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Angiotensin II Induces Diverse Signal Transduction Pathways via Both G_q and G_i Proteins in Liver Epithelial Cells

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Abstract Angiotensin II stimulates a biphasic activation of Raf-1, MEK, and ERK in WB liver epithelial cells. The first peak of activity is rapid and transient and is followed by a sustained phase. Angiotensin II also causes a rapid activation of $p21^{ras}$ in these cells. Moreover, two Src family kinases (Fyn and Yes) were activated by angiotensin II in a time- and concentration-dependent manner. Microinjection of antibodies against Fyn and Yes blocked angiotensin II-induced DNA synthesis and c-Fos expression in WB cells, indicating an obligatory involvement of these tyrosine kinases in the activation of the ERK cascade by angiotensin II. Finally, substantial reduction of the angiotensin II-stimulated activation of Fyn, Raf-1, ERK, and expression of c-Fos by pertussis toxin pretreatment argues that G proteins of the G_i family as well as the G_q family are involved in angiotensin II-mediated mitogenic pathways in WB cells. J. Cell. Biochem. 69:63–71, 1998.

Key words: angiotensin II; G proteins; Src tyrosine kinases; c-Fos

The mitogen-activated protein kinase (MAPK) pathways are stimulated by a variety of hormones and growth factors and play an important role in cellular differentiation and proliferation [Cobb and Goldsmith, 1995; Seger and Krebs, 1995]. Three structurally related MAPK subfamilies have been identified: extracellular signal-regulated kinases (ERKs), and the stress-activated kinases, c-Jun N-terminal kinases and p38 kinases. These kinases can be activated by distinct stimuli, have different downstream targets, and are thought to perform different functions [Cobb and Goldsmith, 1995; Seger and Krebs, 1995]. All three MAPK subfamilies phosphorylate substrates on serine and threonine residues and require phosphorylation on both tyrosine and threonine for full activation. The MAP kinases phosphorylate a wide range of transcription factors and nuclear proteins, such as c-Fos, c-Jun, Elk-1, and c-Myc, suggesting that they provide an important regulatory component of protein and DNA synthesis.

The ERKs are characteristically activated by mitogenic peptides and growth factors, which cause mitogenesis or differentiation, depending on the cell type and the duration of ERK activation. Potent mitogens stimulate a sustained activation of ERK and its translocation to the nucleus, both events being essential for the induction of cell division [Meloche et al., 1992; Lenormand et al., 1993; Mii et al., 1996]. For example, agonists of G protein-coupled receptors, such as thrombin, endothelin, and LPA, have been shown to be potent mitogens and to induce a biphasic, persistent activation of ERK [Kahan et al., 1992; Cook et al., 1993; Wang et al., 1993; Rodrigrez-Fernandez and Rozengurt, 1996]. ERKs are activated by an upstream dual-specificity (Tyr/Thr) kinase (MEK1/2), which in turn is activated by a Ser/Thr kinase of the Raf family (Raf-1, Raf-A, Raf-B) [Kyriakis and Avruch, 1996]. Raf activation occurs upon its translocation to the membrane and interaction with $p21^{ras}$ (Ras), a small GTP-binding protein that is active in its GTP-bound form [Luo et al., 1997]. Agonist-stimulated tyrosine phosphorylation of the adapter protein Shc and its subse-

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quent interaction with the Grb2-Sos complex precede the activation of Ras [Cobb and Goldsmith, 1995; Seger and Krebs, 1995]. This signal transduction pathway appears to be shared by both tyrosine kinase receptors and G protein-coupled receptors [Post and Brown, 1996; van Biesen et al., 1996]. Although G protein-coupled receptors lack an intrinsic tyrosine kinase activity, they can modulate the activation of cytoplasmic tyrosine kinases [Ishida et al., 1995; Simonson et al., 1996; Post and Brown, 1996; van Biesen et al., 1996]. Thus, transient expression of subunits of pertussis toxin-sensitive G_i proteins has been shown to cause c-Src activation, tyrosine phosphorylation of Shc and activation of ERK, suggesting activation of the same Ras/Raf/MEK-dependent pathway as that activated by growth factor receptors having intrinsic tyrosine kinases [van Biesen et al., 1996]. Pertussis toxin-insensitive G_q proteins have also been shown to cause a stimulation of tyrosine kinases. However, the mechanism of this stimulation remains to be elucidated.

