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Angiotensin II Stimulates Calcium-Dependent Activation of c-Jun N-Terminal Kinase

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In GN4 rat liver epithelial cells, angiotensin II (Ang II) and other agonists which activate phospholipase C stimulate tyrosine kinase activity in a calcium-dependent, protein kinase C (PKC)-independent manner. Since Ang II also produces a proliferative response in these cells, we investigated downstream signaling elements traditionally linked to growth control by tyrosine kinases. First, Ang II, like epidermal growth factor (EGF), stimulated AP-1 binding activity in a PKC-independent manner. Because increases in AP-1 can reflect induction of c-Jun and c-Fos, we examined the activity of the mitogen-activated protein (MAP) kinase family members Erk-1 and -2 and the c-Jun N-terminal kinase (JNK), which are known to influence c-Jun and c-Fos transcription. Ang II stimulated MAP kinase (MAPK) activity but only ~50% as effectively as EGF; again, these effects were independent of PKC. Ang II also produced a 50- to 200-fold activation of JNK in a PKC-independent manner. Unlike its smaller effect on MAPK, Ang II was approximately four- to sixfold more potent in activating JNK than EGF was. Although others had reported a lack of calcium ionophore-stimulated JNK activity in lymphocytes and several other cell lines, we examined the role of calcium in GN4 cells. The following results suggest that JNK activation in rat liver epithelial cells is at least partially Ca^{2+} dependent: (i) norepinephrine and vasopressin hormones that increase inositol 1,4,5-triphosphate stimulated JNK; (ii) both thapsigargin, a compound that produces an intracellular Ca^{2+} signal, and Ca^{2+} ionophores stimulated a dramatic increase in JNK activity (up to 200-fold); (iii) extracellular Ca^{2+} chelation with ethylene glycol tetraacetic acid (EGTA) inhibited JNK activation by ionophore and intracellular chelation with 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl-ester (BAPTA-AM) partially inhibited JNK activation by Ang II or thapsigargin; and (iv) JNK activation by Ang II was inhibited by pretreatment of cells with thapsigargin and EGTA, a procedure which depletes intracellular Ca^{2+} stores. JNK activation following Ang II stimulation did not involve calmodulin; neither W-7 nor calmidizolium, in concentrations sufficient to inhibit Ca^{2+}/calmodulin-dependent kinase II, blocked JNK activation by Ang II. In contrast, genistein, in concentrations sufficient to inhibit Ca^{2+}-dependent tyrosine phosphorylation, prevented Ang II and thapsigargin-induced JNK activation. In summary, in GN4 rat liver epithelial cells, Ang II stimulates JNK via a novel Ca^{2+}-dependent pathway. The inhibition by genistein suggests that Ca^{2+}-dependent tyrosine phosphorylation may modulate the JNK pathway in a cell type-specific manner, particularly in cells with a readily detectable Ca^{2+}-regulated tyrosine kinase.

Hormones which activate seven transmembrane receptors such as bombesin, thrombin, and angiotensin II (Ang II) can act as growth factors in some cell types (5, 25, 35). The mitogenic pathways induced by these hormones depend upon the class of G-proteins to which the receptor is coupled and the downstream pathways activated by that G-protein. For example, $G_{\alpha i}$ may activate MEK kinase (MEKK) (29, 34) while $G_{\alpha q}$ activates phospholipase C, stimulating diacylglycerol and calcium-dependent pathways (2). The relative contributions of the individual intracellular signals to the growth response may also differ from cell type to cell type. For example, in some cell types, a calcium signal is thought to be dispensable for growth (6, 44, 48), but in other cell types, a rise in intracellular calcium appears to be part of the proliferative signal (2, 60, 61).

In rat liver epithelial cells, Ang II stimulates proliferation. Until recently, this was presumed to occur through G-protein-dependent activation of protein kinase C (PKC) or calcium/calmodulin-dependent enzymes, including calcium/calmodulin-dependent kinase II. However, work from this laboratory has added another potential mechanism leading to proliferation: an Ang II-dependent stimulation of tyrosine kinase activity (24–26). In both normal (WB) and transformed (GN4) rat liver epithelial cells, treatment with Ang II, vasopressin, and α_1 agonists results in rapid (15 to 60 s) tyrosine phosphorylation of at least five proteins. This hormonally induced increase in tyrosine kinase activity is blocked by calcium chelators which prevent a rise in intracellular calcium and is mimicked by agents that increase intracellular calcium concentrations. The Ang II-dependent increase in tyrosine kinase activity is PKC independent. The tyrosine kinase regulated by calcium in GN4 cells is likely to be a member of a novel family, because it appears to be a cytosolic enzyme of 115 to 120 kDa. This activity is not recognized by JAK1, JAK2, or JAK3 antisera and can be clearly separated from $pp125^{FAK}$ activity (unpublished results). Other G-protein-coupled receptors have also been shown to stimulate tyrosine phosphorylation in several cell types (3, 13, 14, 38, 40, 45, 47, 53, 56, 66).

Tyrosine phosphorylation is a regulatory mechanism frequently associated with cell growth and mitosis. Stimulation of growth factor receptors such as the epidermal growth factor (EGF) receptor results in tyrosine phosphorylation, stimulation of the Ras pathway, and activation of a serine/threonine kinase cascade that includes the mitogen-activated protein (MAP) kinase (MAPK) family members Erk-1 and -2 (for a

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review, see reference 9). Activated MAPK in turn influences gene expression by phosphorylating transcription factors such as Elk-1 (16, 39). Other members of the MAPK family have recently been identified in yeast and mammalian cells; one group, referred to as the stress-activated protein kinases or the c-Jun amino-terminal kinases (JNKs), has been cloned and assigned a physiologic function (12, 23, 32). JNK1 was initially identified as a 46-kDa kinase activity with an unusually high affinity for its substrate, c-Jun. JNK1 and its 54-kDa related JNK2 phosphorylate c-Jun on specific N-terminal serine residues (Ser-63 and Ser-73), leading to enhanced c-Jun transactivation potential (57). Unlike Erk-1 and -2, the JNKs are strongly activated by stimuli other than growth factor receptor tyrosine kinases, including signals as diverse as UV irradiation (12, 23), osmotic shock (15, 19), protein synthesis inhibitors, hydrogen peroxide (32), and tumor necrosis factor alpha (TNFα) stimulation (32, 62, 63). While the pathways of JNK activation have not been fully delineated, these kinases are only slightly activated by agents that primarily activate Raf/MEK/MAPK (32, 43, 62). This suggests separate pathways leading to JNK and MAPK activation, a divergence substantiated by the isolation of SEK (also referred to as JNKK), a dual-specificity kinase that directly activates JNK by phosphorylating the enzyme on a TPY motif (36, 54, 65), while MEK activates Erk-1 and -2 by phosphorylating a TEY motif (49).

