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Angiotensin II Stimulates Protein-Tyrosine Phosphorylation in a Calcium-Dependent Manner

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Cellular responses to epidermal growth factor (EGF) are dependent on the tyrosine-specific protein kinase activity of the cell-surface EGF receptor. Previous studies using WB rat liver epithelial cells have detected at least 10 proteins whose phosphotyrosine (P-Tyr) content is increased by EGF. In this study, we have examined alternate modes of activating tyrosine phosphorylation. Treatment of WB cells with hormones linked to Ca^{2+} mobilization and protein kinase C (PKC) activation, including angiotensin II, [Arg^8]vasopressin, or epinephrine, stimulated rapid (≤15-s) and transient increases in the P-Tyr content of several proteins (p120/125, p75/78, and p66). These proteins, detected by anti-P-Tyr immunoblotting, were similar in molecular weight to a subset of EGF-sensitive P-Tyr-containing proteins (P-Tyr-proteins). The increased P-Tyr content was confirmed by [^{32}P]phosphoamino acid analysis of proteins recovered by anti-P-Tyr immunoprecipitation. Elevating intracellular [Ca^{2+}] with the ionophore A23187 or ionomycin or with the tumor promoter thapsigargin mimicked the effects of hormones on tyrosine phosphorylation, whereas treatment with a PKC-activating phorbol ester did not. In addition, responses to angiotensin II were not diminished in PKC-depleted cells. Ca^{2+} mobilization, measured by fura-2 fluorescence, was coincident with the increase in tyrosine phosphorylation in response to angiotensin II or thapsigargin. Loading cells with the intracellular Ca^{2+} chelator bis-(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) inhibited the appearance of all P-Tyr-proteins in response to angiotensin II, thapsigargin, or ionophores, as well as two EGF-stimulated P-Tyr-proteins. The majority of EGF-stimulated P-Tyr-proteins were not affected by BAPTA. These studies indicate that angiotensin II can alter protein-tyrosine phosphorylation in a manner that is secondary to, and apparently dependent on, Ca^{2+} mobilization. Thus, ligands such as EGF and angiotensin II, which act through distinct types of receptors, may activate secondary pathways involving tyrosine phosphorylation. These results also raise the possibility that certain growth-promoting effects of Ca^{2+}-mobilizing agents such as angiotensin II may be mediated via tyrosine phosphorylation.

Reversible phosphorylation of proteins is a major mechanism by which metabolic processes are regulated. Phosphorylation on serine and threonine residues, which together account for >99% of total cellular protein phosphate, are recognized as key elements in the regulation of such diverse pathways as glycogen metabolism, protein biosynthesis, and cell surface receptor signaling (19). Phosphorylation on tyrosine residues, which accounts for <0.5% of protein-bound phosphate (12), has been linked broadly with the control of cell proliferation. Receptors for several growth factors, including epidermal growth factor (EGF) and platelet-derived growth factor, contain ligand-activated, tyrosine-specific protein kinase domains essential for biological activity (76, 78). Similarly, the protein products of several oncogenes (e.g., *src* and *abl*) exhibit tyrosine kinase activities essential for oncogenic transformation (36, 41).

The identities of relevant substrates for tyrosine kinases remain largely obscure because of the technical challenge of monitoring transient tyrosine phosphorylation events against high backgrounds of serine/threonine phosphorylation and because of the complex and temporally protracted nature of mitogenesis. Nevertheless, several potential substrates for tyrosine kinases in intact cells have been identified, using combinations of ^{32}P labeling, electrophoresis, phosphoamino acid analysis, and immunoprecipitation and immunoblotting with antiphosphotyrosine (anti-P-Tyr) antibodies. One group of substrates are structural proteins, including vinculin, the fibronectin receptor, ezrin, and calpactins I and II (reviewed in reference 9). Several proteins likely to propagate acute growth factor receptor signals also have been identified as tyrosine kinase substrates: phosphatidylinositol kinase, phosphoinositide (PtdIns)-specific phospholipase C-γ(PLCγ), the *ras* GTPase-activating protein, and the serine/threonine kinases Raf-1 and microtubule-associated protein 2 (MAP-2) kinase (56, 76).

Our laboratory has studied the regulation of EGF signalling in the nontransformed rat liver epithelial cell line WB (71), which expresses EGF receptors at levels (2×10^5 to 3×10^5 per cell) comparable to those in hepatocytes. Exposure of WB cells to EGF provokes a cascade of events: EGF receptor tyrosine autophosphorylation and phosphorylation of other cellular proteins (≤5 s); production of Ca^{2+}-mobilizing inositol phosphates, activation of protein kinase C (PKC), and receptor internalization (1 to 2 min) and, after a period of hours, protein and DNA synthesis (38, 49, 71). These cells also express receptors for several hormones (e.g., angiotensin II, [Arg^8]vasopressin, and epinephrine) whose actions are believed to be mediated by non-tyrosine kinase-containing receptors coupled principally to Ca^{2+} mobilization and PKC activation. These hormones increase EGF receptor mRNA and protein levels by a mechanism

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partly independent of PKC (18). Activation of PKC by phorbol esters can inhibit EGF-stimulated PtdIns hydrolysis (38), EGF receptor kinase activity, and tyrosine phosphorylation of PLCγ in WB cells (W. R. Huckle, J. R. Hepler, S. G. Rhee, T. K. Harden, and H. S. Earp, Endocrinology, in press); phorbol ester treatment likewise can uncouple angiotensin II receptors from ligand-stimulated PtdIns hydrolysis (38). While investigating possible heterologous desensitization of the EGF receptor kinase by other PKC-activating agonists in WB cells, we noted that angiotensin II itself stimulated increases in the tyrosine phosphorylation of several proteins. In this work, we report this effect and present evidence that it is dependent on increases in cytosolic Ca^{2+} levels. These findings indicate that hormone receptors not thought to possess intrinsic tyrosine kinase activity may nonetheless signal through tyrosine phosphorylation and suggest a mechanism by which their ligands elicit effects similar to those of recognized growth factors.

