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Calcium-dependent increase in tyrosine kinase activity stimulated by angiotensin II

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ABSTRACT **The cellular effects of numerous hormones and neurotransmitters, including the vasoactive agents angiotensin II (AngII) and [Arg^8]vasopressin, are mediated in part by protein-serine/threonine kinases activated by increase of cytosolic Ca^{2+} concentration. In this study, we have tested the ability of Ca^{2+}-mobilizing agents to activate cellular tyrosine kinases. Treatment of intact GN4 liver epithelial cells with AngII rapidly (≤15 sec) increased tyrosine kinase activity measured either in unfractionated cell lysates or in anti-phosphotyrosine immune complexes from detergent-solubilized cells. Increased phosphorylation of the exogenous substrate poly(Glu^{80}Tyr^{20}) (3- to 4-fold over control) by immunoprecipitated kinases closely paralleled the time- and dose-dependence of the appearance of tyrosine phosphoproteins in intact cells. This effect of AngII was mimicked by thapsigargin, a Ca^{2+}-elevating tumor promoter. The ability of AngII, but not epidermal growth factor, to increase tyrosine kinase activity was blocked in cells loaded with the Ca^{2+} chelator bis-(*O*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. Dephosphorylation of immunoprecipitated proteins by tyrosine phosphatase treatment was accompanied by a 60–70% loss in *in vitro* kinase activity, suggesting that the AngII-sensitive kinase(s) are activated by phosphorylation in intact cells. These findings demonstrate a link between two widely occurring signaling pathways, the tyrosine kinases and the Ca^{2+} second-messenger system, and suggest the possible involvement of Ca^{2+}-activated tyrosine kinases in the endocrine actions of AngII and [Arg^8]vasopressin.**

Reversible phosphorylation of proteins on tyrosine residues is a regulatory mechanism frequently associated with cell growth and mitosis. The receptors for several peptide growth factors, including epidermal growth factor (EGF), platelet-derived growth factor, and insulin-like growth factor I, as well as the transforming proteins of several oncogenes (e.g., *src*, *abl*) contain tyrosine kinase domains essential for growth-promoting activity (1, 2). In comparison to the receptor kinases, which are known to be activated by ligand binding, the pathways regulating nonreceptor tyrosine kinases are poorly understood. Likewise, the biological function of nonreceptor tyrosine kinases in both proliferating and nonproliferating cells is unclear. However, the abundance of tyrosine kinase activity in terminally differentiated cells such as platelets (3) and neurons (4) has suggested non-growth-related roles for tyrosine phosphorylation in processes as diverse as secretion and neural plasticity.

Studies in our laboratory have shown that treatment of WB rat liver epithelial cells with angiotensin II (AngII), [Arg^8]vasopressin, or epinephrine rapidly increases cellular protein-phosphotyrosine [Tyr(*P*)] content in a Ca^{2+}-dependent, protein kinase C-independent fashion (5). These agents are members of a large class of hormones and neurotransmitters that increase cytosolic Ca^{2+} and diacylglycerol levels by activating receptors linked to phospholipase C via a GTP-binding protein (6). In turn, cellular effects of such hormones are believed principally to involve serine/threonine protein kinases, notably the Ca^{2+}/calmodulin-dependent kinases and protein kinase C. However, our findings have suggested the existence of an additional pathway by which intracellular Ca^{2+} acts—modulation of the tyrosine-phosphorylation states of specific proteins (5). Although Ca^{2+}-activated tyrosine phosphorylation recently has been reported in several other cell types, including vascular smooth muscle (7), neutrophils (8), platelets (9, 10), and hippocampal cells (11), the mechanism of this response remains to be elucidated. In the present studies, we have examined the hypothesis that mobilization of Ca^{2+} increases steady-state levels of tyrosine-phosphorylated proteins by activating tyrosine kinases.