In rat liver epithelial cells, treatment with both Ang II and EGF increased tyrosine phosphorylation and proliferation (24–26). We now report that both Ang II and EGF increase AP-1 binding as well as MAPK and JNK activation in a PKC-independent manner. Particularly striking was a 200-fold activation of JNK by Ang II, a stimulation considerably greater than that by EGF. Thus, unlike many ligands which stimulate primarily either JNK (e.g., TNFα) or MAPK (e.g., EGF), Ang II effectively stimulated both JNK and MAPK. In lymphocytes and several other cell lines, a calcium signal did not increase JNK activity (59). In contrast, Ang II-induced JNK activation in GN4 cells is dependent on increases in calcium and is mimicked by agents that increase calcium. Analogous to Ang II's activation of tyrosine kinase activity in GN4 cells, JNK activation was PKC and calmodulin independent and was inhibited by genistein. These parallels suggest that differential expression of a calcium-regulated tyrosine kinase may distinguish those cell types in which a calcium signal, as a single stimulus, can activate JNK.

MATERIALS AND METHODS

Materials. EGF was purified from mouse salivary glands as described previously (55). Human Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was obtained from Sigma. Ang II was prepared in 50 mM acetic acid. EGF was prepared as 100-fold-concentrated solutions in 10 mM NaP_i (pH 7.4)–150 mM NaCl containing 0.1% bovine serum albumin (BSA). Phorbol 12-myristate 13-acetate (TPA; Sigma), thapsigargin (Sigma), bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM; Gibco-BRL), ionomycin, and A23187 (Calbiochem) were prepared as stock solutions in dimethyl sulfoxide (final concentration, ≤0.1% [vol/vol]). Polyclonal anti-ERK/MAPK antiserum K-23 was obtained from Santa Cruz Biotechnology, and enhanced chemiluminescence (ECL) reagents were from Amersham.

Cell culture. GN4 rat liver epithelial cells were grown at 37°C in Richter's improved minimal essential medium with 0.1 μM insulin supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere as described previously (24). Prior to agonist stimulation, cells were serum starved (0.5% FBS) for 20 h and incubated for 18 h with 6 to 10 μM TPA or vehicle to deplete cells of PKC.

Assessment of AP-1 activity. Nuclear extracts were prepared as described by Sadowski and Gilman (52) with modifications. Briefly, confluent GN4 cells (100-mm plate) were serum starved for 24 h (0.5% FBS) and treated for 18 h with either 10 μM TPA or vehicle. Following treatment with EGF (300 ng/ml) or Ang II (1 μM), cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), once with PBS containing 1 mM Na_3VO_4 and 20 mM NaF, and once with hypotonic buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA [ethylene glycol tetraacetic acid], 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg of leupeptin per ml, 4 kallikrein inhibitor units [KIU] of aprotinin per ml). Cells were lysed by addition of 0.5 ml of hypotonic buffer with 0.2% Nonidet P-40, and lysates were scraped into Eppendorf tubes and centrifuged at maximum speed for 20 s. The pelleted nuclei were resuspended in 120 μl of hypotonic buffer with 420 mM NaCl and 20% glycerol added and rocked gently for 30 min at 4°C. Soluble extracted nuclear proteins were isolated by a 20-minute centrifugation at maximum speed.

Gel shift assays were performed as described before (1). Briefly, 10 μg of nuclear extracts was incubated on ice for 15 min with 2 μg of poly (dI · dC) · poly(dI · dC) in binding buffer (10 mM Tris [pH 7.9], 0.5 mM EDTA, 10% glycerol, 1 mM DTT, BSA), and then an excess of end-labeled AP-1 probe (5′TGAGGGTGATTCAGAGGCAGGTGCCCACAGTTTCAC TTC3′; ~10,000 cpm) (17) was added, followed by incubation at room temperature for 20 min. Binding to the radiolabeled probe was inhibited by addition of unlabeled oligonucleotide to an AP-1 sequence. Reaction mixes were run on 5% nondenaturing polyacrylamide gels and autoradiographed.

Preparation of GST fusion protein. pGEX2T-c-Jun (wild type), containing the transactivation domain of c-Jun (amino acids 1 to 223), pGEX2T-c-Jun (Ala-63 and -73), and pGEX2T were described previously (23). This portion of c-Jun contains the JNK binding domain and serine residues 63 and 73, which, when phosphorylated, correlate with increased transcriptional activity of c-Jun. The serine 63 and 73 residues are mutated to Ala in the c-Jun Ala protein, and the mutated fusion protein serves as a control to determine increases in phosphorylation at sites not associated with increased transcriptional activation. The glutathione-*S*-transferase (GST)-protein expression vectors were transformed into *Escherichia coli* DH5α. Proteins were purified as described by Pharmacia. The amounts of purified proteins were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent staining with Coomassie blue. The appearance of a phosphorylated GST-c-Jun doublet is produced by the full-length GST-c-Jun amino acids 1 to 223 [GST-c-Jun(1–223)] and a proteolytic cleavage product containing approximately 80 N-terminal amino acids. This lower band, created during purification of the fusion protein, migrates similarly to a purified GST-c-Jun(1–79) construct.

Preparation of cell extracts. Cells were lysed with ice-cold new lysis buffer (20 mM sodium HEPES [pH 7.3], 50 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, 1 μg of leupeptin per ml, 4 KIU of aprotinin per ml). Lysates were clarified by centrifugation at 14,000 × *g* for 15 min at 4°C.