Angiotensin II (Ang II) is a typical Ca^{2+}-mobilizing hormone involved in the regulation of vasoconstriction, cell growth, and salt and fluid homeostasis [Huckle and Earp, 1994; Duff et al., 1995]. Most of the biological effects of Ang II are mediated by the type one (AT1) receptors, which are coupled to G proteins of both the G_q and G_i families [Wong et al., 1992]. The interaction of Ang II with AT1 receptors causes an activation of G_q family proteins with release of α and $\beta\gamma$ subunits, both of which may activate different isoforms of phospholipase C-β (PLC-β) followed by hydrolysis of phosphotidylinositol 4,5-bisphosphate-generating second messengers, which mediate mobilization of intracellular Ca^{2+} and activation of protein kinase C (PKC) [Hepler et al., 1990; Huckle and Earp, 1994; Duff et al., 1995]. Ang II was reported to stimulate a rapid and transient activation of ERK in rat mesangial cells and human vascular smooth muscle cells, being nonmitogenic for both cell types [Tsuda et al., 1992; Huwiler et al., 1995; Sadoshima et al., 1995]. Ang II stimulation also leads to a rapid increase in protein tyrosine phosphorylation. ERK-1/2, Shc, FAK, PLC-γ1, and annexin 1 have been identified among these phosphorylated proteins [Salles et al., 1993; Marrero et al., 1994; Schorb et al., 1994]. Interestingly, a transient activation of Src family kinases by Ang II was observed in rat cardiac myocytes and vascular smooth muscle cells [Sadoshima and Izumo, 1996; Ishida et al., 1995].

Although Ang II is mostly synthesized in liver, its effects on liver cells are less well characterized than those on cardiac and kidney cells. However, Ang II may play a major role in liver cell development by activating the regulatory mechanisms that control cell division [Duff et al., 1995]. Moreover, epithelial cells exhibit the first response to the hormone, which might be crucial for the hormonal effect on the whole organ. To address this issue, we used WB cells, an epithelial cell line from rat liver [Tsao et al. 1984]. The data presented in this report show that Ang II stimulates the ERK pathway in a biphasic manner, the first phase of activation being rapid and transient followed by a sustained second phase. We also present evidence that Ang II induces a sustained activation of two Src family kinases, Fyn and Yes, in WB cells and that the Ang II-induced activation of Fyn and the ERK cascade is sensitive to inhibition by pertussis toxin, indicating an involvement of members of the G_i family.

MATERIALS AND METHODS

Materials

Antibodies against Fyn (3), Raf-1(C-12), Ras (259), MEK1/2 (12-B), ERK1/2 (D-2), Src (N-16), Yes (3), protein A-agarose, HRP-conjugated antimouse or antirabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A panel of antibodies against some Src family kinases was a gift from Dr. A. Tsygankov (Temple University, Philadelphia, PA). [γ-P^{32}]ATP was purchased from NEN Research Products (Boston, MA). Plasmids encoding an inactive GST-MEK-1 and ERK-2 were provided by Drs. Michael J. Weber (University of Virginia, Charlottesville, VA) and Melanie H. Cobb (University of Texas, Dallas, TX), respectively. Ang II, enolase, and myelin basic protein (MBP) were obtained from Sigma Chemical (St. Louis, MO). Genistein, pertussis toxin, and [4-(2-aminoethyl)benzenesulfonylfluoride, HCl] (AEBSF) were purchased from Calbiochem (La Jolla, CA). Leupeptin, aprotinin, and pepstatin A were obtained from Boehringer Mannheim (Indianapolis, IN). Other chemicals were standard analytical grade from Fisher Scientific (Fair Lawn, NJ).

Cell Culture

WB cells (passage 20-30) were cultured at 37°C in 5% CO_2 humidified atmosphere in Richter's improved MEM containing 0.1 µM insulin (Irvine Scientific, Santa Ana, CA), supplemented with 10% fetal bovine serum (GibcoBRL, Grand Island, NY). Subculture was performed by detaching cells with 0.25% trypsin in isotonic buffer (GibcoBRL) followed by seeding the cells at a 1:10 dilution onto 100-mm plastic culture plates (Falcon). WB cells were used within 1 day after reaching confluence. Before stimulation with 1 µM Ang II, the cells were preincubated in medium without serum for at least 30 min at 37°C. When needed, pertussis toxin was added to cell cultures at a concentration of 10 ng/ml and incubated for 20 h in serum-free medium.

