mean ± standard deviation from six independent experiments. *, $p < 0.05$ versus control.

(Yokohama, Japan) and the calcium-sensitive fluor Fura-2 as described previously (25).

Raf-1 and MEK activity assay. Equal amounts of cell lysates were immunoprecipitated with anti-Raf-1 antibody or anti-MEK1&2 antibody, and the immunoprecipitates were used for an *in vitro* kinase assay. *In vitro* kinase activity was measured by incubating the immunoprecipitates with 100 ng/ml wild-type MEK and 500 ng/ml GST-KNERK1 (Raf-1 assay) or with GST-KNERK1 (MEK assay) in 30 μl of kinase buffer (20 mM Tris-Cl, pH 7.5, 10 mM magnesium chloride, 20 mM β-glycerophosphate, 200 μM sodium orthovanadate, 2 mM EGTA, and 10 μCi of [γ-^{32}P]ATP) for 20 min at 30° (23). Then, the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the phosphoproteins were visualized by autoradiography.

PKC assay. Serum-starved cells were washed twice with PBS and stimulated with 100 nM AII or 100 ng/ml TPA for 1 min. Then, the cells were rinsed with ice-cold PBS and scraped in 1 ml/dish of buffer A (25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). The cells were homogenized 10 times using a 15-ml Dounce homogenizer, and the lysates were centrifuged at 100,000 × *g* for 30

min. The supernatants (cytosolic fractions) were collected, and the membrane pellets were suspended in 1 ml of buffer A containing 1% Triton X-100. The suspensions were rocked for 30 min at 4° and then centrifuged at 100,000 × *g* for 30 min. The supernatants were used for membrane fractions. Equal amounts of cytosolic and membrane fractions were used for Western blotting.

Results and Discussion

To determine the mechanism by which the AT_1 receptor transduces its biological signal, we developed stable CHO cell lines expressing the recombinant human AT_1 receptor. These cells bound ^{125}I-[Sar^1,Ile^8]AII with a high affinity. The cell line with the highest binding activity, CHO-AT_1, was used in these experiments. The CHO-AT_1 cells expressed AT_1 receptors with a K_d for AII of ~1 nM (data not shown), which is comparable to the binding affinity that has been reported (7).

Because the AT_1 receptor has been reported to couple to G_i/G_o (5, 7), we examined the effect of AII on adenylyl cyclase activity. Activation of the AT_1 receptor inhibited forskolin-induced adenylyl cyclase activation by 25.6% (Fig. 1), which is comparable to the result described previously (5). However, after pretreatment of the cells with PTX, AII stimulated adenylyl cyclase activity in CHO-AT_1 cells, indicating that the AT_1 receptor couples to PTX-sensitive G proteins, most likely G_i, and PTX-insensitive G proteins, such as G_s or G_q. AII-induced activation of adenylyl cyclase has been shown in cultured vascular smooth muscle cells (28) and bovine adrenal cells (29), and activation of receptors coupled to polyphosphoinositide turnover has been shown to enhance agonist-stimulated cAMP accumulation in bovine adrenal cells (30). Because G_s activation by AII is not known, the AII-induced activation of adenylyl cyclase is presumably mediated through activation of the G_q family. Thus, the activation of adenylyl cyclase by AII in CHO-AT_1 cells seems to be regulated by G_i and the G_q family.

Because the c-*fos* gene activation is closely related to the mechanism of AII-induced cell proliferation (13), we next examined its regulation in CHO-AT_1 cells. To check whether the c-*fos*-luciferase reporter system (31) can be used to assess AT_1 receptor signaling in CHO-AT_1 cells, we determined the time course and dose dependency of c-*fos* gene activation by AII. After cotransfection of the reporter plasmid p2FTL and pSV-β-gal as an internal control, the cells were serum starved for 24 hr and stimulated with 100 nM AII for various times. After stimulation with AII, the c-*fos*-luciferase/β-gal ratio reached a peak at 6 hr and then declined (data not shown). AII activated c-*fos* gene expression in a dose-dependent manner with an EC_{50} of ~7 nM (Fig. 2), which is consistent with the K_d value of this receptor for AII. AII increased the luciferase/β-gal ratio to ~7-fold the base-line value and reached a plateau at 100 nM.