EXPERIMENTAL PROCEDURES

Anti-Tyr(*P*) Immunoprecipitation. Confluent cultures of GN4 cells in 60-mm dishes were washed once with serum-free medium and were stimulated with various agents at 37°C for the times indicated. Cells were lysed with ice-cold lysis buffer [20 mM Hepes (pH 7.3) containing 500 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, 10% (vol/vol) glycerol, and 10 μg of phenylmethylsulfonyl fluoride, 20 μg of leupeptin, and 100 kallikrein inhibitor units of aprotinin per ml], and lysates were clarified by centrifugation at 14,000 × *g* for 15 min at 4°C. Tyrosine-phosphorylated proteins were precipitated by incubation with monoclonal anti-Tyr(*P*) antibody [qualitatively similar results were obtained with antibodies PY20 (1 μg per 500 μg of total protein; ICN) and PT-66 (1 μl per 500 μg; Sigma)], 5 μg of rabbit anti-mouse IgG, and 10 μl of pansorbin (Calbiochem) for 3 hr at 4°C. Immune complexes were collected by centrifugation at 14,000 × *g* for 3 min at 4°C, were washed once with lysis buffer and twice with 2-fold-concentrated tyrosine kinase assay buffer (100 mM sodium Hepes, pH 7.6/60 mM $MgCl_2$/2 mM $MnCl_2$/0.2 mM Na_3VO_4/0.2% Nonidet P-40), and then were resuspended with 70 μl of this buffer. In some experiments, an aliquot (10–20 μl) of the washed immune complex was removed for anti-Tyr(*P*) immunoblotting after SDS/PAGE on 8% gels as described (12). Efficient anti-Tyr(*P*) recovery of tyrosine phosphoproteins and kinase activity required high NaCl concentration (500 mM) in the cell lysis buffer. We postulate that Tyr(*P*) residues are dissociated from endogenous binding domains [e.g., src-homology domains (13)] at high ionic strength and thereby are made accessible for immunoprecipitation, since association of several WB cell tyrosine phosphoproteins with phospholipase C-γ_1 is reversed at high ionic strength (14).

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Abbreviations: EGF, epidermal growth factor; AngII, angiotensin II; Tyr(*P*), phosphotyrosine; BAPTA, bis-(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Immune Complex Tyrosine Kinase Assay. Routinely, 10-μl aliquots of immune complex suspension were preincubated for 5 min at 4°C with 160 μg of the synthetic tyrosine kinase substrate poly(Glu^{80}Tyr^{20}) (15) or the control substrate poly-(Glu). Reactions (80-μl total reaction volume) were initiated by addition of 5 μM [γ-^{32}P]ATP (5 μCi per reaction; 1 Ci = 37 GBq). After 4 min at 25°C, 50 μl of the reaction mix was spotted onto Whatman 3MM paper. The papers were washed with trichloroacetic acid, air-dried, and assayed by liquid scintillation for acid-insoluble ^{32}P (16). Tyrosine kinase activity was defined as ^{32}P incorporation in cpm occurring in the presence of poly(Glu^{80}Tyr^{20}) minus that occurring in the presence of poly(Glu). This procedure allows estimation of poly(Glu^{80}Tyr^{20}) phosphorylation above the background of endogenous protein phosphorylation. The rates of tyrosine phosphorylation so measured were linear for at least 15 min and were proportional to the amount of cell lysate used for immunoprecipitation.

Intracellular Ca^{2+} Measurements. GN4 cells grown on 22-mm diameter glass coverslips were incubated with 5 μM fura-2/AM (acetoxymethyl ester) for 25 min at 37°C and then were washed and incubated in a balanced salt solution (10 mM Hepes/138 mM NaCl/5.3 mM KCl/1.3 mM $CaCl_2$/0.8 mM $MgCl_2$/0.35 mM Na_2HPO_4/0.44 mM KH_2PO_4/5.6 mM glucose, pH 7.3) for 5 min prior to addition of AngII. Microscopic fields containing 50–60 cells were monitored for fluorescence emission at 505 nm under excitation at 340 and 380 nm by using a SPEX analytical system (17).