Raf-1 Assay

After stimulation, WB cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 1 mM AESBF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml pepstatin) for 30 min on ice. After removal of cell debris, the protein concentration was determined by Coomassie Plus protein assay (Pierce, Rockford, IL) and normalized. The cell lysates were incubated with antibody against Raf-1 for 1 h on ice followed by an incubation with Protein A-agarose with rotation for 1 h at 4°C. An irrelevant rabbit antibody was used as a negative control. The agarose beads were washed three times with the lysis buffer and twice with kinase buffer (10 mM PIPES, pH 7.0, 10 mM $MgCl_2$). The reactions were carried out by addition of 5 µCi [γ-^{32}P]ATP and 10 µg/ml GST-MEK in the kinase buffer at 30°C for 10 min and stopped by addition of Laemmli sample buffer and heating at 95°C for 5 min. The kinase assay samples were subjected to 10% SDS-PAGE followed by gel drying and exposure to X-ray film at -86°C. The results were analyzed by densitometry of the autoradiograms (in the linear range of the exposure) using a ScanJet 3c scanner (Hewlett Packard, Rockford, IL) and SigmaGel software (Jandel) The graphics were created using SigmaPlot software (Jandel)

MEK and ERK Assay

Stimulation of WB cells with Ang II and immunoprecipitations of MEK and ERK were carried out as described above by using anti-MEK1/2 or anti-ERK1/2 antibodies, respectively. Immunoprecipitates were washed once with lysis buffer, twice with modified RIPA buffer (10 mM MOPS, pH 7.0, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1.0% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4) and twice with kinase buffer. GST-ERK-2 (25 µg/ml) and MBP (5 µg/ml) were used as substrates for MEK or ERK assays, respectively. The reactions were initiated by addition of 5 µCi [γ-^{32}P]-ATP and carried out at 30°C for 15 min. The kinase assay samples were subjected to 12% SDS-PAGE and analyzed as described for the Raf-1 assay.

Src Family Kinase Assay

Src family kinases were immunoprecipitated from WB cell lysates prepared as described above by using the appropriate antibodies. Immunoprecipitates were washed as described for the Raf-1 assay, and the kinase reaction was initiated by addition of acid-denatured enolase (2 µg) and 5 µCi [γ-^{32}P]-ATP at 30°C for 5 min. The assay samples were subjected to 10% SDS-PAGE and analyzed as described above.

Ras Activation Assay

Assays for detection of activated Ras were carried out as described by Taylor and Shalloway [1996]. Briefly, pGEX-RBD plasmid (a gift from Dr. David Shalloway, Cornell University, Ithaca, NY), which encodes Raf-1(1-149)-GST fusion protein, was produced in *Escherichia coli*, and the fusion protein was purified on glutathione-agarose beads. Quiescent WB cells were stimulated with 1 µM Ang II and lysed according to Taylor and Shalloway [1996]. Cell lysates were incubated with GST-RBD beads; the bound proteins were eluted with SDS-PAGE sample buffer and were subjected to Western blotting with anti-Ras antibody. Amount of eluted Ras was analyzed by densitometry using a ScanJet 3c scanner.

DNA Synthesis Assay

Microinjection of antibodies and studies of Ang II-induced DNA synthesis in WB cells were carried out as reported previously [Baffy et al., 1995]. WB cells were injected with antibodies against Fyn and Yes (Santa Cruz Biotechnology) and stimulated with 1 µM Ang II for 24 h. The incorporation of BrdU was measured by

using a commercial kit from Calbiochem (Cambridge, MA). Control experiments showed no difference between DNA synthesis in cells treated with Ang II alone or with Ang II and irrelevant antibodies.

c-Fos Induction Assay

WB cells seeded on coverslips were serum starved for at least 24 h and stimulated with 1 μM Ang II for the indicated times. Subsequently, cells were fixed in an ethanol/glacial acetic acid mixture (95:5) for 30 min at 4°C. After washing three times with ice-cold PBS, cells were permeabilized with 0.2% Triton X-100/PBS for 10 min and incubated with 3% goat serum for 1 h at room temperature. Then, cells were incubated with a polyclonal anti-c-Fos antibody (Santa Cruz Biotechnology) for 1 h at room temperature. After washing three times with PBS, a fluorescein-labeled secondary antibody (Amersham Life Science, Cleveland, OH) was added to cells for 30 min. After washing with PBS several times, the coverslips were mounted on a microscope slide with an antifade oil (Molecular Probe, Eugene, OR). The proportion of cells expressing c-Fos were counted under microscopic visualization with controls being performed by using irrelevant antibodies.