We next examined which G protein is involved in c-*fos* gene activation by AII. We pretreated CHO-AT_1 cells for 24 hr with TPA to inhibit TPA-sensitive PKC, which is downstream of the G_q family, and with PTX to inactivate G_i/G_o. We then determined the effect of TPA and PTX on c-*fos* gene activation induced by AII. Pretreatment with TPA abolished AII- and TPA-induced c-*fos* gene activation (Fig. 3). In contrast, PTX did not significantly affect AII-induced c-*fos* activation in CHO-AT_1 cells. In control nontransfected CHO cells, AII did not stimulate the c-*fos* gene induction. These results indicate that c-*fos* gene activation by AII occurs preferentially through a G_q family activation pathway and is probably mediated by PKC activation.

To confirm that PKC mediates AII activation of the c-*fos* gene in CHO-AT_1 cells, we assessed whether AII-induced activation of MAP kinases, which are upstream of c-*fos*, is PKC dependent. For this experiment, we determined the level of phosphorylation of MBP in lysates of cells expressing the AT_1 receptor after treatment with AII. AII and TPA stimulated MAP kinases in CHO-AT_1 cells by ~2.5- and ~5-fold, respectively (Fig. 4). Pretreatment with TPA abolished AII- and TPA-mediated MAP kinase activation. However, pretreatment with PTX had no effect. AII did not stimulate MAP kinases in control CHO cells. These results indicate that MAP kinase activation and c-*fos* activation by AII are PKC dependent.

To show that pretreatment with TPA does not affect upstream signaling, we assessed PLC activity by measuring changes in the intracellular Ca^{2+} increase after AII stimulation in CHO-AT_1 cells. We observed that the intracellular Ca^{2+} response to AII was not affected by pretreatment with TPA (Fig. 5). In addition, PTX pretreatment had no effect on AII-induced intracellular Ca^{2+} response (data not shown), indicating that this Ca^{2+} response is mediated by the G_q family. Thus, PLC activity is not affected by TPA pretreatment. We also found that binding of ^{125}I-[Sar^1,Ile^8]AII was not affected in this cell line by pretreatment with TPA (data not shown). Thus, pretreatment with TPA does not seem to affect upstream signaling.

Sequential activation of Raf and MEK has been proposed

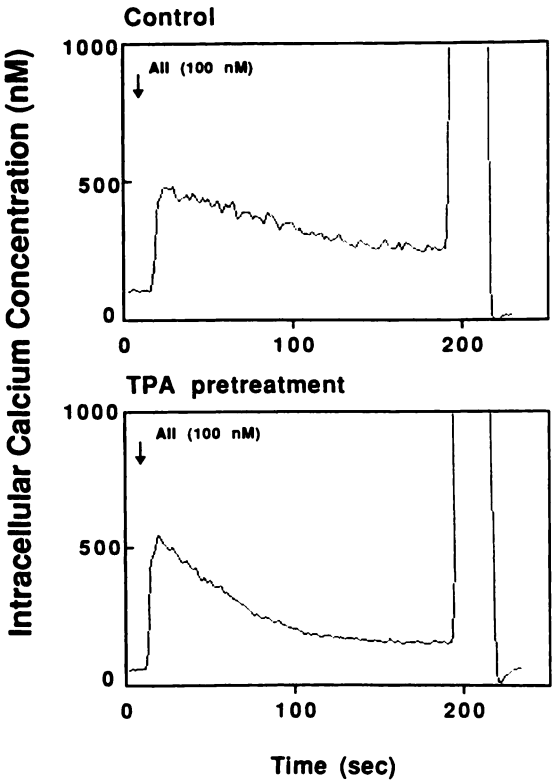

Fig. 5. AII-induced increases in cytoplasmic calcium in CHO-AT_1 cells. After pretreatment with vehicle (control) or 100 ng/ml TPA for 24 hr, the cells were collected by being incubated in 2 mM EDTA-PBS for 10 min at 37°. Then, AII-induced cytoplasmic calcium transients were measured as described in Experimental Procedures.