Autophosphorylation and Phosphoamino Acid Analysis. Aliquots of washed immune complex suspensions were incubated for 10 min at 25°C in the presence of 25 μCi of [γ-^{32}P]ATP without poly(Glu^{80}Tyr^{20}) and then were subjected to SDS/PAGE, electrophoretic transfer to Immobilon membranes (Millipore), and autoradiography. Regions of the membrane containing the ^{32}P-labeled species of interest were excised and subjected to acid hydrolysis, two-dimensional electrophoresis, and autoradiography (18). ^{32}P-labeled phosphoserine, Ser(^{32}P); phosphothreonine, Thr(^{32}P); and Tyr(^{32}P) were located by comigration with ninhydrin-stained standards.

RESULTS AND DISCUSSION

In a recent report, we showed that AngII treatment of WB rat liver epithelial cells increases the phosphorylation states of several proteins in a Ca^{2+}-dependent fashion (5). In the present studies, we have evaluated the effects of Ca^{2+}-mobilizing agents on cellular tyrosine kinases, by measuring tyrosine kinase activities in cell-free preparations. In initial experiments, unfractionated lysates from control and AngII-treated cells were assayed by measuring incorporation of ^{32}P into an acid-insoluble synthetic tyrosine kinase substrate, poly(Glu^{80}Tyr^{20}). However, high background activities of serine/threonine kinases toward endogenous lysate proteins prevented reliable assessment of relative tyrosine kinase activities. We have circumvented this problem by preparing anti-Tyr(*P*) immunoprecipitates from AngII-treated cells prior to assay with poly(Glu^{80}Tyr^{20}). This procedure was designed to detect activated tyrosine kinases which, if similar to a number of known receptor and nonreceptor tyrosine kinases (1, 2, 19–23), autophosphorylate when activated. Anti-Tyr(*P*) immunoblotting of anti-Tyr(*P*) immunoprecipitates from control and treated cells revealed that recovery of tyrosine-phosphorylated proteins was specific and efficient: no tyrosine phosphoproteins were precipitated when nonimmune mouse IgG was substituted for monoclonal anti-Tyr(*P*) (e.g., see Fig. 4 *Left*) or when specific immunoprecipitation was blocked by 1 mM phenylphosphate, and no Tyr(*P*)-containing proteins were detectable in supernatants after one round of immunoprecipitation (not shown).

Initially, studies of tyrosine kinase activation were conducted with the WB and GN4 cell lines. The latter was derived previously from WB cells by serial treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (24). GN4 cells exhibited an ≈3-fold greater AngII-stimulated increase in endogenous protein-tyrosine phosphorylation compared with WB cells, whereas responses to EGF were of similar magnitude in the two lines (Fig. 1 *Upper*). EGF treatment of the two cell types stimulated similar (≈3-fold) increases in tyrosine kinase activity measured by using anti-Tyr(*P*) immunoprecipitates of cell lysates (Fig. 1 *Lower*). As anticipated, the activity recovered from EGF-treated cells is due in part to the presence of activated EGF receptors, based on the appearance of a 170-kDa tyrosine-phosphorylated species in anti-Tyr(*P*) immunoprecipitates that is recognized by anti-EGF receptor antibodies. AngII treatment of both WB and GN4 cells also stimulated increases in immune complex tyrosine kinase activity (Fig. 1 *Lower*), with GN4 cells again being more responsive than WB cells (340% vs. 160% of control activity, respectively). These results are consistent with a link between endogenous protein-tyrosine phosphorylation and kinase activation in response to AngII and prompted us to use GN4 cells in subsequent studies.