RESULTS

Ang II Induces a Sustained Activation of the ERK Pathway

Because the ERK cascade plays a crucial role in growth factor-induced mitogenesis, the kinetics of activation of Raf-1, MEK, and ERK by Ang II were studied in WB cells, where Ang II has been shown to be mitogenic [Huckle and Earp, 1994]. Quiescent WB cells were stimulated with 1 μM Ang II for various times up to 90 min and lysed. The resulting cell lysates were used for immunoprecipitation studies. Immune complex kinase assays showed a biphasic activation of Raf-1, MEK, and ERK (Fig. 1). The activity of Raf-1 peaked 30 s after stimulation with Ang II, reaching a level fourfold above control values. Subsequently, the activity of Raf-1 declined rapidly to reach a sustained level twofold above the control. Analysis of MEK and ERK activity following Ang II stimulation revealed a more biphasic pattern of activation, with an initial rapid phase followed by a second sustained phase. The activation of MEK and ERK peaked at 1 min (3- to 3.5-fold over control) and remained elevated (two- to threefold over control) for the next 90 min after stimulation.

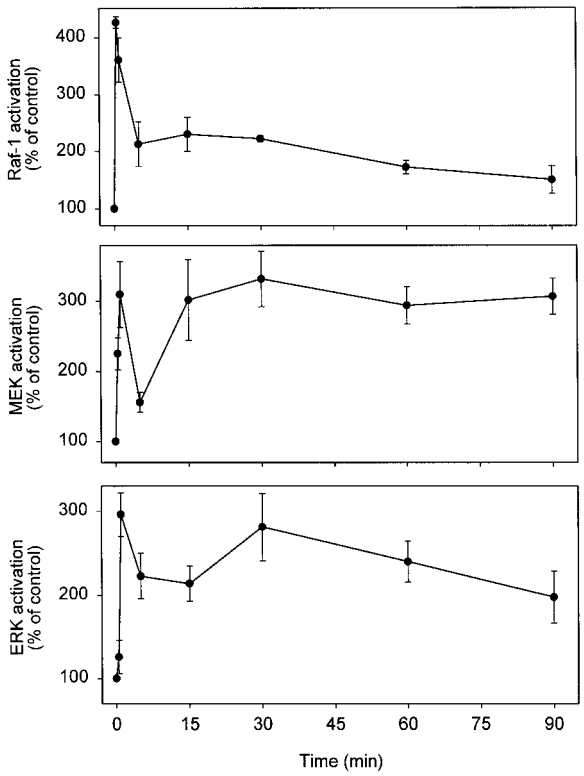

Fig. 1. Time course of Raf-1, MEK, and ERK activation by Ang II in WB cells. Quiescent WB cells were stimulated with 1 μM Ang II for various time intervals and lysed. The protein kinases were immunoprecipitated by incubation with the appropriate antibodies and Protein A-agarose and immune complex kinase assays for Raf-1, MEK, and ERK were carried out as described in Methods. The assay samples were resolved by SDS-PAGE. The gels were dried, and phosphoprotein bands were visualized by autoradiography and analyzed by densitometry. Kinase activity in the nonstimulated cells was set for 100%. Results represent means ± SE from four independent experiments.

The small molecular weight G protein, Ras, is a major upstream regulator of Raf-1 [Kyriakis and Avruch, 1996; Luo et al., 1997]. Ras activity was measured in WB cells following treatment with 1 μM Ang II for various times from 0.5 to 90 min. Cell lysates were incubated with Ras-binding domain (RBD) of Raf-1 immobilized on agarose beads, and the level of cellular Ras bound to RBD was used as assessment of Ras activation [Taylor and Shalloway, 1996]. The hormone induced a rapid increase in Ras activation, which remained elevated for up to 60 min (Fig. 2). Taken together, these results show that Ang II stimulates Ras and the ERK pathway in a rapid and sustained fashion.

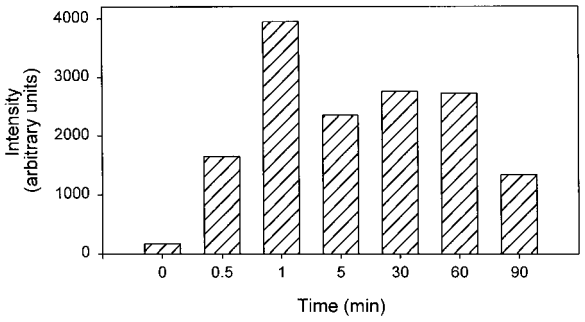

Fig. 2. Effect of Ang II on Ras activity in WB cells. WB cells were serum starved overnight and stimulated with Ang II for 0–90 min. GTP-Ras was affinity precipitated with GST-RBD as described in Methods. Bound proteins were eluted with SDS-PAGE sample buffer and blotted with anti-Ras antibody. Bound Ras was analyzed by densitometry. The data represent one of two independent experiments.