MATERIALS AND METHODS

Materials. EGF was purified from mouse salivary glands as described previously (58). Human sequence angiotensin II (Asp Arg Val Tyr Ile His Pro Phe), [Arg^8]vasopressin, (−)-epinephrine (Sigma), and EGF were prepared as 100-fold-concentrated solutions in 10 mM NaP_i–150 mM NaCl (pH 7.4) containing 0.1% bovine serum albumin. Phorbol 12-myristate 13-acetate (PMA; Sigma), thapsigargin (LC Services), bis-(*O*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM; GIBCO-BRL), ionomycin, A23187 (Calbiochem), and fura-2, tetraacetoxymethyl ester (fura-2-AM; Molecular Probes) were prepared as stock solutions in dimethyl sulfoxide; final vehicle concentration was ≤0.1% (vol/vol). Polyclonal anti-P-Tyr antibodies were prepared as described previously (49) except that affinity purification was performed by chromatography on immobilized phosphotyramine.

Cell cultures. WB cells were maintained at 37°C in Richter improved minimal essential medium containing 10% fetal bovine serum and 0.1 μM insulin in a humidified 5% CO_2 atmosphere as described previously (38). Seven to ten days before each experiment, cells of passages 19 to 26 were seeded onto plastic culture dishes (Costar) or glass cover slips (Fisher). Except where indicated, cells were used for experiments 1 to 2 days after reaching confluence. Before addition of EGF, angiotensin II, or other agents, cells were washed once with Eagle minimal essential medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (EMEM-HEPES, pH 7.3) and were allowed to equilibrate in this medium for 15 min at 37°C.

Anti-P-Tyr immunoblotting. Cell treatments and anti-P-Tyr immunoblotting were performed essentially as described previously (49), using WB cells grown in 35-mm-diameter culture dishes. Briefly, treatment incubations were terminated by rapid aspiration of the medium and addition of 250 μl ice-cold RIPA buffer (49). Cell extracts were scraped from culture dishes and solubilized by addition of 125 μl of threefold-concentrated sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and heating to 100°C for 3 min. Proteins were separated by electrophoresis on 7 or 10% polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed by sequential incubation with affinity-purified anti-P-Tyr antibodies and ^{125}I-protein A (2 to 10 μCi/μg; Dupont-New England Nuclear), followed by autoradiography. Molecular weights of P-Tyr-containing proteins (P-Tyr-proteins) were estimated by using prestained standards calibrated by comparison with the migration of nonprestained standards (Sigma). Relative intensities of autoradiographic bands were estimated by scanning densitometry (LKB Ultroscan XL). The appearance of all autoradiographic bands could be completely blocked by addition of 1 mM phenylphosphate or P-Tyr during the antibody incubation. In some experiments, blots were probed with monoclonal anti-P-Tyr antibody PY20 (29) (1 μg/ml; ICN ImmunoBiologicals), followed by rabbit anti-mouse immunoglobulin G (1 μg/ml; Jackson ImmunoResearch) and ^{125}I-protein A.

Phosphoamino acid analysis. Confluent WB cell cultures in 60-mm-diameter dishes were prelabeled by incubation (2 h, 37°C) with 1 mCi of $H_3{}^{32}PO_4$ (>8,500 Ci/mmol; Dupont-New England Nuclear) in 3 ml of phosphate-free EMEM-HEPES supplemented with 0.5% fetal bovine serum. After treatment with EGF or other agents, the medium was aspirated, and cells were scraped from the dishes in 0.3 ml of ice-cold lysis buffer (20 mM HEPES [pH 7.3], 50 mM NaF, 10% [vol/vol] glycerol, 1% [wt/vol] Triton X-100, 1 mM sodium *o*-vanadate, 10 μg of phenylmethylsulfonyl fluoride per ml). Lysates were tumbled for 30 min at 4°C and then centrifuged at 12,000 × *g* for 15 min at 4°C. Supernatants were transferred to conical 15-ml polypropylene tubes, brought to 1% SDS and 100 mM 2-mercaptoethanol, and heated to 100°C for 5 min. Samples were chilled on ice and diluted to 3 ml with lysis buffer. P-Tyr-proteins were immunoprecipitated by incubation (2 h, 4°C) with 1 μg of monoclonal anti-P-Tyr antibody PY20, 5 μg of rabbit anti-mouse immunoglobulin G, and 10 μl of Pansorbin (Calbiochem). Immunoprecipitates were recovered by centrifugation and washed by sequential suspension and centrifugation in lysis buffer plus (i) 0.1% SDS, (ii) 0.5 M NaCl, and (iii) 0.1% SDS. Immunoprecipitates then were solubilized for SDS-PAGE on 7% polyacrylamide gels. Phosphoproteins were transferred to Immobilon membranes and were located by autoradiography. Bands of interest were excised from the Immobilon blot and subjected to acid hydrolysis (43), two-dimensional electrophoresis (12), and autoradiography.