as the major pathway for MAP kinase activation (28–30). Therefore, we tried to determine whether in AT_1 receptor signal transduction in CHO-AT_1 cells, Raf-1 and MEK are signaling intermediates between PKC and MAP kinases. Raf, a serine/threonine kinase, is thought to play a central role in the transduction of growth signals and is activated by growth factors such as PDGF (35) and epidermal growth factor (10). PKC is known to activate Raf by direct phosphorylation (14, 15). Therefore, we investigated whether AII can activate Raf-1 and MEK by a PKC-dependent mechanism in CHO-AT_1 cells. Raf-1 activity was assayed by incubation of Raf-1 immunoprecipitates with MEK and a kinase negative mutant of ERK1 (KNERK1). Phosphorylation of the KNERK1 was then determined. As shown in Fig. 6A, AII and TPA activated Raf-1. This activation of Raf-1 by AII and TPA was blocked by inhibition of PKC. These results are direct evidence that AII can activate Raf-1 via a PKC-dependent mechanism. A recent study showed that B-Raf may play an important role in MEK activation (36). However, we could not show B-Raf activation by AII in CHO-AT_1 cells (data not shown).

To determine whether AII can also activate MEK by a PKC-dependent mechanism in CHO-AT_1 cells, MEK activity was assayed by incubating MEK immunoprecipitates with KNERK1. Phosphorylation of the KNERK1 was then determined. We found that AII and TPA activated MEK and that this activation of MEK by AII and TPA was blocked by inhibition of PKC (Fig. 6B), indicating that AII can also activate MEK by a PKC-dependent mechanism. These results show that Raf-1 and MEK are signaling intermediates between PKC and MAP kinases.

It has been demonstrated that Raf-1 is a substrate for a variety of PKC isozymes, in particular, α, β, and γ (14, 15). Because we have shown that TPA-sensitive PKC is responsible for Raf-1 activation, we tried to determine which isoform of PKC is activated by AII among the TPA-sensitive PKC isoforms (α, β, γ, δ, ϵ). When PKC is activated, it is translocated from the cytosol to the membrane (37). Therefore, after stimulating the cells, we fractionated the cell lysates into cytosolic and membrane fractions. Each PKC isoform was then identified by Western blotting. Among TPA-sensitive PKC isoforms tested, we found that only PKCα and PKCϵ were detected by Western blotting in CHO-AT_1 cells and that AII and TPA induced translocation of PKCα and PKCϵ from the cytosol to the membrane, although stimulation of PKC by AII is weaker than that by TPA (Fig. 7). Both PKC isoforms almost disappeared from the cells after pretreatment with TPA. Based on the report of Raf-1 activation by PKCα (14, 15), these results suggest that PKCα is responsible for AII-induced Raf-1 activation.

Because Hirai *et al.* (38) reported that PKCδ and PKCϵ activate Ras, we explored the possibility that PKCϵ-mediated Ras activation is involved in MAP kinase and c-*fos* gene activation in CHO-AT_1 cells. To test whether Ras is involved in AT_1 receptor signaling in CHO-AT_1 cells, we inhibited the function of Ras using a dominant negative mutant of Ras (N^{17}Ras). First, we examined whether N^{17}Ras can affect MAP kinase activation by AII in CHO-AT_1 cells. After transfecting pcDNAI-HA-tagged ERK2 in the presence of an N^{17}Ras construct (pcDNA3-N^{17}Ras) or a control vector (pcDNA3) in CHO-AT_1 cells and CHO cells that express PDGFβ receptors (CHO-PDGFβR) (39), we stimulated the cells with appropriate agonists and then measured MAP