In vitro JNK assay. In vitro kinase assays were carried out as previously described with GST-c-Jun linked to Sepharose beads as the substrate (23). Briefly, 50 μg of cell extracts was incubated with 10 μg of GST, GST-c-Jun, or GST-c-Jun(Ala) and 300 μl of HEPES binding buffer (20 mM HEPES [pH 7.7], 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5% Triton X-100). The mixture was then rotated for 2 h in a microcentrifuge tube. After the incubation, protein was pelleted by centrifugation at 14,000 × *g* for 2 min. JNK binds to the transactivation domain of c-Jun and is precipitated with the GST-c-Jun. After four washes in 1 ml of HEPES binding buffer, the pelleted beads were resuspended in 30 μl of kinase buffer (20 mM HEPES [pH 7.6], 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM Na_3VO_4, 2 mM DTT) containing 0.5 μCi of [γ-^{32}P]ATP (40 μM ATP). Samples were incubated for 20 min at 30°C and chilled, and excess kinase buffer was removed. Phosphorylated proteins were eluted by boiling with 7 μl of 3× concentrated SDS sample buffer (26) and resolved on an SDS–10% PAGE gel, followed by autoradiography and phosphorimage analysis. The consistency of GST-c-Jun protein recovery was monitored by examination of the Coomassie blue-stained protein in each gel lane.

MAPK activation assays. Activation of MAPK was determined as described previously (62). Briefly, 10 μl of 3× sample buffer was added to 45 μg of cell lysate, and extracts were resolved on an SDS–15% PAGE gel. Proteins were then transferred to nitrocellulose membranes for analysis by Western blotting (immunoblotting) with the rabbit anti-ERK/MAPK antiserum K-23 (Santa Cruz Biotechnology) to detect p42 MAPK and p44 MAPK. Blots were probed with a secondary donkey anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase, and immunoreactive proteins were visualized by using ECL reagents (Amersham).

Phosphotyrosine immunoblotting. Cells incubated for 15 min with or without genistein at various doses were treated with EGF (300 ng/ml) or Ang II (0.1 μM). At 1 min, the cells were lysed in detergent-containing buffer, clarified by centrifugation in a microcentrifuge, boiled in SDS sample buffer, separated by 8% PAGE, and transblotted to nitrocellulose (26). Antiphosphotyrosine immunoblotting was performed with monoclonal antibody PT66 (Sigma) as described before (26).

RESULTS

Ang II increases PKC-independent AP-1 binding. We examined the changes affecting gene expression associated with the Ang II-dependent tyrosine phosphorylation and proliferative responses. First, the effect of Ang II treatment on AP-1 DNA

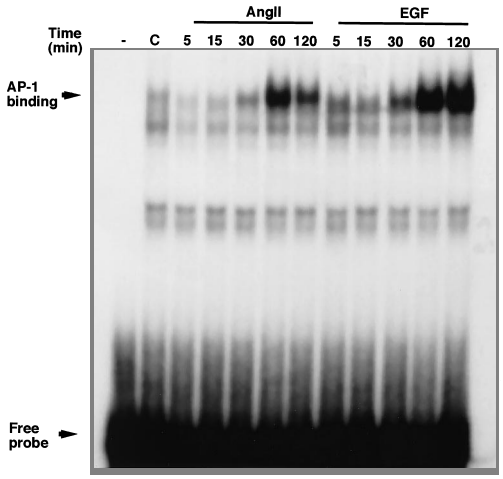

FIG. 1. Ang II and EGF induce AP-1 binding. GN4 cells were treated with 10 μM TPA for 18 h. Nuclear extracts were prepared from cells stimulated with Ang II or EGF for the indicated times. Extracts were incubated with labeled AP-1 oligonucleotide probe and subjected to nondenaturing gel electrophoresis followed by autoradiography, as described in Materials and Methods. AP-1 binding is indicated by the upper arrow, and free probe is indicated by the lower arrow. The nonspecific binding in the mid-gel region (not inhibited by unlabeled AP-1 oligonucleotides) indicates the even loading of the gel. Data are representative of three experiments.

binding was assessed by a gel shift assay as described in Materials and Methods. AP-1 binding was increased within 30 min of Ang II stimulation (data not shown). Ang II stimulation can activate PKC, which by itself increases AP-1 binding. To determine if Ang II-dependent AP-1 activity was due to PKC activation, cells were treated for 18 h with phorbol ester (10 μM TPA), which depletes TPA-sensitive PKC activity (22) and abolishes the PKC-dependent activation of MAPK in these cells (see Fig. 2 below). In serum-starved, TPA-treated cells, EGF-induced AP-1 binding was detected beginning at 30 min and remained elevated for 120 min. Similar to EGF, Ang II-induced AP-1 binding was detected at 30 min; however, AP-1 activity peaked at 60 min and declined by 120 min (Fig. 1). Ang II-induced AP-1 binding at 60 min was slightly less than EGF-induced binding at 60 min.

Ang II stimulation leads to PKC-independent activation of MAPK. MAPK activation can lead to increased AP-1 binding via increases in c-Fos expression (see reference 30 for a review). To determine if Ang II stimulation increases MAPK activation, GN4 cells were stimulated with Ang II or EGF. After lysis, the phosphorylation state of MAPK was assessed in a gel mobility shift assay by subjecting cell extracts to Western blot analysis with anti-Erk-1 and -2 antibodies. Phosphorylation of MAPK was detected as a shift in mobility and is highly

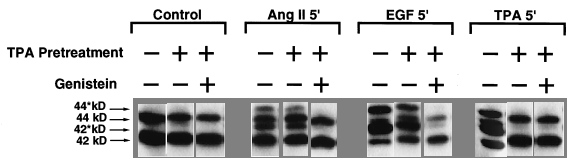

FIG. 2. Ang II stimulation activates both Erk-1 and -2. GN4 cells were (+) or were not (−) treated with 10 μM TPA for 18 h to deplete PKC or with genistein for 15 min to inhibit tyrosine kinase activity. Cells were stimulated for 5 min with vehicle, Ang II (1 μM), EGF (300 ng/ml), or TPA (100 nM). Lysates were run on SDS-PAGE gels and MAPK immunoblotted. Unphosphorylated Erk-1 and -2 are indicated by the arrows marked 44 and 42 kD, respectively. Asterisks denote the mobility-shifted form of these two Erks.