The effects of AngII treatment on tyrosine kinase activity were rapid (≤15 sec) and transient (maximal at 45–60 sec;

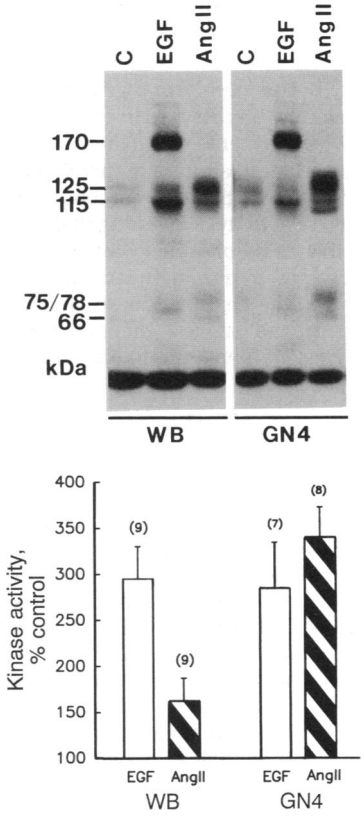

FIG. 1. Increased tyrosine phosphorylation and tyrosine kinase activity in WB and GN4 cells. Confluent cultures of WB and GN4 cells were treated with EGF at 300 ng/ml for 1 min or with 1 μM AngII for 45 sec for WB cells or 1 min for GN4 cells under conditions that allowed maximal increase of both tyrosine phosphorylation and recovered kinase activity compared with control cells (lanes C). (*Upper*) WB and GN4 cells treated as indicated were lysed with radioimmunoprecipitation assay (RIPA) buffer and were subjected to anti-Tyr(*P*) immunoblotting as described (12). (*Lower*) Tyrosine kinase activity toward poly(Glu^{80}Tyr^{20}) was assayed in anti-Tyr(*P*) immune complexes derived from WB or GN4 cells as described in text. The results shown (mean ± SEM from the number of experiments indicated in parentheses) are expressed as a percentage of the control (untreated) tyrosine kinase activity.

Fig. 2). This increase corresponded temporally to the appearance of endogenous tyrosine-phosphorylated proteins in intact GN4 cells (not shown). Both responses to AngII were dose-dependent, with half-maximal effects occurring at ≈1 nM. No measurable tyrosine kinase activity was immunoprecipitated in the presence of phenylphosphate or when anti-Tyr(*P*) antibodies were replaced by nonimmune IgG.

To validate the results obtained with immunoprecipitates, we performed [^{32}P]ATP-autokinase assays using unfractionated cell lysates prepared in kinase assay buffer containing 1% Triton X-100. Anti-Tyr(*P*) immunoprecipitation of the *in vitro* phosphorylated lysates, followed by SDS/PAGE and phosphoamino acid analysis, revealed an ≈2-fold increase in total protein-associated Tyr(^{32}*P*) content in lysates of AngII-treated cells compared to controls. These results indicate that relative tyrosine kinase activities measured in anti-Tyr(*P*) immune complexes are representative of those in cell lysates.

AngII is known to rapidly increase intracellular Ca^{2+} and diacylglycerol concentrations in target cells (5, 25–27). We have shown that Ca^{2+} mobilization but not protein kinase C activation is necessary for elevation of tyrosine phosphorylation in response to AngII (5). To evaluate the role of Ca^{2+} in AngII-stimulated tyrosine kinase activation, GN4 cells were loaded with the Ca^{2+} chelator bis-(*O*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (28) prior to AngII treatment. In BAPTA-loaded cells, the AngII-stimulated increase in cytosolic Ca^{2+}, measured by fura-2 fluorescence, was reduced by >90% (Fig. 3 *Upper*). BAPTA pretreatment also completely abolished the AngII-stimulated increase in immune complex tyrosine kinase activity but had little effect on immune complex tyrosine kinase activity from EGF-treated cells (Fig. 3 *Lower*). This lack of an effect of BAPTA on EGF receptor kinase activity is consonant with the known Ca^{2+}-independence of this enzyme (29) and indicates the absence of toxic or nonspecific effects of BAPTA on GN4 cells in this context. To further evaluate the involvement of Ca^{2+} in tyrosine kinase activation, GN4 cells were treated with the tumor promoter thapsigargin, which promotes Ca^{2+} release from endoplasmic reticulum (30). Treatment with 2 μM thapsigargin for 5 min increased immune complex tyrosine kinase activity to 245 ± 38% of vehicle controls (mean ± SEM, n = 5 separate experiments). In contrast, treatment with the protein kinase C activator phorbol myristate acetate at 300 nM for 10 min had no significant effect on recovery of tyrosine kinase activity.