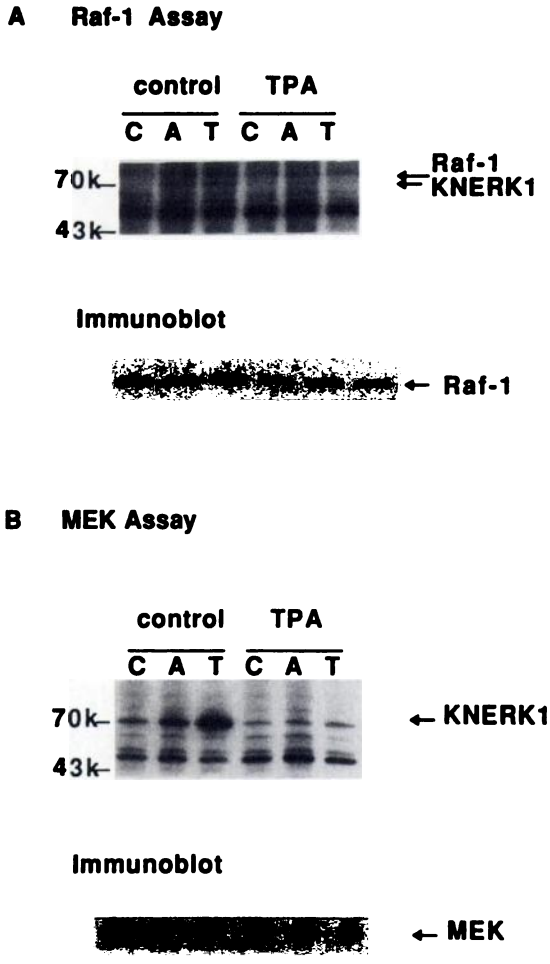

Fig. 6. Activation of Raf-1 (A) and MEK (B) by AII in CHO-AT_1 cells. CHO-AT_1 cells were serum starved in the presence of vehicle (control) or 100 ng/ml TPA for 24 hr and then stimulated with vehicle (*C*), 100 nM AII (*A*), or 100 ng/ml TPA (*T*) for 5 min. Equal amounts of cell lysates were immunoprecipitated with anti-Raf-1 or anti-MEK1&2 antibody, and the immunoprecipitates were used for an *in vitro* kinase assay. The phosphoproteins were visualized by autoradiography. *Arrow*, position of phosphorylated KNERK1. A, Autophosphorylated Raf-1 (*arrow*). The amounts of the immunoprecipitate were assessed by Western blotting. A representative autoradiogram from four independent experiments is shown. *Left*, molecular mass standards (70 and 43 kDa).

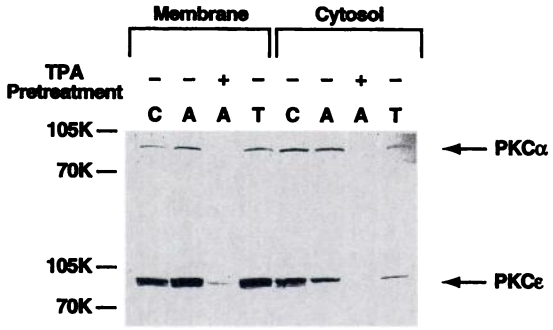

Fig. 7. Translocation of PKC induced by AII. CHO-AT_1 cells were serum starved in the presence of vehicle or 100 ng/ml TPA for 24 hr and stimulated with vehicle (cDNA, *C*), 100 nM AII (*A*), or 100 ng/ml TPA (*T*) for 1 min. Equal amounts of cytosolic and membrane proteins were immunoprecipitated with anti-PKCα or PKCϵ antibody and analyzed by Western blotting. A representative data from three independent experiments is shown. *Left*, molecular mass standards (105 and 70 kDa).