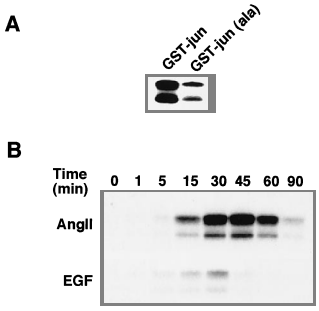

FIG. 3. Ang II stimulation activates JNK. (A) TPA-treated GN4 cells were stimulated with Ang II (1 μM) for 30 min, and cell lysates were used for the in vitro kinase assay with GST-c-Jun or GST-c-Jun(Ala) as the substrate. (B) TPA-treated GN4 cells were stimulated with Ang II (1 μM) or EGF (300 ng/ml) for the indicated times. Cell lysates were used for in vitro kinase assays with GST-c-Jun as the substrate as described in Materials and Methods.

correlated with increased MAPK activity. Ang II stimulation resulted in activation of MAPK at 5 min (Fig. 2).

PKC can activate the MAPK pathway, and Ang II activates PKC. In order to determine if PKC mediated Ang II-induced MAPK activation, cells were depleted of PKC by overnight TPA treatment. The right panel of Fig. 2 demonstrates that a short (5 min) TPA treatment stimulated MAPK in GN4 cells. This stimulation was abolished by an 18-h TPA treatment, confirming the loss of TPA-sensitive isoforms of PKC. In contrast, EGF- or Ang II-induced MAPK activation was equivalent or only slightly diminished in PKC-depleted cells, indicating that both Ang II and EGF increase MAPK activity at least partly by PKC-independent mechanisms. These results were confirmed by MAP kinase assays in which Erk-1 was immunoprecipitated with specific antibodies and used in an immune complex kinase activity with myelin basic protein as the substrate (data not shown).

In TPA-treated cells, Ang II stimulated both Erk-1 and -2 within 1 min; activation was nearly maximal at 5 min (data not shown). The percent stimulation of MAPK phosphorylation by Ang II was variable but was always less than that produced by EGF. In general, the activation induced by Ang II was approximately 50% of that by EGF. Activation of MAPK by Ang II requires tyrosine kinase activity. Stimulation of MAPK by both Ang II and EGF was blocked by the tyrosine kinase inhibitor genistein (Fig. 2, second and third panels). This concentration of genistein (600 μM) totally inhibited Ang II-stimulated tyrosine kinase activity (see Fig. 9B).

Ang II stimulation leads to PKC-independent activation of JNK. Activation of JNK can also increase AP-1 binding by increasing the transactivation potential of c-Jun and ATF-2, leading to induction of c-Jun (12, 18). Therefore, we tested the effect of Ang II on JNK activation in a solid-state kinase assay with GST fused to the N-terminal 223 amino acids of c-Jun as a substrate. In our preparation, a portion of this fusion protein is proteolyzed, cleaving approximately all but the first 79 amino acids of the c-Jun N terminus. Both forms of the fusion protein contain Ser-63 and Ser-73, which are phosphorylated by JNK. A similar fusion protein with Ser-63 and Ser-73 of c-Jun mutated to alanine [c-Jun(Ala)] was used to assess the specificity of the JNK kinase activity. JNK binds with high affinity to the c-Jun N terminus and can then be precipitated with the GST-Jun substrate and separated from nonspecific kinases by a low-speed centrifugation. Serum-starved, TPA-treated cells were stimulated with Ang II for 1 to 90 min and lysed. Ang II increased JNK kinase activity, and the activity was greater

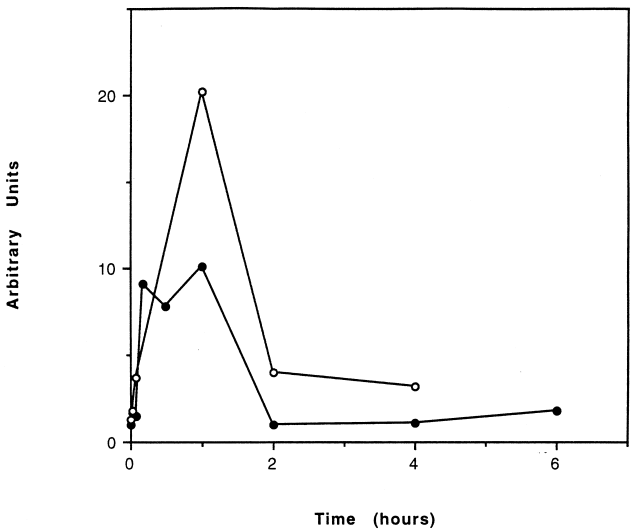

FIG. 4. TPA treatment enhances Ang II activation of JNK. GN4 cells were treated for the indicated times (○) or not treated (●) with TPA (10 μM) and then stimulated with Ang II (1 μM). Cell extracts were used in an in vitro kinase assay with GST-c-Jun as a substrate. Incorporation of [γ-^{32}P]ATP was quantitated by phosphorimage analysis and is expressed in arbitrary units.