The successful recovery of tyrosine kinase activity from AngII-treated cells by anti-Tyr(*P*) immunoprecipitation suggests that this activity may reside in one or more of the tyrosine-phosphorylated species detected by immunoblotting (Fig. 1). To investigate the possible presence of autophosphorylating kinases, anti-Tyr(*P*) immune complexes were

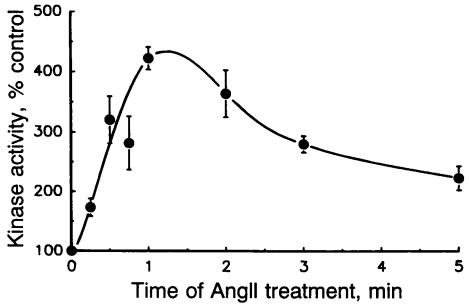

FIG. 2. Time-course of AngII-stimulated tyrosine kinase activity. Tyrosine kinase activity was assayed in anti-Tyr(*P*) immune complexes derived from GN4 cells treated with AngII for the times indicated. The results shown (mean ± SEM from three experiments) are expressed as a percentage of control (untreated) tyrosine kinase activity (960 ± 180 fmol of *P*/min per mg of total protein).

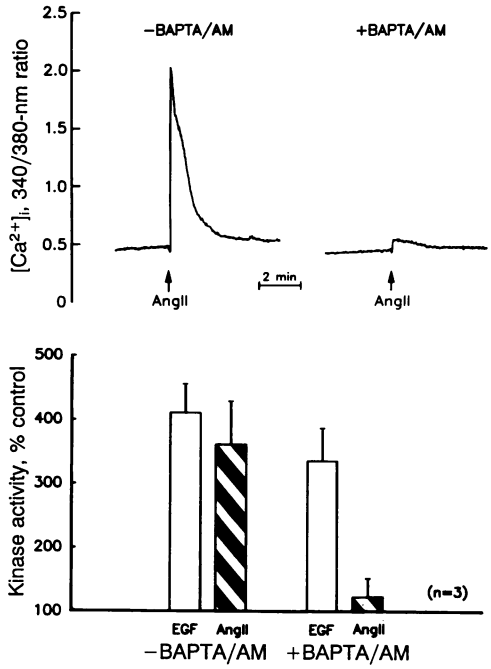

FIG. 3. Inhibition of Ca^{2+} mobilization and tyrosine kinase activation by BAPTA. (*Upper*) Fura-2 fluorescence was monitored in GN4 cells pretreated with either vehicle (0.1% dimethylsulfoxide) or 25 μM BAPTA acetoxymethyl ester (BAPTA/AM) for 15 min. Cells were stimulated with 1 μM AngII at the times indicated by the arrows. Ca^{2+} concentration is reported as the 340/380-nm fluorescence ratio. (*Lower*) Tyrosine kinase activity was assayed in anti-Tyr(*P*) immune complexes derived from GN4 cells that had been pretreated with BAPTA/AM or vehicle as in *Upper* and then treated for 1 min with EGF at 300 ng/ml or 1 μM AngII. The results shown represent mean ± SEM from three experiments.

incubated with [γ-^{32}P]ATP in the absence of poly(Glu^{80}-Tyr^{20}), and phosphorylated proteins were detected by autoradiography after SDS/PAGE. The resulting patterns of *in vitro* phosphorylation strongly resembled those detected by anti-Tyr(*P*) immunoblotting of immune complexes, with protein species of apparent molecular masses 66 (p66), 75/78 (p75/78), 93 (p93), and 115/125 (p115/125) kDa becoming phosphorylated (Fig. 4 *Left*). Phosphoamino acid analysis of the p66, p75/78, and p115/125 species revealed that *in vitro* phosphorylation was detectable only on tyrosine (Fig. 4 *Right*). Treatment of GN4 cells with 1 μM [Arg^8]vasopressin for 1 min produced a similar pattern of activated autophosphorylation (not shown). These results suggest that the proteins recovered by anti-Tyr(*P*) immunoprecipitation are subject to further phosphorylation *in vitro*, either as autophosphorylating kinases or as kinase substrates. Whether the proteins phosphorylated *in vitro* are identical to those phosphorylated in intact cells and which, if any, of the detectable species contain kinase activity remain to be determined.