Measurement of intracellular Ca^{2+}. Calcium monitoring was performed essentially as described previously (77). WB cells were grown on 22-by-22-mm glass cover slips to approximately 80% confluence. Cells were chilled to 4°C for 5 min in EMEM-HEPES and then loaded with the fluorescent Ca^{2+} indicator fura-2 (34) by incubation with 5 μM fura-2-AM for 15 min at 37°C. After three washes with EMEM-HEPES, the cover slips were mounted on the stage of a digital video fluorescence microscope. Fluorescence (340- and 380-nm excitation; >440-nm emission) was monitored at 15- to 30-s intervals before and after treatment of cells with angiotensin II or thapsigargin. Free [Ca^{2+}] was calculated by the ratio method, with correction for background fluorescence (80).

RESULTS

Angiotensin II and [Arg^8]vasopressin increase protein-tyrosine phosphorylation in WB cells. As reported previously (49), EGF stimulated rapid (≤5-s) increases in the tyrosine phosphorylation of several proteins, the most prominent being the 170-kDa EGF receptor (Fig. 1). Treatment with angiotensin II provoked increases in the tyrosine phosphorylation of several species similar in molecular weight to those affected by EGF: p120/125, p75/78, and p66 (Fig. 1). On shorter autoradiographic exposures, the species of 120/125 and 75/78 kDa could be resolved as doublet bands (Fig.

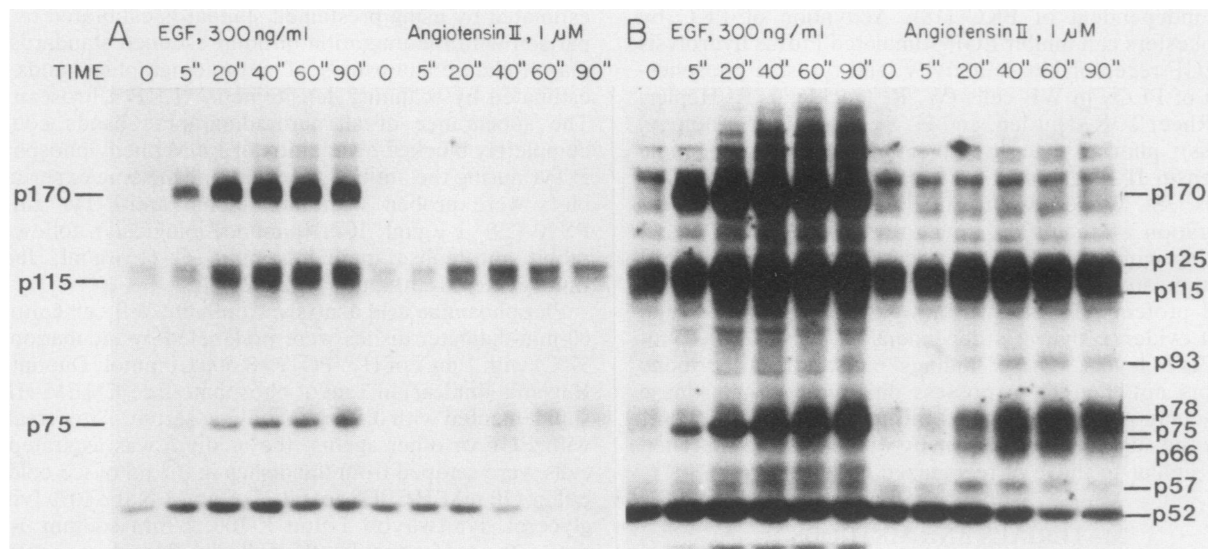

FIG. 1. EGF- and angiotensin II-stimulated tyrosine phosphorylation. WB cells were treated with EGF (300 ng/ml) or angiotensin II (1 μM) for the times indicated (in seconds), and tyrosine phosphorylation was assessed by anti-P-Tyr immunoblotting after SDS-PAGE on 7% polyacrylamide gels as described in Materials and Methods. Autoradiographs shown (representative of three separate experiments) were exposed for 2 (A) or 6 (B) days at −70°C.

1A). Angiotensin II-stimulated increases in less prominent species, P-Tyr-p57, -p93, and -p115, were evident upon longer autoradiographic exposures (Fig. 1B). Similar patterns of tyrosine phosphorylation were observed when blots were probed with either purified polyclonal anti-P-Tyr antibodies or monoclonal antibody PY20 (not shown). The response to angiotensin II was both rapid and transient, with maximal increases in P-Tyr-p66, -p75/78, and -p120/125 occurring between 30 and 60 s of treatment. Phosphorylation of the p75/78 doublet persisted above basal levels for >5 min, although an increase in the intensity of the upper band, and concomitant decrease in the intensity of the lower band, occurred over 5 min of treatment. In contrast, increased P-Tyr-p66 was no longer detected after 2 min of angiotensin II treatment. Elevated tyrosine phosphorylation was detectable at angiotensin II concentrations as low as 1 nM; the angiotensin II effects could be blocked by the receptor antagonist [Sar^1, Val^5, Ala^8]angiotensin.

Although both EGF and angiotensin II affected the tyrosine phosphorylation of several proteins similar in molecular weight (p57, p66, p75/78, p93, and p120/125), other species (p170/EGF receptor, p59, and p40) were unique to EGF-treated cells. Similarly, PLCγ is phosphorylated on tyrosine in response to EGF but not angiotensin II (38a). Initial attempts to separate EGF- and angiotensin II-dependent substrates into cytosolic and membrane fractions by conventional procedures were complicated by rapid losses of P-Tyr, even in the presence of phosphatase inhibitors. Homogenization and fractionation at pH 9.5, however, inhibited loss of P-Tyr from EGF- and angiotensin II-sensitive proteins and revealed that all angiotensin II-stimulated bands, as well as the EGF-sensitive bands of similar molecular weight (p120, p75, and p66), partitioned into the supernatant fraction (105,000 × *g*; W. R. Huckle, C. A. Prokop, and H. S. Earp, unpublished results).