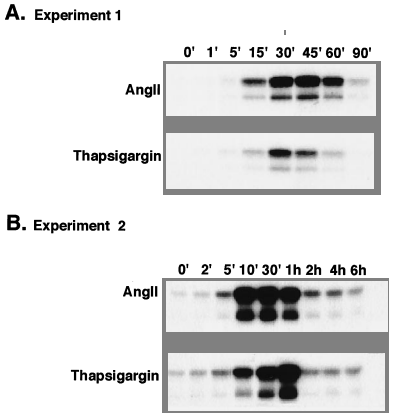

FIG. 5. Thapsigargin treatment activates JNK. TPA-treated GN4 cells were stimulated with Ang II or thapsigargin (1 μM) for the indicated times (in minutes or hours). Cell lysates were used in an in vitro kinase assay with GST-c-Jun as the substrate. Panels A and B represent two different experiments, showing the range and time course of JNK activation by thapsigargin.

when assayed with the wild type than with the Ala-mutated substrate (Fig. 3A). As shown in Fig. 3B, Ang II stimulation of JNK kinase activity peaked between 30 and 60 min and declined by 90 to 120 min. The fold activation of JNK by Ang II stimulation was dependent on the background activity but ranged from 40- to 200-fold. This massive fold activation is due in part to the low background of JNK activity in these epithelial cells, a background that appears to differ from the higher basal activity seen in cultured fibroblasts and other cell types reported in the literature. The induction by Ang II was generally four- to sixfold greater than activation by EGF (Fig. 3B). The significant activation of both JNK and MAPK (Fig. 2 and 3) by Ang II was somewhat surprising, given the fact that many ligands predominantly activate only one of the MAPK family members (32, 62).

Norepinephrine and vasopressin are two agonists that bind to G-protein-coupled receptors, leading to increases in intracellular Ca^{2+} levels and tyrosine phosphorylation in GN4 cells. A 30-min treatment with either agonist (norepinephrine [10 μM] or vasopressin [1 μM]) increased JNK kinase activity by an amount comparable to that by Ang II (data not shown). These data suggest that in GN4 cells, JNK activation is a common downstream effector to agonists which increase intracellular Ca^{2+} levels.

In other cell types, PKC does not substantially activate JNK (43); however, we investigated its role in GN4 cells because Ang II, norepinephrine, and vasopressin all activate PKC. Short (5 min) TPA stimulation consistently activated MAPK (e.g., see Fig. 2); however, when assayed in the same cell lysates, JNK activity was not significantly increased (data not shown). In fact, in cells depleted of PKC by TPA treatment, JNK activation in response to Ang II was greater than that in non-TPA-treated cells (Fig. 4). This is probably due to an absence of PKC-dependent downregulation of the Ang II response at the receptor or G-protein level, a phenomenon that we had previously observed in rat liver epithelial cells (22).

A calcium signal activates JNK in GN4 cells. Ang II stimulation leads to transient increases in intracellular calcium levels, and this calcium signal mediates Ang II-dependent increases in tyrosine kinase activity (24, 26). Stimulation of tyrosine kinase activity in GN4 cells is mimicked by increasing intracellular calcium levels by using either calcium ionophores or thapsigargin. Additionally, norepinephrine and vasopressin, which increase intracellular calcium, also increase JNK activity, suggesting that calcium may participate in JNK activation. To define the role of calcium in JNK activation, TPA-treated GN4 cells were stimulated for the indicated times with Ang II or thapsigargin (an agent that prevents reuptake of intracellular calcium into storage vessicles and thus produces an intracellular calcium signal [25]). JNK activity increased in response to thapsigargin but was slightly delayed compared with that after Ang II treatment (Fig. 5A and B), as for the thapsigargin-induced intracellular calcium signal (25). In multiple experiments in which Ang II and thapsigargin were directly compared, the thapsigargin effect ranged from approximately 40 to 120% (e.g., Fig. 5A and B) of that of Ang II, suggesting that Ang II-induced calcium increases may be in part responsible for JNK activation. In contrast, when MAPK and JNK activation were analyzed with the same cell lysates, thapsigargin only minimally activated MAPK at 5 or 30 min, while Ang II clearly activated MAPK (unpublished results).

To further test for the role of calcium in JNK activation, TPA-treated GN4 cells were incubated with the calcium ionophore A23187 for the indicated times (Fig. 6A). A23187 stim-

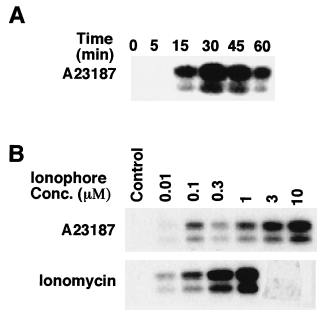

FIG. 6. Calcium ionophores activate JNK. (A) TPA-treated GN4 cells were stimulated with A23187 (10 μM) for the indicated times, and JNK activity was assessed as described in Materials and Methods. (B) TPA-treated cells were stimulated with 10 μM A23187 or ionomycin for 30 min, and JNK activity was assessed as described in Materials and Methods.

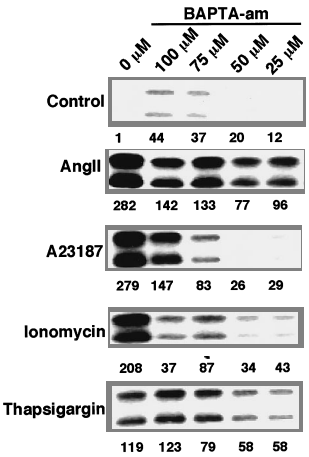

FIG. 7. JNK activation is calcium dependent. TPA-treated cells were incubated (30 min) with the indicated concentrations of BAPTA-AM (25 to 100 μM) and then stimulated (30 min) with vehicle, Ang II (1 μM), A23187 (10 μM), ionomycin (1 μM), or thapsigargin (1 μM). JNK activation was assessed as described in Materials and Methods and quantitated by phosphorimage analysis. Activation is expressed as fold activation over that in vehicle-treated cells.

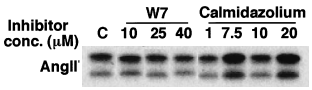

FIG. 8. JNK activation is not mediated by calmodulin. TPA-treated cells were incubated with the indicated concentrations of W7 and calmidazolium (5 min) or vehicle and then stimulated with Ang II (1 μM, 30 min). Control cells (C) were incubated without inhibitor. JNK activity was assessed as described in Materials and Methods.

ulated JNK, with increased kinase activity detectable at 15 min, peaking between 30 and 45 min, and declining by 60 min. JNK activation by A23187 (or ionomycin) was comparable to that by Ang II (up to 200-fold; see Fig. 7). The dose response of JNK activation to A23187 and ionomycin is shown in Fig. 6B. Both A23187 and ionomycin increase JNK kinase activity at 0.01 μM; an effect of ionomycin was seen at doses as low as 0.001 μM. The maximal effect seen at 1 μM ionomycin was lost at higher doses, presumably because of a toxic effect. The highest doses of ionophore appeared to be toxic, as they prevented full activation of MAPK. The toxic effect (total inhibition of JNK activation) by 3 μM or greater ionomycin was observed in three separate experiments.