Ang II Stimulates Fyn and Yes in WB Cells

Tyrosine phosphorylation is one of the earliest signaling events in the response of cells to numerous growth factors and hormones, including Ang II [Post and Brown, 1996; van Biesen et al., 1996]. Genistein, an inhibitor of protein tyrosine kinases (PTK), completely inhibited the Ang II-mediated stimulation of Raf-1 (by 93 ± 9% of control at 60 min after addition of Ang II, n = 3–4, $P < 0.05$). Ang II-stimulated activation of MEK and ERK was also inhibited by genistein by 48 ± 7% and 72 ± 5%, respectively (at 60 min after addition of Ang II, n = 3–4, $P < 0.05$), indicating the potential involvement of PTK in Ang II signaling. A similar inhibition of Raf-1, MEK, and ERK activities was observed in experiments using herbimycin A (data not shown). The ability of Ang II to activate Src family kinases was examined by an immune complex kinase assay using antibodies against Src family kinases. Three tyrosine kinases were identified in WB cells, namely, Fyn, Src, and Yes. Figure 3 shows that Fyn and Yes were rapidly activated by Ang II. The activation of both kinases was observed at Ang II concentrations as low as 0.1 nM, and their activities were sustained for at least 90 min (Fig. 3 and data not shown). In contrast, Src was found to be constitutively activated in WB cells, and its activity did not change with Ang II stimulation (O.M. Tsygankova, unpublished data).

To investigate whether Fyn and Yes were required for Ang II-induced mitogenesis, quiescent WB cells were microinjected with antibodies against Fyn and Yes, followed by incubation with Ang II and BrdU, as described in Methods.

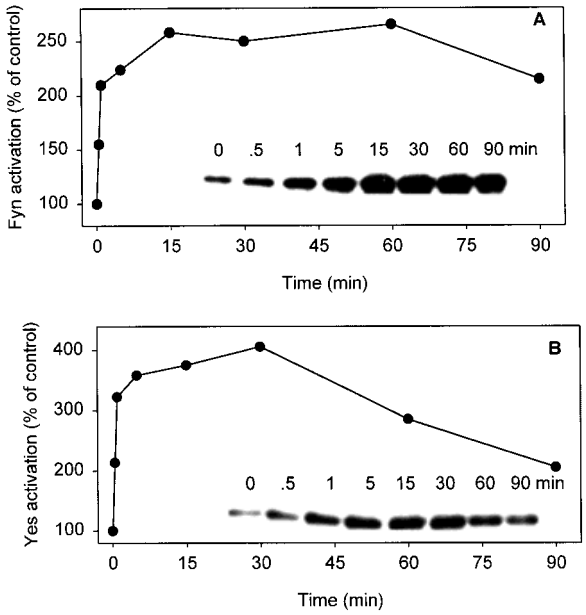

Fig. 3. Time course of Ang II-induced activation of Fyn and Yes in WB cells. Quiescent WB cells were stimulated with 1 μM Ang II for various time intervals, lysed, and the normalized cell extracts were used for immunoprecipitation of Fyn (**A**) and Yes (**B**). Immunoprecipitates were washed, and immune complex kinase assays were carried out as described in Materials and Methods. The inserts show phosphorylation of enolase by Fyn (A) or Yes (B). The data represent one of four independent experiments.

Figure 4 shows that Ang II caused a threefold increase in DNA synthesis, which was abrogated in cells that were microinjected with antibodies against Fyn or Yes and then treated with Ang II. These results are consistent with the suggestion that activation of Fyn and Yes are required for Ang II-stimulated DNA synthesis in WB cells.

Involvement of G_i Proteins in Ang II Signaling in WB Cells

It is known that Ang II receptors are coupled to both G_i and G_q members of the G protein family [Jard et al., 1981; Wong et al., 1992]. Ang II-mediated breakdown of inositol phospholipids by phospholipase C enzymes is pertussis toxin insensitive in WB cells and presumably is mediated by activation of G_q [Hepler et al., 1990]. Less is known about the involvement of G_i proteins in Ang II signaling in these cells, but by analogy with hepatocytes it may be assumed that their activation is associated with an inhibition of adenylyl cyclase [Jard et al., 1981]. The βγ subunits released from heterotrimeric G proteins may also play an important role