Treatment of WB cells with another peptide hormone, [Arg^8]vasopressin (Fig. 2), or with the adrenergic receptor agonist epinephrine (not shown) produced increases in tyrosine phosphorylation similar to that seen in response to angiotensin II. This effect of epinephrine was not obtained with the β-adrenergic receptor-selective agonist isoproterenol or with the membrane-permeant compound dibutyryl cyclic AMP (Huckle et al., unpublished results).

Increases in cytosolic calcium mimic the effects of angiotensin II on tyrosine phosphorylation. Activation of the angiotensin receptor is recognized to produce PKC-activating diacylglycerols and Ca^{2+}-mobilizing inositol phosphates. To test the role of cytosolic Ca^{2+} in the angiotensin II-stimulated increase in tyrosine phosphorylation, WB cells were treated with other agents that increase intracellular Ca^{2+} levels (Fig. 3). The tumor promoter thapsigargin (67), which

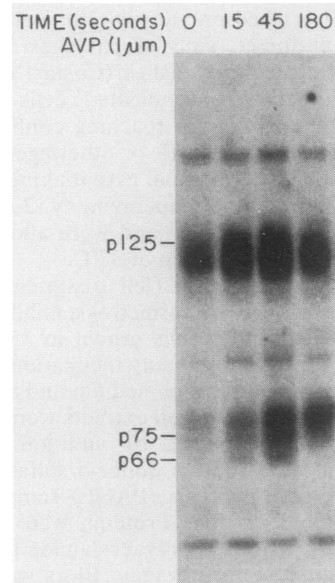

FIG. 2. [Arg^8]vasopressin-stimulated increase in tyrosine phosphorylation. WB cells were treated with 1 μM [Arg^8]vasopressin (AVP) for the times indicated; tyrosine phosphorylation then was assessed by immunoblotting as described in Materials and Methods.

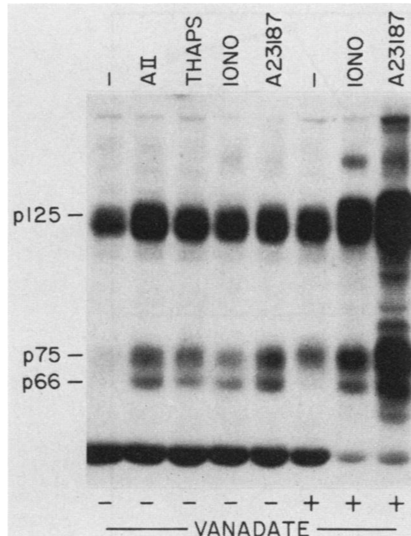

FIG. 3. Effects of Ca^{2+}-elevating agents on WB cell tyrosine phosphorylation. WB cells were treated with various Ca^{2+}-mobilizing agents for periods yielding maximal tyrosine phosphorylation response: angiotensin II (A II; 1 μM, 45 s), thapsigargin (THAPS; 2 μM, 5 min), ionomycin (IONO; 10 μM, 1 min), and A23187 (10 μM, 5 min) in the absence of *o*-vanadate or ionomycin (10 μM, 2.5 min) or A23187 (10 μM, 5 min) in the presence of 200 μM *o*-vanadate (15-min preincubation). Results shown are representative of three separate experiments.

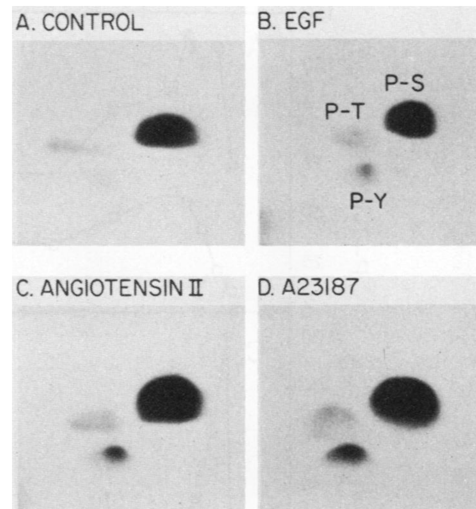

FIG. 4. Phosphoamino acid analysis of ^{32}P-p75/78. WB cells were prelabeled with $^{32}P_i$ for 2 h before treatment with vehicle (A), 300 ng of EGF per ml (B), 1 μM angiotensin II (C), or 10 μM A23187 plus 200 μM *o*-vanadate (D). ^{32}P-proteins were recovered by monoclonal anti-P-Tyr immunoprecipitation and subjected to SDS-PAGE and phosphoamino acid analysis as described in Materials and Methods. Results (representative of three similar experiments) show two-dimensional electrophoretic separations of phosphoamino acids derived from the p75/78 species. In the same experiment, the p120/125 region from angiotensin II- and A23187-treated cells likewise showed increased [^{32}P]Tyr content. The [^{32}P]Tyr spots from control, EGF-, angiotensin II-, and A23187-treated samples shown contained 17, 25, 57, and 99 cpm above blank, respectively, determined by liquid scintillation counting.

is postulated to allow the release of Ca^{2+} from inositol trisphosphate-sensitive stores (65), produced a pattern of protein-tyrosine phosphorylation strongly resembling that seen in response to angiotensin II, as did the calcium ionophores A23187 and ionomycin. The phosphatase inhibitor *o*-vanadate alone (200 μM, 15 min) produced small increases in tyrosine phosphorylation. *o*-Vanadate enhanced the response to either ionophore, but its effect on A23187 action was more pronounced, allowing the detection of several species in the 50- to 90-kDa range not seen in angiotensin II-treated cells (Fig. 3).