Activation of JNK following Ang II stimulation is dependent upon increases in intracellular calcium. Ang II-dependent increases in tyrosine phosphorylation (26) and JNK activation (see Fig. 5 and 6) were mimicked by thapsigargin and ionophore treatment. Since increased tyrosine kinase activity following Ang II, thapsigargin, or ionophore stimulation is inhibited by intracellular or extracellular calcium chelation (24, 26), we sought to determine if JNK activation was also calcium dependent. Three approaches were used: (i) incubation of cells with BAPTA-AM, a membrane-permeable calcium chelator, (ii) a 30-min incubation with thapsigargin in the presence of EGTA, a treatment that depletes intracellular calcium stores, and (iii) incubation of cells with EGTA prior to ionophore treatment.

TPA-treated (18 h) GN4 cells were incubated for 10 min with 25, 50, 75, and 100 μM BAPTA-AM and then stimulated with Ang II, A23187, ionomycin, or thapsigargin. The acetoxymethyl ester of BAPTA can enter the cell, where the ester moiety is cleaved, leaving BAPTA to chelate intracellular calcium. BAPTA-AM (25 to 50 μM) was sufficient to block both Ang II-induced calcium transient and subsequent increases in tyrosine kinase activity in GN4 cells (24, 26). Surprisingly, BAPTA-AM treatment of GN4 cells resulted in a dose-dependent activation of JNK, particularly at 75 to 100 μM BAPTA-AM (Fig. 7); presumably, a toxic effect of BAPTA-AM is responsible. When GN4 cells were treated with agonists that raise intracellular calcium (Ang II, thapsigargin, and ionophore), JNK was activated 100- to 300-fold. Treatment with BAPTA-AM at 25 to 50 μM reduced the response to calcium ionophores by approximately 90% and the response to Ang II and thapsigargin by up to 60 to 70% (Fig. 7). The inhibition by BAPTA-AM was relatively specific; BAPTA-AM pretreatment, at concentrations sufficient to block a rise in intracellular calcium and to inhibit Ang II-dependent JNK activation, did not block activation of JNK by UV irradiation (100 J/m^2) (data not shown). The failure to fully block Ang II and thapsigargin-induced JNK activation with BAPTA-AM was seen consistently in all experiments and indicates either that Ang II and thapsigargin have calcium-independent as well as calcium-dependent mechanisms for JNK activation or that there is a complex interaction between low-dose BAPTA and Ang II with respect to basal JNK activity.

To further examine the Ca^{2+} dependence of Ang II-induced JNK activation, GN4 cells were incubated with thapsigargin and EGTA for 30 min prior to Ang II stimulation. Depleting intracellular calcium reduced the response to Ang II by 50%, similar to the 60% reduction in Ang II-stimulated JNK activation by BAPTA (data not shown). Moreover, when extracellular calcium was chelated with EGTA before addition of A23187 or ionomycin, JNK activation was reduced by greater than 90% (data not shown).

Calcium-dependent activation of JNK is not mediated via calmodulin but is inhibited by genistein. A major mechanism for calcium signal transduction is via the large class of enzymes activated by calcium and calmodulin, including the calcium/calmodulin-dependent protein kinase II (see reference 21 for a review). In order to determine if calmodulin mediates Ang II activation of JNK, GN4 cells were incubated with two well-studied calmodulin inhibitors at concentrations sufficient to block the activation of calcium calmodulin kinase II. Incubation with W7 (10 to 40 μM) or calmidazolium (7.5 to 20 μM) failed to inhibit Ang II-dependent JNK activation (Fig. 8) or MAPK activation (data not shown). When GN4 cells were treated with Ang II, an increase in calcium calmodulin-dependent kinase II was detected, as assessed by phosphorylation of a pseudosubstrate peptide (autocamtide 3) in an in vitro kinase assay (20). Incubation of GN4 cells with W7 (25 to 40 μM) and calmidazolium (10 to 20 μM) prior to Ang II stimulation blocked this increase in calcium calmodulin kinase II activity (data not shown), suggesting that W-7 and calmidazolium inhibit calmodulin-dependent processes at doses that do not block JNK activation.

In contrast to the lack of efficacy of calmodulin inhibitors, genistein (100 to 300 μM) consistently inhibited the activation of JNK by Ang II or thapsigargin (Fig. 9A). In four experiments, the activation of JNK was inhibited by 50 to 100 μM genistein and was abolished by 300 to 400 μM genistein. Parallel assessment of MAPK in these and other experiments (e.g., Fig. 2) showed that genistein also blocked MAPK activation by either Ang II or EGF. Figure 9B shows that incubation of GN4 cells with 300 μM genistein blocked Ang II- and EGF-dependent tyrosine phosphorylation.

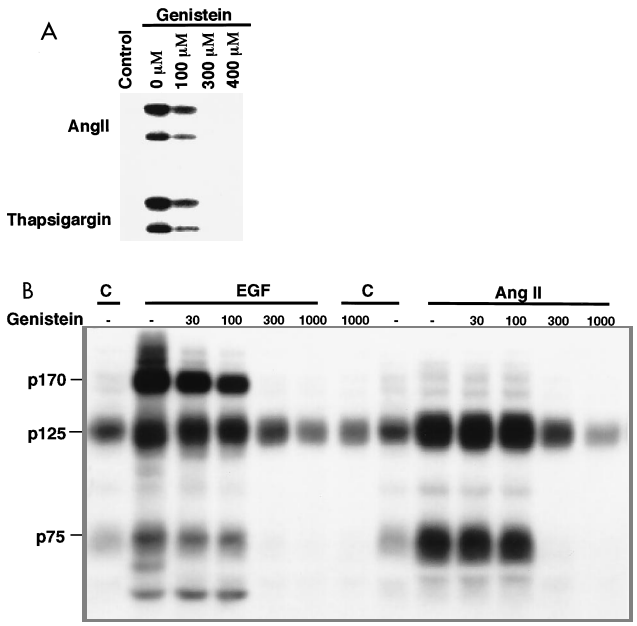

FIG. 9. JNK activation requires the action of tyrosine kinases. (A) TPA-treated cells were incubated with the indicated concentrations of genistein or vehicle (5 min) and then stimulated (30 min) with Ang II (1 μM) or thapsigargin (1 μM). JNK activation was assessed as described in Materials and Methods. (B) Activation of a tyrosine kinase following Ang II stimulation is inhibited by genistein. Cells were incubated with the indicated concentrations of genistein or vehicle (—) for 15 min and then stimulated (1 min) with Ang II (1 μM) or EGF (300 ng/ml). Lysates were subjected to immunoblotting with monoclonal antiphosphotyrosine antibody as described in Materials and Methods.