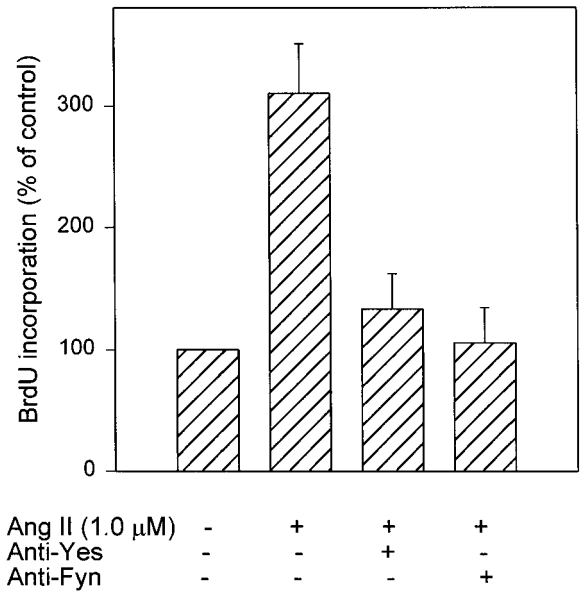

Fig. 4. Antibodies against Fyn and Yes inhibit Ang II-stimulated DNA synthesis in WB cells. WB cells were serum-starved for 24 h before injection. Approximately 100–140 cells were injected with antibodies against Fyn or Yes in each experiment. After incubation in serum-free medium for 1 h, WB cells were treated with 1 μM Ang II for 24 h to stimulate DNA synthesis. The number of nontreated cells incorporated BrdU was taken as 100%. The data represent the means ± SE of three independent experiments.

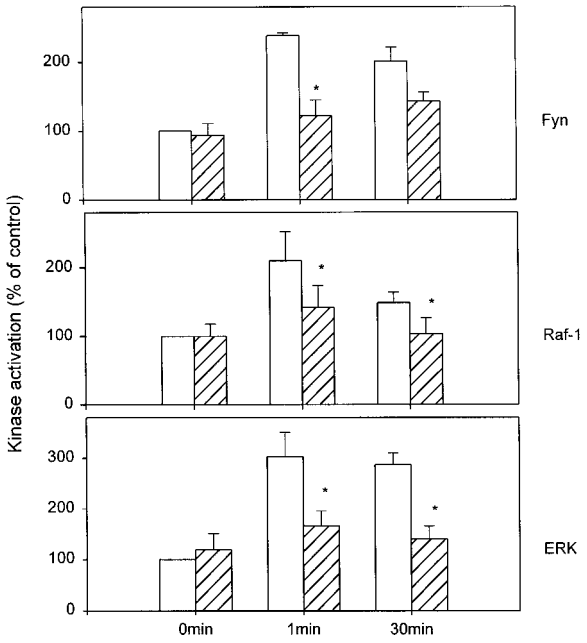

Fig. 5. Effect of pertussis toxin on Ang II-induced activation of Fyn, Raf-1, and ERK. Quiescent WB cells were treated with (striped bars) or without (open bars) 10 ng/ml of pertussis toxin for 20 h and then stimulated with 1 μM Ang II for 1 and 30 min. Cell lysates were immunoprecipitated with appropriate antibodies, and immune complex kinase assays were carried out as described in Methods. Kinase activity in nonstimulated cells was taken as 100%. Results represent means ± SE of four independent experiments. The statistical analysis was performed by using a paired *t*-test. $*P < 0.05$.

in modulating signal transduction pathways responsible for cell growth, because agonists linked to G_i protein-coupled receptors are believed to mediate the activation of Src family kinases and the ERK pathway by release of βγ subunits [Post and Brown, 1996; van Biesen et al., 1996]. To determine whether G_i proteins are involved in Ang II-mediated activation of the ERK pathway in WB cells, the cells were treated with pertussis toxin before stimulation with Ang II. Control experiments showed that pretreatment of WB cells with 10 ng/ml of pertussis toxin for 20 h was sufficient for complete ADP-ribosylation of G_i protein subunits (data not shown). As shown in Figure 5, pertussis toxin pretreatment significantly ($P < 0.05$) inhibited both the first phase (1 min) and the second phase (30 min) of the Ang II-induced activation of Raf-1 and ERK. These results suggest that G_i proteins are likely to be involved in the ERK activation in WB cells. Interestingly, pertussis toxin pretreatment did not affect Ang II-induced activation of Yes (data not shown) but did inhibit Fyn activation (Fig. 5), suggesting that Fyn and Yes may be activated through G_i and G_q proteins, respectively.