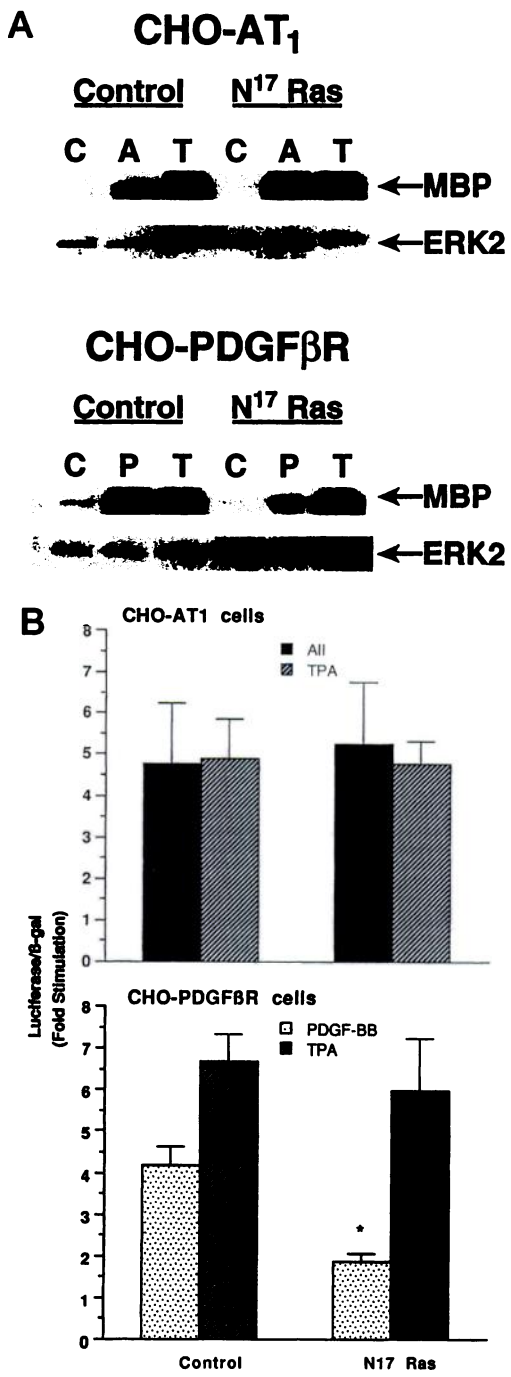

Fig. 8. Effect of N^{17}Ras on MAP kinase and c-*fos* gene activation by AII in CHO-AT$_1$ cells and by PDGF-BB in CHO-PDGFβR cells. *Top*, After CHO-AT$_1$ and CHO-PDGFβR cells were transiently transfected with 1 μg/ml pcDNAI-HA tagged ERK2 in the presence of 1 μg/ml pcDNA3 (control) or pcDNA3-N^{17}Ras (N^{17}Ras), they were serum starved for 24 hr and stimulated with vehicle (*C*), 100 nM AII (*A*), 1 nM PDGF-BB (*P*), or 100 ng/ml TPA (*T*) for 5 min. Phosphorylated MBP were visualized by autoradiography. The amount of the immunoprecipitate was assessed by Western blotting. A representative autoradiogram and Western blot from three independent experiments are shown. *Bottom*, After CHO-AT$_1$ and CHO-PDGFβR cells were transiently transfected with p2FTL and pSV-β-gal (0.2 μg/well each) in the presence of 0.2 μg of pcDNA3 (control) or pcDNA3-N^{17}Ras (N^{17}Ras), they were serum starved for 24 hr and stimulated with 100 nM AII, 1 nM PDGF-BB, or 100 ng/ml TPA for 6 hr. Data are expressed as fold increase over basal of luciferase/β-gal ratio after stimulation with AII, PDGF-BB, and TPA and mean ± standard deviation of three independent experiments with triplicate determinations. *, $p < 0.05$ versus control.