DISCUSSION

Growth control is a complex process regulated in a cell type-specific manner by a number of receptor classes. Most studies emphasize the transmembrane growth factor receptors bearing intrinsic tyrosine kinases (e.g., EGF and platelet-derived growth factor [PDGF] receptors), but in many cell types, members of the cytokine receptor family or G-protein-coupled receptors modulate proliferation. The role of calcium as an intracellular signal mediating proliferation by various receptor classes has been debated for several years, in part because its role may vary from cell type to cell type. A number of authors have linked cell growth and calcium signals (see references 2 and 61 for reviews). However, elimination of the phospholipase C activation by mutation of its specific SH2 docking site on the EGF and fibroblast growth factor receptors does not significantly alter EGF- and fibroblast growth factor-induced mitogenesis (6, 44, 48). In contrast, an extensive study of PDGF receptor signaling via SH2-containing proteins does support a role for phospholipase C activation in PDGF-dependent growth in certain cells (60). Prevention of a serum-induced rise in calcium with BAPTA inhibited proliferation in fibroblasts (61), and a role for G-protein-linked receptors that elevate intracellular calcium has been firmly established during in vivo growth of liver cells (liver regeneration following partial hepatectomy) (10, 41, 50). Therefore, calcium appears to play a role in the growth of some, but perhaps not all, cells.

Whatever the stimulus, diverse growth-signaling pathways appear to converge on MAPK family members and the immediate-early gene response. Recent data show a multiplicity within members and regulation of the MAPK pathway. Cell type specificity may play a role in regulation of MAPKs by the dual-specificity kinases (MEKs and SEKs) that control the MAPKs. For example, in mammals alone, more than five members of the MAPK family, four members of the MEK/SEK family, and three to four members of the MEKK family have recently been identified or postulated (4, 11, 54, 65). While the precise role of each subfamily or isoform is still being clarified, these enzymes provide the flexibility for differential activation of transcription by different factors (e.g., c-Fos, c-Jun, and ATF-2) and in different cell types. This manuscript reports a novel cell type-specific regulatory pathway, calcium-dependent JNK activation, which may be modulated via a calcium-regulated tyrosine kinase.

Our initial observation of calcium-dependent tyrosine phosphorylation resulted from studies of the Ang II and EGF receptors in rat liver epithelial cells. In intact cells, Ang II and its calcium signal activate a cytosolic tyrosine kinase, producing an activated, tyrosine-autophosphorylated kinase within 15 to 30 s of hormone addition. Although activation requires a calcium signal, the tyrosine kinase(s) is not directly activated by calcium in cell lysates, implying that its regulation involves intermediaries. We have used antibodies, expression cloning, and degenerate PCR techniques to identify 20 different tyrosine kinases in rat liver epithelial cells, but none of the identified kinases appears to be the cytosolic calcium-regulated enzyme. Most notably, the soluble $p125^{FAK}$ is not the major calcium-regulated kinase activity in these cells, although FAK is tyrosine phosphorylated and slightly (50%) activated by Ang II. In other experiments, we have used antisera to JAK1, JAK2, and JAK3 to demonstrate that none of these enzymes are activated in Ang II-treated rat liver epithelial cells. (unpublished results). We have purified the major calcium-regulated activity, a tyrosine-phosphorylated protein of 115 to 120 kDa with significant tyrosine kinase activity. We have separated this putative novel tyrosine kinase from $p125^{FAK}$ and hope in the future to determine whether this kinase is specifically involved in the JNK activation pathway.

The demonstration that Ang II stimulated tyrosine phosphorylation and proliferation in these cells (25) led us to investigate the effect of Ang II on traditional signaling pathways associated with proliferation. In this paper, we demonstrate that Ang II increases AP-1 binding as well as MAPK and JNK activity independent of PKC. The increases in AP-1 binding and MAPK and JNK activity temporally followed activation of a calcium-dependent tyrosine kinase. Genistein, in concentrations sufficient to inhibit the calcium-dependent tyrosine kinase, prevented Ang II-dependent activation of MAPK and JNK. While not extensively studied, genistein at these concentrations does not appear to directly inhibit the kinase activity of MEK (64), the dual-specificity kinase homologous to the JNK-activating SEK. Thus, it is reasonable to assume that genistein's inhibition of Ang II-induced JNK activation implicates a tyrosine kinase in this pathway.

Activation of JNK is independent of typical PKC isoforms. Ang II stimulation leads to activation of phospholipase Cβ, resulting in increased inositol 1,4,5-triphosphate and diacylglycerol. Subsequent elevations in diacylglycerol and calcium activate various PKC isoforms, and diacylglycerol alone can stimulate other PKC isoforms (46, 58). To determine if PKC is involved in Ang II-induced JNK activation, we treated cells with TPA, which downregulates TPA-sensitive PKCs. The data are definitive: TPA-sensitive PKCs are not involved in activation of JNK by Ang II; however, PKC ζ and λ are not activated by phorbol esters and would not be downregulated by TPA treatment (37). Thus, our experiments do not preclude involvement of TPA-insensitive isoforms. The Ang II-dependent JNK activation is slower (barely detectable at 5 min, detectable at 15 min, and maximal at 30 to 45 min) than Ang II activation of

either tyrosine kinase (15 to 60 s) or MAPK (1 to 5 min). The slow JNK response to Ang II suggests that this pathway involves more (or decidedly slower) steps than the MAPK pathway. JNK activation by TNFα is also slower than the initial TNFα ceramide signaling (63). Current evidence suggests that the final steps for some JNK activators involve stimulation of MEKK (43, 65) and SEK/JNKK (36, 54). Whether cytokine- or calcium-dependent processes use the same or different upstream regulators remains to be determined, as does the reason for the slower JNK response to Ang II. However, the time lag between Ang II-dependent MAPK and JNK activation presumably does provide flexibility in regulation and duration of the transcriptional response.