Taken together, the present findings support the hypothesis that AngII activates a Ca^{2+}-dependent process, resulting in increase of tyrosine kinase activity. The appearance of tyrosine-phosphorylated endogenous proteins in response to AngII closely paralleled changes in immune complex tyrosine kinase activity in terms of time- and dose-dependence and Ca^{2+} requirements. We have considered several alternative explanations for our results. It is conceivable, for example, that an apparent increased recovery of tyrosine kinase activity by immunoprecipitation might reflect a decreased level of tyrosine phosphatase activity recovered from treated cells. However, anti-Tyr(*P*) immunoprecipitates from control or AngII-treated cells contained no detectable tyrosine phosphatase activity when heat-denatured ^{32}P-autophosphory-

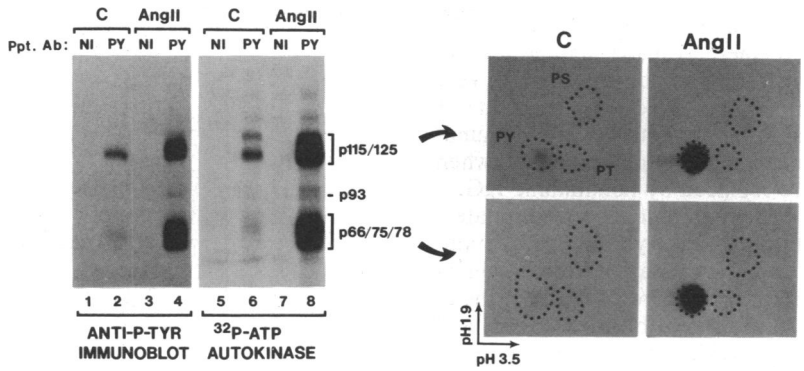

FIG. 4. *In vitro* phosphorylation of endogenous proteins in anti-Tyr(*P*) immune complexes. (*Left*) Lysates from control GN4 cells (lanes C) or from GN4 cells treated with 1 μM AngII for 1 min were immunoprecipitated with nonimmune mouse IgG (lanes NI) or monoclonal anti-Tyr(*P*) (lanes PY). Aliquots (25 μl) of the immune complex suspension were subjected to SDS/PAGE, electrophoretic transfer to nitrocellulose, and anti-Tyr(*P*) immunoblotting (lanes 1–4). For autokinase assay, 25-μl aliquots of the same suspensions were incubated for 10 min at 25°C in the presence of 25 μCi of [γ-^{32}P]ATP and then subjected to SDS/PAGE, electrophoretic transfer to Immobilon (Millipore), and autoradiography (lanes 5–8). (*Right*) The regions of Immobilon membrane containing the ^{32}P-labeled p66/75/78, and p115/125 species from anti-Tyr(*P*) immunoprecipitates of control (*Left*, lane 6) or AngII-treated (*Left*, lane 8) cells were excised and subjected to phosphoamino acid analysis. Locations of ninhydrin-stained phosphoserine (PS), phosphothreonine (PT), and Tyr(*P*) (PY) standards are indicated. The hydrolyzed p66/75/78 regions shown yielded 13 and 176 cpm of Tyr(^{32}*P*) (above blank) from control and AngII-treated cells, respectively, while the corresponding p115/125 regions yielded 25 and 245 cpm.

lated rat liver EGF receptor was used as a substrate. Furthermore, there was no detectable loss of Tyr(*P*) from immunoprecipitated proteins when either immune complexes or eluted phosphoproteins were incubated for up to 30 min in the presence of all kinase assay components except ATP. Therefore, it is unlikely that decreased recovery of phosphatase activity rather than increased kinase activity can account for the effects of AngII treatment on phosphorylation rates in the immune complex assay.