Studies on Ang II-Induced c-Fos Expression in WB Cells

Growth factor-stimulated mitogenesis is associated with a rapid induction of the immediate early genes (*c-fos, c-myc, c-jun*). Moreover, transcription of *c-fos* is mediated by activated ERK [Karin et al., 1997]. Therefore, it was of interest to investigate whether Ang II signaling involves *c-fos* induction in WB cells and whether G_i proteins participate in this process. Quiescent WB cells were stimulated with 1 μM Ang II for different times and stained with antibodies against c-Fos. The number of cells expressing c-Fos was increased three times within 15 min of stimulation and reached a maximum at 30 min, indicating that Ang II stimulates a rapid induction of c-Fos synthesis in WB cells (Fig. 6). Pertussis toxin pretreatment caused an inhibition of c-Fos expression by 20–50% over the 2-h time period of the experiment (Fig. 6), indicating that G_i proteins partially mediate *c-fos* induction by Ang II in these cells.

To investigate whether Fyn and/or Yes mediate Ang II-induced expression of c-Fos in WB

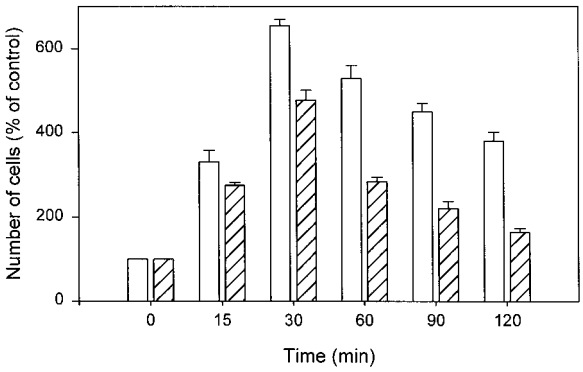

Fig. 6. Pertussis toxin inhibits Ang II-induced c-Fos expression in WB cells. WB cells were serum starved for 24 h in the presence (striped bars) or absence (open bars) of 10 ng/ml pertussis toxin and then stimulated by 1 μM Ang II for the indicated time. After fixation, cells were stained with antibodies against c-Fos as described in Methods. The number of cells expressing c-Fos before Ang II stimulation was taken as 100%. The results represent means ± SE from three independent experiments.

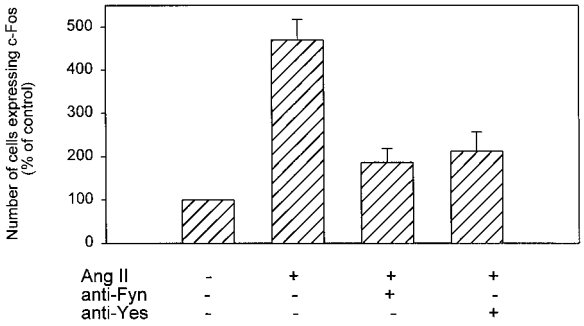

Fig. 7. Antibodies against Fyn and Yes inhibit Ang II-stimulated c-Fos expression. WB cells were serum starved for 24 h before injection. Approximately 100–140 cells were injected with antibodies against Fyn or Yes in each experiment. After incubation in serum-free medium for 2 h, WB cells were treated with 1 μM Ang II for 45 min, and c-Fos induction was assayed as described in Methods. The number of nontreated cells showing c-Fos expression was set for 100%. The data represent the means ± SE of three independent experiments.

cells, antibodies against Fyn and Yes were microinjected into the cells, which were then incubated in serum-free medium, stimulated with Ang II, and stained with anti-c-Fos antibody. In cells microinjected with either anti-Fyn or anti-Yes antibody, Ang II-stimulated expression of c-Fos was significantly inhibited (Fig. 7), indicating that both kinases participate in pathways leading to *c-fos* transcription.

DISCUSSION

The present studies show that Ang II induces activation of Raf-1, MEK, and ERK in a biphasic and sustained fashion in WB cells (Fig. 1). The sustained activation of the ERK cascade was sensitive to inhibition by genistein or herbimycin A, arguing that PTKs are involved in its regulation. This conclusion was supported by the observation that Ang II stimulated a prolonged activation of Fyn and Yes (Fig. 3), whereas antibodies against these kinases completely inhibited Ang II-induced DNA synthesis in WB cells (Fig. 4). Taken together, these data suggest that the tyrosine kinase activities of both Fyn and Yes are an essential component of the Ang II-stimulated pathway(s) leading to a sustained activation of the ERK cascade and subsequent mitogenesis. A requirement of Src family kinases for proliferation has also been shown in cells stimulated with growth factors (PDGF, NGF, EGF) [Kremer et al., 1991; Roche et al., 1995; Broome and Hunter, 1996]. However, to date, an obligatory requirement of Src family kinases for proliferation mediated via G protein-coupled receptors has not been fully evaluated [Roche et al., 1995]. A relevant finding is that the expression of Csk, a negative regulator of Src, led to inhibition of ERK activation induced by stimulation of G_i protein-coupled receptors in COS-7 cells [Luttrell et al., 1996]. In addition, the involvement of Src family kinases in nuclear signaling has been shown in vascular cells, in which a dominant negative mutant of Src blocked endothelin-stimulated c-Fos expression [Simonson et al., 1996].