Calcium dependence of JNK activation by Ang II. Since others had not observed activation of JNK by calcium ionophore in lymphocytes and several test cell lines (59), a key question was whether this PKC-independent action of Ang II was mediated by an inositol 1,4,5-triphosphate-induced intracellular calcium signal. These data strongly suggest that Ang II-dependent JNK activation in GN4 cells is in part calcium dependent. First, Ang II as well as other G-protein-coupled receptors that raise intracellular calcium (e.g., norepinephrine and vasopressin) activate JNK. Second, thapsigargin, which increases intracellular calcium and activates a calcium-regulated tyrosine kinase in GN4 cells, generally stimulates JNK 50 to 75% as well as Ang II. Third, calcium ionophores activate JNK with the same time course and with the same efficacy as Ang II. Activation of JNK by ionophore is clearly calcium dependent because this effect is blocked by the calcium chelators EGTA and BAPTA-AM. However, the calcium signal produced by ionophore is generally greater in magnitude and duration than that produced by Ang II, and we cannot rule out a toxic or "stress" effect of the prolonged calcium signal as adjunctive to ionophore activation of JNK. Fourth, the effects of Ang II and thapsigargin are significantly inhibited by BAPTA-AM. While useful, the BAPTA-AM experiments point out potential pitfalls in the use of cell-permeable chelators as a sole criterion for calcium dependence. BAPTA-AM treatment alone resulted in increases in JNK activation, especially at higher doses. Nevertheless, at lower doses, BAPTA-AM clearly attenuated the effect of Ang II and thapsigargin but did not inhibit stress activation of JNK by UV irradiation of GN4 cells. Furthermore, depletion of intracellular calcium stores by thapsigargin and EGTA treatment also inhibited but did not totally abolish the effect of Ang II. This leaves open the possibility of a calcium-independent component of the Ang II-dependent JNK activation (perhaps by signaling via the βγ subunits of the heterotrimeric G-protein activated by Ang II). However, taken together, these data provide strong evidence that Ang II-dependent JNK activation is at least partly calcium dependent.

In contrast, the calcium dependence of Ang II's stimulation of MAPK in GN4 cells was less definitive. Ang II is a reasonable activator of MAPK in GN4 cells, being approximately 50% as efficacious as EGF, and others have shown that Ang II or other G-protein-coupled receptors increase in MAPK activity in several cell types (13, 28, 33, 42, 45, 47, 51). While thapsigargin activates JNK to 50 to 75% of the level stimulated by Ang II, Ang II is much more effective in activating MAPK than is thapsigargin. Additionally, BAPTA's effect on Ang II-dependent MAPK activation was less definitive than its effect on JNK. BAPTA alone stimulated MAPK activity almost as well as Ang II (unpublished results). Mechanisms other than calcium have been suggested for G-protein-linked receptor activation of MAPK, e.g., release of the βγ subunits from the heterotrimeric G-protein, which may activate the Ras pathway by an unknown mechanism (8, 27, 31). The Ang II activation of MAPK appears to be less calcium dependent than that of JNK in GN4 cells, implying that MAPK activation by Ang II involves components in addition to calcium.

JNK activation: multiple cell type-specific pathways? The rapidly growing literature on JNK indicates that it is most potently activated by pathways other than traditional growth factor receptor tyrosine kinases. The strongest activators, UV irradiation and TNFα, as well as stresses such as hydrogen peroxide and heat shock involve signal transduction pathways that fail to fully activate MAPK (32, 62). The pathway to JNK activation by these diverse stimuli has not been fully elucidated but does not universally involve calcium as a single stimulus. For example, UV irradiation activates JNK in lymphocytes, but the calcium ionophore A23187 alone does not activate JNK. (However, ionophore with TPA was synergistic in lymphocytes in JNK activation.) Calcium ionophore also failed to elevate JNK activity in several fibroblasts and epithelial cell lines tested (59).

In contrast to the failure of ionophores as a single agent to stimulate JNK in lymphocytes, ionophores produced up to a 200-fold activation of JNK in GN4 cells. Ang II and thapsigargin produce similar activation. Our hypothesis is that the expression of a calcium-regulated tyrosine kinase in liver epithelial cells can link a calcium signal to the JNK activation pathway. The corollary suggests that the calcium-regulated kinase is not present or acts in a different manner in lymphocytes and in some other cell lines. While proof of this hypothesis will require isolation and subsequent expression of the calcium-regulated tyrosine kinase(s), the supporting evidence (albeit circumstantial) can be summarized as follows: (i) Ang II activates a tyrosine kinase and JNK in a PKC-independent, calcium-dependent manner; (ii) thapsigargin and ionophore stimulate both the calcium-regulated tyrosine kinase and JNK activity; (iii) activation of the calcium-regulated tyrosine kinase and JNK by these agonists does not involve a calmodulin-dependent pathway; and (iv) activation of both the calcium-regulated tyrosine kinase and JNK is inhibited by genistein.

While the manuscript was in preparation, another group reported that G_q-coupled muscarinic acetylcholine receptors overexpressed in NIH 3T3 cells can activate JNK. This work did not examine the calcium dependence of this effect (7). Whether a calcium-regulated tyrosine kinase is involved or not, the present data define a novel means for JNK activation in certain cell types, a G-protein-mediated, calcium-dependent, PKC-independent process that acts via a mechanism other than calmodulin. Further studies should define the role of the calcium-regulated tyrosine kinase in JNK and MAPK activation as well as its role in cell type-specific growth regulation.

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