To corroborate the results obtained by anti-P-Tyr immunoblotting, $^{32}P_i$-labeled WB cells were treated with EGF, angiotensin II, or A23187 plus *o*-vanadate. [^{32}P]Tyr-containing proteins were recovered by anti-P-Tyr immunoprecipitation, separated by SDS-PAGE, and transferred to Immobilon membranes. Autoradiography revealed patterns of increased phosphorylation similar to those observed by using anti-P-Tyr immunoblotting. These experiments (Fig. 4) confirmed that proteins in the molecular weight ranges detected by immunoblotting (Fig. 1 to 3) did contain elevated P-Tyr content as a consequence of treatment with angiotensin II or A23187.

To test the potential involvement of PKC in angiotensin II-dependent tyrosine phosphorylation, WB cells were treated with PMA, an exogenous activator of PKC. Acute PMA treatment (300 nM, 0.25 to 10 min) produced small increases in P-Tyr-p120/125 and P-Tyr-p75/78 in two of four replicate experiments and no effects in the remaining experiments (not shown).

Angiotensin II-stimulated increases in tyrosine phosphorylation are coupled to calcium mobilization. To examine the temporal relationship between Ca^{2+} mobilization and tyrosine phosphorylation, cytosolic Ca^{2+} levels were monitored in cells loaded with the fluorescent indicator fura-2. The Ca^{2+} responses to angiotensin II or thapsigargin were coincident with the measurable changes in tyrosine phosphorylation (Fig. 5). Angiotensin II stimulated more rapid (≤5-s) increases in [Ca^{2+}], accompanied by transient increases in P-Tyr-protein levels. The Ca^{2+} increase in response to thapsigargin was maximal after 2 to 3 min and persisted, along with elevated P-Tyr-proteins, for ≥5 min. Thapsigargin stimulates a similarly persistent elevation of [Ca^{2+}] in hepatocytes (67).

If the increases in tyrosine phosphorylation in response to angiotensin II, ionophores, or thapsigargin are secondary to Ca^{2+} mobilization, blockade of the Ca^{2+} increase provoked by these agents should inhibit their capacity to increase tyrosine phosphorylation. Treatment of WB cells with angiotensin II in the presence of 5 mM EGTA had minimal effects on the response to angiotensin II (not shown), consistent with the preferred utilization of Ca^{2+} from intracellular rather than extracellular sources. To block increases in cytosolic Ca^{2+} irrespective of its origin, WB cells were incubated with the membrane-permeant compound BAPTA-AM, which is hydrolyzed to the active chelator BAPTA upon entry into cells (72, 73). The concentrations of BAPTA-AM used were sufficient to inhibit EGF- or angiotensin II-induced increases in intracellular [Ca^{2+}] in hepatocytes, as measured by digital video fluorescence microscopy (B. Herman, unpublished data). BAPTA inhibited the increases in all angiotensin II- and thapsigargin-stimulated P-Tyr-proteins (p66, p75, and p125; Fig. 6); on longer exposure of autoradiographs from 7% polyacrylamide gels, blockade of the minor angiotensin II-sensitive species, P-Tyr-p93 and -p57, also was noted in BAPTA-loaded cells (not shown). In contrast, BAPTA loading did not inhibit EGF receptor autophosphorylation (p170), the increases in