Secondly, it might be argued that the presence of precipitating antibodies in the immune complex assay could artifactually alter tyrosine kinase activities. To test this possibility, Tyr(*P*) proteins were specifically eluted from immune complexes prior to kinase assay. Washed anti-Tyr(*P*) immunoprecipitates from control and AngII-treated GN4 cells were resuspended in 2-fold concentrated assay buffer containing 1 mM *p*-nitrophenyl phosphate. After 30 min at 4°C, eluted proteins were separated from the immune complex by centrifugation for 5 min at 14,000 × *g* and 4°C. *p*-Nitrophenyl phosphate eluted >95% of the detectable Tyr(*P*) proteins from anti-Tyr(*P*) antibodies. There was no detectable change in tyrosine kinase activity in eluates compared with noneluted immune complexes assayed either by autophosphorylation or poly(Glu^{80}Tyr^{20}) phosphorylation (not shown), indicating that the precipitating antibodies do not influence the activity of the recovered kinases.

Finally, the possibility exists that increased recovery of tyrosine kinase activity merely reflects an increase in the pool of phosphorylated tyrosine kinase molecules by a process that is not a consequence of kinase activation. To assess the relationship between the phosphorylation state of recovered kinases and their activities, Tyr(*P*) proteins eluted from immune complexes were dephosphorylated by treatment with recombinant human T-cell tyrosine phosphatase (37-kDa form) (31). Tyrosine phosphatase treatment removed virtually all detectable tyrosine phosphate from eluted proteins (Fig. 5 *Left*). This dephosphorylation was accompanied by a 60–70% loss in the kinase activity measured subsequently with poly(Glu^{80}Tyr^{20}) in the presence of vanadate (Fig. 5 *Right*). The ability of vanadate to inhibit the T-cell phosphatase was confirmed by the complete blockade of protein dephosphorylation when phosphatase incubations contained vanadate (Fig. 5 *Left*); inhibition of dephosphorylation was accompanied by retention of kinase activity (Fig. 5 *Right*). Therefore, we conclude that the loss of kinase activity stems from dephosphorylation of immunoprecipi-