kinase activity. N^{17}Ras inhibited PDGF-induced MAP kinase activation but did not inhibit AII-induced MAP kinase activation (Fig. 8, *top*). N^{17}Ras did not affect TPA-induced MAP kinase activation in both cell lines. Next, we examined the effect of N^{17}Ras on c-*fos* gene activation. p2FTL and pSV-β-gal were cotransfected in the presence of pcDNA3-N^{17}Ras or pcDNA3 in CHO-AT$_1$ cells and CHO-PDGFβR cells. The effect of N^{17}Ras on AII- and PDGF-induced c-*fos* activation was then determined. N^{17}Ras inhibited PDGF-BB-induced c-*fos* activation by 70% in CHO-PDGFβR cells but did not inhibit AII-induced c-*fos* gene activation in CHO-AT$_1$ cells (Fig. 8, *bottom*). This result is consistent with the result of MAP kinase activation by AII and PDGF. These results suggest that Ras is not involved in the AT$_1$ receptor signaling cascades that lead to activation of MAP kinase and the c-*fos* gene. In addition, we found that N^{17}Ras did not affect MAP kinase and c-*fos* gene activation by TPA in either cell type. Furthermore, c-*fos* activation by a constitutively active mutant of Ras was not blocked by inhibition of PKC (data not shown). Thus, Ras and PKC seem to activate the c-*fos* gene through independent pathways in CHO cells. However, in other cell lines, the relationship of Ras and PKC in signal transduction may be different (40–42). Although we have shown that Ras is not involved in the signaling pathway leading to the activation of MAP kinases and the c-*fos* gene, Ras might be involved in the activation of c-*jun* through MEKK and c-*jun* amino-terminal kinase in AT$_1$ receptor signaling (21).

In contrast to our finding, Sadoshima *et al.* (43) showed that MAK kinase activation by AII was not blocked by TPA pretreatment in cardiac myocytes. We also found MAP kinase activation by AII was not inhibited by TPA or PTX pretreatment in rat aortic smooth muscle cells in culture.[3] Considering the fact that cAMP stimulates mitogenesis in Swiss 3T3 fibroblasts (44), whereas this nucleotide inhibits the proliferation of smooth muscle cells (45), signaling events in fibroblastic cell lines might not always be applicable to smooth muscle cells. Because most of the signal transduction pathways have been elucidated in fibroblastic cell lines, we should be aware of the cell specificity of signaling pathways. However, it is possible the signaling pathway in CHO cells can be applied to other cell types that express the AT$_1$ receptor.

In conclusion, we found that AII activated Raf-1, MEK, MAP kinase, and the c-*fos* gene in a PKC-dependent manner in CHO-AT$_1$ cells. Ras does not seem to be involved in the activation of MAP kinase and the c-*fos* gene by AII in this cell line, although we could not rule out the possibility that Ras might be involved in the activation of c-*jun* through MEKK and c-*jun* amino-terminal kinase. In that process, PTX-sensitive G_i/G_o might be involved. Recently, Giasson and Meloche (46) demonstrated that the p70 S6 protein kinase is involved in AII-induced hypertrophy in vascular smooth muscle cells. The relative contribution of the p70 S6 kinase and proto-oncogenes such as c-*fos* to smooth muscle proliferation remains to be clarified.

[3] H. Arai and J. A. Escobedo, unpublished observations.

Acknowledgments

We thank Drs. Akira Kikuchi, Bert Pronk, Mike Kavanaugh, and Israel Charo and Ms. Mimi Zeiger for helpful discussions and critical reading of the manuscript.