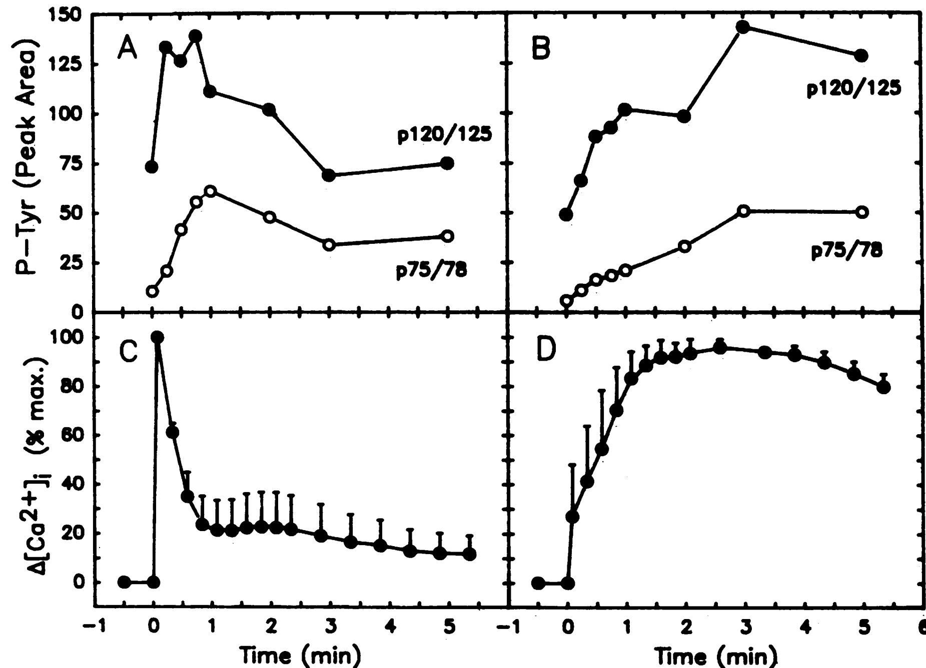

FIG. 5. Time courses of angiotensin II- or thapsigargin-stimulated tyrosine phosphorylation and Ca^{2+} mobilization. (A and B) WB cells in 35-mm-diameter culture dishes were treated with 1 μM angiotensin II (A) or 2 μM thapsigargin (B) for the times indicated, and tyrosine phosphorylation was assessed by immunoblotting. Relative intensities of autoradiographic bands in the p75/78 and p120/125 regions were estimated by scanning densitometry. Data shown (representative of three experiments) are expressed as densitometric peak area (arbitrary units). (C and D) WB cells grown on 22-by-22-mm cover slips were loaded with fura-2 and monitored for free intracellular $[Ca^{2+}]$ by digital video fluorescence microscopy as described in Materials and Methods. Each time point is the mean free $[Ca^{2+}]$ across a microscopic field containing five to six cells selected at random. Results are expressed as the mean ± standard error (of three separate experiments) of percent maximal change in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) observed in response to 1 μM angiotensin II (C) or 2 μM thapsigargin (D). Preaddition and peak intracellular $[Ca^{2+}]$ values for angiotensin II-treated cells were 275 ± 36 and 1,002 ± 65 nM, respectively; corresponding values for thapsigargin-treated cells were 320 ± 35 and 926 ± 185 nM, respectively.

P-Tyr-p115, -p75, and -p59 seen in response to EGF, or the increases in P-Tyr-p100 and -p40 noted in longer exposures (not shown). However, BAPTA was noted to inhibit the EGF-stimulated increases in P-Tyr-p120 and -p66 (Fig. 6). These results were obtained reproducibly in four separate experiments. The persistence of EGF receptor phosphorylation in the presence of BAPTA is consistent with the reported insensitivity of the EGF receptor kinase to Ca^{2+} (21) and serves as a control for nonspecific or toxic effects of BAPTA on WB cells. Additional experiments using BAPTA-loaded cells confirmed that the marked potentiation of A23187 action by *o*-vanadate was indeed Ca^{2+} dependent (not shown).

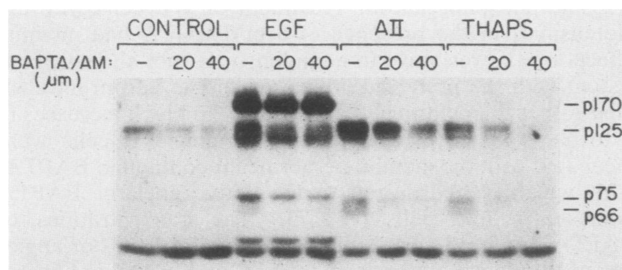

FIG. 6. Effects of BAPTA on tyrosine phosphorylation in WB cells. WB cells were loaded with BAPTA by preincubation with the indicated concentrations of BAPTA-AM for 15 min at 37°C. Cells were treated with EGF (300 ng/ml, 1 min), angiotensin II (AII; 1 μM, 45 s), or thapsigargin (THAPS; 2 μM, 5 min), conditions that elicit maximal tyrosine phosphorylation responses. Immunoblotting was performed after electrophoresis on 10% polyacrylamide gels, on which the diffuse angiotensin II-sensitive species of p120/125 and p75/78 kDa migrated as single bands. Results shown are representative of four separate experiments.

As an additional test of the involvement of PKC (a major intracellular target of mobilized Ca^{2+}) in angiotensin II-stimulated tyrosine phosphorylation, WB cells were treated with a high concentration of PMA for 18 h before challenge with angiotensin II. This pretreatment protocol reduces WB cell PKC activity to ≤4% of control levels (38) and eliminates the ability of phorbol esters to inhibit EGF- or angiotensin II-stimulated PtdIns hydrolysis (38) and EGF-stimulated tyrosine phosphorylation of PLCγ (Huckle et al., in press). In PKC-depleted cells, there was no reduction in the ability of angiotensin II to increase tyrosine phosphorylation compared with results for control cells (Fig. 7). In fact, PKC depletion prolonged the angiotensin II-stimulated elevation of tyrosine phosphorylation, although increases in magnitude were not apparent.

DISCUSSION

The peptide hormone angiotensin II exerts effects on a variety of tissues, including vascular smooth muscle (vasoconstriction), the adrenal gland (synthesis and release of aldosterone and catecholamines), and the liver (glycogenolysis). Activation of angiotensin II receptors typically is associated with the generation of Ca^{2+}-mobilizing inositol phosphates and PKC-activating diacylglycerols from PtdIns

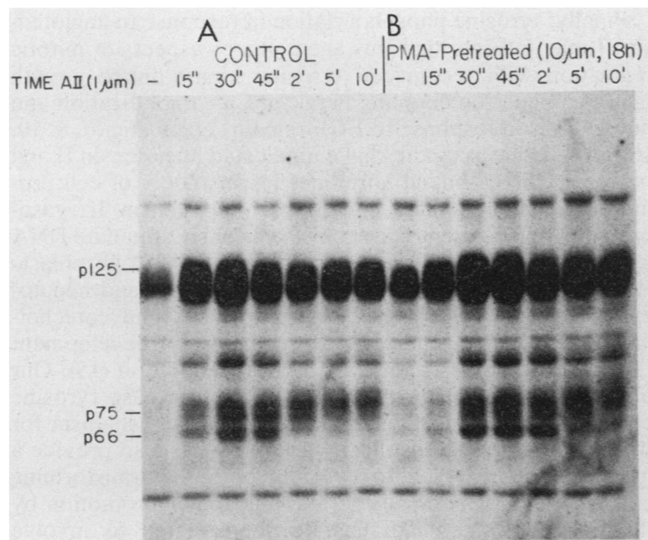

FIG. 7. Effect of PKC depletion on angiotensin II-stimulated tyrosine phosphorylation. WB cell cultures were treated with 0.1% dimethyl sulfoxide (A) or 10 μM PMA (B) for 18 h before addition of angiotensin II (AII) for the times indicated (", seconds; ', minutes). Tyrosine phosphorylation then was assessed by anti-P-Tyr immunoblotting. Results shown are representative of three separate experiments.