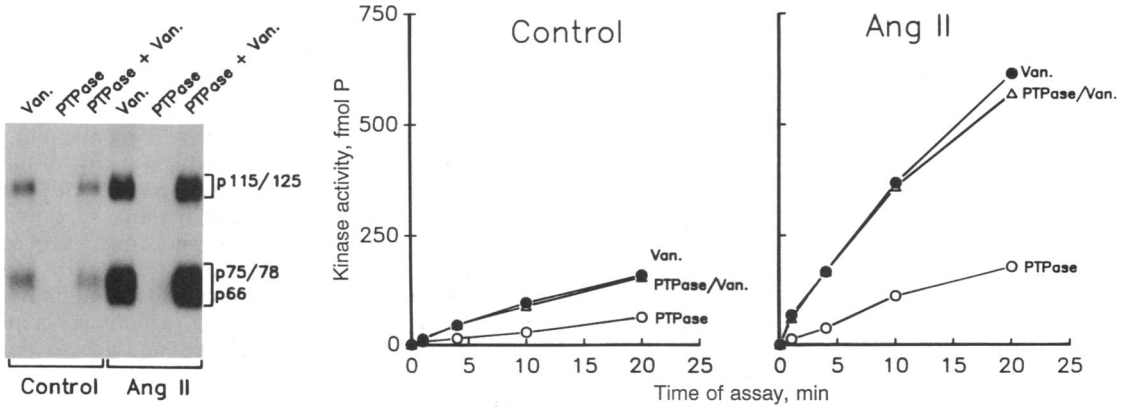

FIG. 5. Loss of tyrosine kinase activity after tyrosine dephosphorylation. Anti-Tyr(*P*) immunoprecipitates from control and AngII-treated GN4 cells were washed twice with 25 mM imidazole (pH 7.2) and eluted from anti-Tyr(*P*) by incubation with this buffer containing 0.05% Triton X-100, 0.1% 2-mercaptoethanol, and 1 mM *p*-nitrophenyl phosphate. Eluted proteins were incubated with 1 mM sodium orthovanadate (lanes Van.), recombinant T-cell tyrosine phosphatase (lanes PTPase; ≈1 ng/μg of total cell protein), or vanadate plus phosphatase for 3 min at 25°C. Vanadate (1 mM) was then added to phosphatase-only samples, and aliquots of all samples were subjected to anti-Tyr(*P*) immunoblotting (*Left*) or *in vitro* kinase assay with poly(Glu^{80}Tyr^{20}) (*Right*).

tated proteins rather than from residual activity of T-cell phosphatase toward poly(Glu^{80}Tyr^{20}) or from other contaminating hydrolases. This finding indicates that the tyrosine phosphorylation of tyrosine kinases (or persistently associated regulatory proteins) recovered from AngII-treated cells both reflects and dictates increased kinase activity. Furthermore, these results validate our rationale for seeking evidence of kinase activation that occurred in intact cells by examining anti-Tyr(*P*) immunoprecipitates.

The sensitivity of AngII-stimulated tyrosine kinase activities to tyrosine dephosphorylation strongly suggests that phosphorylation of these kinases is a major factor in their mechanism of activation. It remains to be established whether this increased kinase phosphorylation is due to autophosphorylation or activation of an intermediating kinase. In either event, these putative enzymes could be directly regulated in a positive manner by increased intracellular Ca^{2+}, as suggested for a reported Ca^{2+}/calmodulin-activated tyrosine kinase (32) or a soybean serine/threonine kinase that contains a Ca^{2+}-binding domain (33). Alternatively, modulation of tyrosine phosphatase activity may be an event more proximal to AngII-stimulated Ca^{2+} mobilization. Thus, Ca^{2+} might maintain one or more tyrosine kinases in an activated (i.e., tyrosine-phosphorylated) state by inhibiting their dephosphorylation. Such a mechanism would require the presence of a basal level of phosphorylating (i.e., kinase-activating) activity in untreated cells, a criterion that is satisfied by the recovery of measurable phosphatase-sensitive tyrosine kinase activity from control cells (Fig. 5). A tyrosine phosphatase that is inhibited by Ca^{2+} has been isolated from bovine brain (34), and Ca^{2+}-mediated inhibition of the leukocyte tyrosine phosphatase CD45 has been reported (35), but the involvement of an analogous mechanism in WB or GN4 cells is untested.

The identity of the kinase(s) involved in AngII-stimulated increases in tyrosine phosphorylation remains unknown. We must consider as candidates known tyrosine kinases, such as the insulin receptor (21, 22) and members of the src family (23, 36, 37), whose activity appears to be regulated positively by autophosphorylation. We have found that immunoprecipitation with antibodies recognizing the common C-terminal peptide of src-related kinases did not decrease AngII-stimulated tyrosine kinase activity subsequently recovered with anti-Tyr(*P*) (unpublished data), suggesting that the AngII-responsive enzymes are not members of this kinase family. In addition, preliminary subcellular fractionation studies indicate that at least part of the AngII-stimulated activity is cytosolic, suggesting that this activity cannot wholly be attributed to transmembrane kinases such as the insulin receptor. Ongoing studies seek to identify the AngII-responsive kinases and to determine whether their mode of activation is common to many cell types. Given the widespread occurrence of receptor-mediated Ca^{2+} mobilization, we anticipate that Ca^{2+}-dependent tyrosine kinase activation may prove to be of fundamental interest, and elucidation of its mechanism may provide new insights into both the acute and growth-regulating actions of Ca^{2+}-mobilizing hormones and neurotransmitters.

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