The present report extends these findings with the demonstration that pertussis toxin pretreatment inhibited Ang II-induced activation of Fyn but did not affect the activation of Yes (Fig. 5 and data not shown). These data suggest that Src family kinases may be regulated by different G proteins that are coupled to Ang II receptors in WB cells. Nevertheless, both kinases appear to be essential for Ang II-induced c-Fos expression (Fig. 7), indicating that signal transduction pathways initiated by different G proteins merge upstream of *c-fos* transcription. These results represent the first demonstration that two different Src kinases may be activated by a single agonist, whose receptors are linked to different G proteins. Activation of several Src family kinases has been shown in lymphocytes after antigen stimulation and also in fibroblasts following PDGF treatment [Chow and Veillette, 1995; Roche et al., 1995]. For example, both Fyn and Lck are activated during T-cell activation and are essential for T-cell receptor signaling, but there is an evidence that they mediate different signal

transduction pathways [Chow and Veillette, 1995]. T-cell activation includes many responses similar to those stimulated by hormones and growth factors, namely, a rapid increase in protein tyrosine phosphorylation, activation of PLC, mobilization of intracellular Ca^{2+}, activation of Ras, and the ERK cascade [Weiss and Littman, 1994]. Moreover, T-cell receptors do not possess intrinsic tyrosine kinase activity and recruit cytoplasmic PTK to propagate the signal, resembling G protein-coupled receptors in this respect. An involvement of several cytoplasmic tyrosine kinases with distinct functions appears to be quite common for cellular signaling.

How G proteins mediate the activation of Src family kinases remains to be elucidated. G_q proteins are believed to regulate the activation of protein kinases via α_q-dependent mechanisms [Post and Brown, 1996; van Biesen et al., 1996]. Recently, it was shown that Bruton's tyrosine kinase can be directly activated by α_q subunit [Bence et al., 1997]. Yes may be activated by a similar mechanism in WB cells. G_i proteins are known to regulate a hormone-stimulated activation of the ERK pathway through $\beta\gamma$- and PTK-dependent mechanisms [Post and Brown, 1996; van Biesen et al., 1996]. Moreover, Src family kinases were activated and formed complexes with tyrosine phosphorylated Shc upon stimulation of G_i protein-coupled receptors [Ptasznik et al., 1995; Luttrell et al., 1996]. These events were blocked by overexpression of $\beta\gamma$ subunits or Csk, suggesting that $\beta\gamma$ subunits mediate Src activation and its interaction with Shc [Luttrell et al., 1996]. In WB cells, Ang II induces a rapid tyrosine phosphorylation of Shc and promotes formation of its complex with Grb2 (O.M. Tsygankova, unpublished data). These findings suggest that Fyn can be involved in the activation of Ras, which leads, in turn, to the activation of Raf-1 and, as a consequence, triggers the entire ERK pathway. However, pertussis toxin-insensitive Ang II-induced activation of Fyn and its interaction with Shc were observed in cardiac myocytes [Salles et al., 1993], suggesting that G_q proteins can also mediate Ras activation. Thereby, it remains to be elucidated, whether G_i and G_q proteins can both mediate Ang II-stimulated Ras activation in WB cells.

To summarize, the present data show that Ang II induces a biphasic and sustained activation of the ERK pathway in WB cells. The results also indicate that both pertussis toxin-sensitive and -insensitive G proteins are involved in Ang II signaling. The action of G proteins appears to be mediated by tyrosine protein kinases, two of which, the Src family kinases Fyn and Yes, are activated in response to Ang II and are required for Ang II-stimulated DNA synthesis. Furthermore, Fyn and Yes participate in the distinct signal transduction pathways. Only Fyn activation is pertussis toxin-sensitive, suggesting that its activation requires $\beta\gamma$ subunits released from G_i proteins, whereas Yes may be activated through G_q or other pertussis toxin-insensitive G proteins.

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