(reviewed in reference 33). Cellular responses to these intracellular messengers are thought to be mediated in part by the targets of PKC and Ca^{2+}/calmodulin-dependent protein kinases (19). The results of this study on WB cells suggest that angiotensin II, as well as [Arg^8]vasopressin and epinephrine, also may influence cell function through tyrosine phosphorylation. These agents increased the tyrosine phosphorylation of several proteins, as detected by immunoblotting with either affinity-purified polyclonal or monoclonal anti-P-Tyr antibodies and confirmed by [^{32}P]phosphoamino acid analysis.

Previous studies in WB cells (49) revealed two classes of proteins whose tyrosine phosphorylation is increased by EGF: (i) those phosphorylated within 5 to 15 s of EGF addition (EGF receptor, p115, p100, p75, p66, p57, and p52) and (ii) those not detected until 45 to 90 s after EGF addition (p120, p78, and p38). The first group of proteins also was phosphorylated, albeit with slower kinetics, when EGF was added to cells at 4°C (a condition that blocks EGF-stimulated PtdIns hydrolysis and receptor endocytosis), whereas the latter group was not detected at 4°C. We postulated that the temperature-sensitive phosphorylations reflected a requirement for dynamic intracellular events, such as EGF receptor internalization or phosphorylation by another tyrosine kinase activated secondarily by EGF. Indeed, these results indicate that WB cells contain an angiotensin II-activated pathway that can increase protein-tyrosine phosphorylation in a manner apparently independent of the EGF receptor kinase. Molecular weight profiles and preliminary subcellular fractionation studies suggest that angiotensin II and EGF may increase the tyrosine phosphorylation of overlapping sets of proteins, but more definitive evidence that EGF and angiotensin II act on identical proteins is needed.

The receptor for angiotensin II is not well characterized biochemically, although Jackson et al. have reported that the *mas* oncogene may encode an angiotensin receptor capable of coupling to Ca^{2+} mobilization (42). The predicted amino acid sequence of neither the *mas* product nor the α_1-adrenergic receptor cDNA (13) indicates the presence of a tyrosine kinase domain. Instead, available evidence suggests that receptors of this type activate PtdIns hydrolysis through a receptor-associated GTP-binding protein (20, 33, 38a). Consistent with this proposed mechanism, increases in tyrosine phosphorylation stimulated by angiotensin II, [Arg^8] vasopressin, or epinephrine were mimicked by NaF (Huckle et al., unpublished results), a G-protein activator which stimulates inositol trisphosphate production in WB cells (38). Furthermore, similar patterns of tyrosine phosphorylation also were provoked by the calcium-mobilizing agent thapsigargin and the Ca^{2+} ionophores A23187 and ionomycin. All angiotensin II-stimulated increases in P-Tyr-proteins were blocked by loading cells with the Ca^{2+} chelator BAPTA but not by depleting cellular PKC activity. The effects of angiotensin II on tyrosine phosphorylation thus appear to be secondary to, and dependent on, Ca^{2+} mobilization but not PKC activation. The inhibition of EGF-stimulated tyrosine phosphorylation of p120 and p66 by BAPTA suggests that these events likewise may be Ca^{2+} dependent. The failure of BAPTA to inhibit EGF-stimulated increases in P-Tyr-p75 suggests that this protein is not identical to the p75 affected by angiotensin II or that distinct kinases or phosphorylation sites are utilized in response to EGF and angiotensin II.

Recently, several agents not usually associated with tyrosine kinase activity have been found to increase levels of tyrosine phosphorylation. These include thrombin (22, 30, 46), collagen (50), and vasopressin (32) in platelets, fMet-LeuPhe in neutrophils (39), muscarinic agonists in hippocampal slices (64), and phytohemagglutinin in Jurkat T cells (69). Disparate findings have been reported regarding the signaling pathways involved in this atypical tyrosine phosphorylation. Several studies using phorbol esters (22, 23, 64) have indicated a possible role for PKC in tyrosine phosphorylation. In permeabilized neutrophils, GTPγS-stimulated increase in P-Tyr-protein levels was not mimicked by exogenous diacylglycerols (51), but the role of Ca^{2+} was not examined. Other investigators have reported that immunoglobulin E-stimulated tyrosine phosphorylation in RBL-2H3 basophils proceeds in a Ca^{2+}-independent manner (3). In contrast, our studies in WB cells establish a link between hormone-induced Ca^{2+} mobilization and increased levels of tyrosine phosphorylation.