References

1. Gibbons, G. H., and V. J. Dzau. Angiotensin converting enzyme inhibition and vascular hypertrophy in hypertension. *Cardiovasc. Drugs Ther.* **4:**237-242 (1990).
2. Sasaki, K., Y. Yamano, S. Bardhan, N. Iwai, J. J. Murray, M. Hasegawa, Y. Matsuda, and T. Inagami. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature (Lond.)* **351:**230-233 (1991).
3. Murphy, T. J., R. W. Alexander, K. K. Griendling, M. S.Runge, and K. E. Bernstein. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature (Lond.)* **351:**233-236 (1991).
4. Iwai, N., and T. Inagami. Identification of two subtypes in the rat type I angiotensin II receptor. *FEBS Lett.* **298:**257-260 (1992).
5. Ohnishi, J., M. Ishido, T. Shibata, T. Inagami, K. Murakami, and H. Miyazaki. The rat angiotensin II AT1A receptor couples with three different signal transduction pathways. *Biochem. Biophys. Res. Commun.* **186:**1094-1101 (1992).
6. Griendling, K. K., B. Lassegue, and R. W. Alexander. The vascular angiotensin (AT1) receptor. *Thromb. Haemost.* **70:**188-192 (1993).
7. Schorb, W., G. W. Booz, D. E. Dostal, K. M. Conrad, K. C. Chang, and K. M. Baker. Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts. *Circ. Res.* **72:**1245-1254 (1993).
8. Molloy, C. J., D. S. Taylor, and H. Weber. Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. *J. Biol. Chem.* **268:**7338-7345 (1993).
9. Butcher, R. D., C. Schollmann, and D. Marme. Angiotensin II mediates intracellular signalling in vascular smooth muscle cells by activation of tyrosine-specific protein kinases and c-raf-1. *Biochem. Biophys. Res. Commun.* **196:**1280-1287 (1993).
10. App, H., R. Hazan, A. Zilberstein, A. Ullrich, J. Schlessinger, and U. Rapp. Epidermal growth factor (EGF) stimulates association and kinase activity of Raf-1 with the EGF receptor. *Mol. Cell Biol.* **11:**913-919 (1991).
11. Naftilan, A. J., R. E. Pratt, and V. J. Dzau. Induction of platelet-derived growth factor A-chain and c-*myc* gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83:**1419-1424 (1989).
12. Taubman, M. B., B. C. Berk, S. Izumo, T. Tsuda, R. W. Alexander, and B. Nadal-Ginard. Angiotensin II induces c-fos mRNA in aortic smooth muscle: role of Ca^{2+} mobilization and protein kinase C activation. *J. Biol. Chem.* **264:**526-530 (1989).
13. Naftilan, A. J., G. K. Gilliland, C. S. Eldridge, and A. S. Kraft. Induction of the proto-oncogene c-*jun* by angiotensin II. *Mol. Cell Biol.* **10:**5536-5540 (1990).
14. Kolch, W., G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marme, and U. R. Rapp. Protein kinase C *alpha* activates RAF-1 by direct phosphorylation. *Nature (Lond.)* **364:**249-252 (1993).
15. Sozeri, O., K. Vollmer, M. Liyanage, D. Frith, G. Kour, G. E. D. Mark, and S. Stabel. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. *Oncogene* **7:**2259-2262 (1992).
16. Crespo, P., N. Xu, J. L. Daniotti, J. Troppmair, U. R. Rapp, and J. S. Gutkind. Signaling through transforming G protein-coupled receptors in NIH 3T3 cells involves c-Raf activation: evidence for a protein kinase C-independent pathway. *J. Biol. Chem.* **269:**21103-21109 (1994).
17. Howe, L. R., and C. J. Marshall. Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring p21ras and p74raf-1. *J. Biol. Chem.* **268:**20717-20720 (1993).
18. Alblas, J., E. J. van Corven, P. L. Hordijk, G. Milligan, and W. H. Moolenaar. Gi-mediated activation of the p21ras-mitogen-activated protein kinase pathway by *alpha* 2-adrenergic receptors expressed in fibroblasts. *J. Biol. Chem.* **268:**22235-22238 (1993).
19. van Corven, E. J., P. L. Hordijk, R. H. Medema, J. L. Bos, and W. H. Moolenaar. Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc. Natl. Acad. Sci. USA* **90:**1257-1261 (1993).
20. Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science (Washington D. C.)* **260:**315-319 (1993).
21. Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R. J. Davis, G. L. Johnson, and M. Karin. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science (Washington D. C.)* **266:**1719-1723 (1994).
22. Sadoshima, J., and S. Izumo. Signal transduction pathways of angiotensin II-induced c-*fos* gene expression in cardiac myocytes *in vitro*: roles of phospholipid-derived second messengers. *Circ. Res.* **73:**424-438 (1993).