While the mechanism by which Ca^{2+} increases tyrosine phosphorylation in WB cells has not been determined, the most straightforward possibility is direct activation of a tyrosine kinase by Ca^{2+} or by a Ca^{2+}-binding regulatory protein. This mechanism would be analogous to regulation of serine/threonine protein kinases by Ca^{2+}/calmodulin (19). A related possibility is that Ca^{2+}-dependent enzymes activate a tyrosine kinase, perhaps by phosphorylation (54) or proteolysis. Alternatively, Ca^{2+} might modulate tyrosine phosphorylation by promoting substrate interaction with a tyrosine kinase. Precedent for such a model exists in that lipocortin I (p35; calpactin II) requires Ca^{2+} in order to bind to phospholipid membranes and to serve as a substrate for the EGF receptor tyrosine kinase (17, 21). Similarly, phosphorylation of calpactin I (p36; lipocortin II) by the pp60^{c-src} tyrosine kinase is markedly enhanced in the presence of Ca^{2+} and phospholipid (14, 28).

A third possible mechanism by which Ca^{2+} might modulate tyrosine phosphorylation is inhibition of tyrosine phosphatase activity. Recent studies of protein-tyrosine phosphatases have demonstrated that these enzymes are greater

in number and likely are subject to closer regulation than once was appreciated (40, 70). In our studies, treatment with *o*-vanadate alone was accompanied by detectable increases in the tyrosine phosphorylation of p75/78 and p120/125 (Fig. 3). Thus, p75/78 and p120/125 may continually undergo phosphorylation and dephosphorylation such that phosphatase inhibition alone might permit some accumulation of their phosphorylated forms. However, angiotensin II, thapsigargin, and Ca^{2+} ionophores clearly have effects on tyrosine phosphorylation greater in magnitude than those induced by *o*-vanadate alone, and the response to angiotensin II or ionophores plus *o*-vanadate is (at least) additive. Moreover, the more rapid dephosphorylation of P-Tyr-p66 relative to other angiotensin II-sensitive substrates, even in the presence of *o*-vanadate (not shown), suggests that inhibition of a single phosphatase cannot account wholly for the effects of angiotensin II on tyrosine phosphorylation.

o-Vanadate has been reported to have other cellular effects, including activation of the insulin receptor kinase (66), a G-protein coupled to PLC (53), and Ca^{2+} influx (48), which could partially mimic the effects of angiotensin II. The potentially pleiotropic effects of *o*-vanadate may contribute to the marked synergism between A23187 and *o*-vanadate (Fig. 3). Alternatively, the synergism may arise from enhanced phosphatase inhibition by supraphysiological intracellular $[Ca^{2+}]$ induced by A23187 or from undetermined actions of *o*-vanadate metabolites (44). Regardless of its molecular mechanism, the marked interaction between A23187 and *o*-vanadate indicates that WB cells contain high levels of latent tyrosine-phosphorylating activity.

Although tyrosine phosphorylation has been studied principally in the context of cell proliferation, evidence from several systems suggests that this protein modification also may regulate acute metabolic responses to stimuli. A leading example is the insulin receptor tyrosine kinase (75), which appears to be essential for insulin-stimulated responses such as glucose uptake and glycogen synthesis (11). In other systems, tyrosine phosphorylation has been implicated in secretion from adrenal chromaffin cells (52), platelets (47), and basophils (3) and in superoxide production in neutrophils (31). The very presence of tyrosine kinases and phosphatases in cells which are characteristically nonproliferative (e.g., neutrophils; 5) or terminally differentiated (e.g., neural retina; 62) implies that tyrosine phosphorylation may participate in a broad spectrum of cellular activities.

Alternatively, angiotensin II-stimulated tyrosine phosphorylation, in addition to serine/threonine phosphorylation, may be involved in longer-term anabolic responses. Angiotensin II increases levels of mRNAs for the EGF receptor in WB cells (18), angiotensinogen in hepatocytes (2, 45), and catecholamine-synthesizing enzymes in adrenal medullary cells (63). Both angiotensin II (26) and $[Arg^8]$vasopressin (25) stimulate hypertrophy of vascular smooth muscle cells. Angiotensin II activates ribosomal S_6 kinase (60) and protein synthesis in smooth muscle cells in a Ca^{2+}-dependent fashion (4). These last findings are especially notable in light of the recent report that MAP-2 kinase requires phosphorylation on both tyrosine and threonine residues in order to activate S_6 kinase (1). Thus, MAP-2 kinase (pp42), which can be phosphorylated on tyrosine in response to EGF, platelet-derived growth factor, phorbol esters, or thrombin (56), becomes another candidate for angiotensin II-stimulated tyrosine phosphorylation. Although we have not detected a P-Tyr-p42 in lysates of angiotensin II-treated WB cells, this protein, like P-Tyr-PLCγ (Huckle et al., in press), might escape detection without enrichment from lysates.

Finally, tyrosine phosphorylation in response to angiotensin II may indicate that this agent shares aspects of mitotic regulation with recognized tyrosine kinase-linked growth factors. While the coupling between Ca^{2+} mobilization and mitogenesis in response to EGF remains enigmatic (6, 8, 10, 16, 74), numerous studies have implicated angiotensin II and other Ca^{2+}/PKC-linked hormones as regulators of cell proliferation under defined conditions. Angiotensin II, vasopressin, and α_1-adrenergic agonists variously stimulate DNA synthesis in adrenal cortex (27, 55), mouse 3T3 fibroblasts (57, 59, 61), vascular smooth muscle cells (7), and hepatocytes (15). Accordingly, peptide hormones and catecholamines have been implicated as regulators of development (37, 79), angiogenesis (24), and liver regeneration (15). Our observation that Ca^{2+}-mobilizing agents increase tyrosine phosphorylation indicates another potential mechanism for their growth-promoting effects. These results also provide a new context in which to evaluate both the transforming activity of the *mas* oncogene (42) and tumor promotion by thapsigargin (35), which has been postulated to involve chronic elevation of intracellular $[Ca^{2+}]$ (68).

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