23. Kikuchi, A., and L. T. Williams. The post-translational modification of ras p21 is important for Raf-1 activation. *J. Biol. Chem.* **269:**20054-20059 (1994).
24. Wong, Y. H., A. Federman, A. M. Pace, I. Zachary, T. Evans, J. Pouyssegur, and H. R. Bourne. Mutant *alpha* subunits of Gi2 inhibit cyclic AMP accumulation. *Nature (Lond.)* **351:**63-65 (1991).
25. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440-3450 (1985).
26. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680-685 (1970).
27. Faure, M., T. A. Voyno-Yasenetskaya, and H. R. Bourne. cAMP and *beta gamma* subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* **269:**7851-7854 (1994).
28. Kubalak, S. W., and J. G. Webb. Angiotensin II enhancement of hormone-stimulated cAMP formation in cultured vascular smooth muscle cells. *Am. J. Physiol.* **264:**H86–H96 (1993).
29. Langlois, D., M. Begeot, M. C. Berthelon, C. Jaillard, and J. M. Saez. Angiotensin II potentiates agonist-induced 3′:5′-cyclic adenosine monophosphate production by cultured bovine adrenal cells through protein kinase C and calmodulin pathways. *Endocrinology* **131:**2189-2195 (1992).
30. Wheeler, M. B., and J. D. Veldhuis. Facilitative actions of the protein kinase-C effector system on hormonally stimulated adenosine 3′,5′-monophosphate production by swine luteal cells. *Endocrinology* **125:**2414-2420 (1989).
31. Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsien, G. N. Gill, and M. G. Rosenfeld. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature (Lond.)* **328:**820-823 (1987).
32. Dent, P., W. Haser, T. A. Haystead, L. A. Vincent, T. M. Roberts, and T. W. Sturgill. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. *Science (Washington D. C.)* **257:**1404-1407 (1992).
33. Crews, C. M., A. Alessandrini, and R. L. Erikson. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science (Washington D. C.)* **258:**478-480 (1992).
34. Kyriakis, J. M., H. App, X. F. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, and J. Avruch. Raf-1 activates MAP kinase-kinase. *Nature (Lond.)* **358:**417-421 (1992).
35. Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF *beta*-receptor. *Cell* **58:**649-657 (1989).
36. Jaiswal, R. K., S. A. Moodie, A. Wolfman, and G. E. Landreth. The mitogen-activated protein kinase cascade is activated by B-Raf in response to nerve growth factor through interaction with p21ras. *Mol. Cell Biol.* **14:**6944-6953 (1994).
37. Nishizuka, Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Washington D. C.)* **258:**607-614 (1992).
38. Hirai, S., Y. Izumi, K. Higa, K. Kaibuchi, K. Mizuno, S. Osada, K. Suzuki, and S. Ohno. Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/*Jun* by PKC *delta*. *EMBO J.* **13:**2331-2340 (1994).
39. Escobedo, J. A., P. J. Barr, and L. T. Williams. Role of tyrosine kinase and membrane-spanning domains in signal transduction by the platelet-derived growth factor receptor. *Mol. Cell Biol.* **8:**5126-5131 (1988).
40. de Vries-Smits, A. M., B. M. Burgering, S. J. Leevers, C. J. Marshall, and J. L. Bos. Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature (Lond.)* **357:**602-604 (1992).
41. Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis. ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell* **68:**1041-1050 (1992).
42. Marquardt, B., D. Frith, and S. Stabel. Signalling from TPA to MAP kinase requires protein kinase C, raf and MEK: reconstitution of the signalling pathway *in vitro*. *Oncogene* **9:**3213-3218 (1994).
43. Sadoshima, J., Z. Qiu, J. P. Morgan, and S. Izumo. Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes: the critical role of Ca^{2+} dependent signaling. *Circ. Res.* **76:**1-15 (1995)
44. Rozengurt, E. Cyclic AMP:a growth-promoting signal for mouse 3T3 cells. *Adv. Cyclic Nucleotide Res.* **14:**429-442 (1981).
45. Loesberg, C., R. Van Wijk, J. Zandbergen, W. G. Van Aken, J. A. Van Mourik, and P. G. De Groot. Cell cycle-dependent inhibition of human vascular smooth muscle cell proliferation by prostaglandin E1. *Exp. Cell Res.* **160:**117-125 (1985).
46. Giasson, E., and S. Meloche. Role of p70 S6 protein kinase in angiotensin II-induced protein synthesis in vascular smooth muscle cells. *J. Biol. Chem.* **270:**5225-5231 (1995).

Send reprint requests to: Dr. Jaime A. Escobedo, Chiron Corporation, 4560 Horton St, Emeryville, CA 94608-2916.