Synergistic Activation of Tyrosine Phosphorylation by *o*-Vanadate plus Calcium Ionophore A23187 or Aromatic 1,2-Diols†

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ABSTRACT: We have shown previously that treatment of WB rat liver epithelial cells with the Ca^{2+} ionophore A23187 provokes a rapid increase in protein–tyrosine phosphorylation that faithfully reproduces the Ca^{2+}-dependent response seen with angiotensin II. In the presence of the tyrosine phosphatase inhibitor *o*-vanadate (2.0-200 μM), the tyrosine phosphorylation response to A23187 was increased >10-fold in magnitude. This synergistic effect of A23187 and vanadate is clearly distinct from the combined effect of angiotensin II and vanadate, which was merely additive. Chelation of either extracellular or intracellular Ca^{2+} abolished the synergistic response to ionophore and vanadate, indicating its Ca^{2+} dependence. That divergent pathways were involved in the angiotensin II and the A23187/vanadate responses was shown definitely by studies of GN4 cells, a transformed line derived from WB cells by carcinogen treatment. GN4 cells are 2–3-fold more responsive than WB cells to angiotensin II-dependent tyrosine kinase activation, yet they completely lacked the synergistic tyrosine phosphorylation response to A23187/vanadate. To test the role of arachidonic acid metabolites in the A23187/vanadate response, cells were pretreated with either indomethacin or nordihydroguaiaretic acid (NDGA). Neither compound was inhibitory, but surprisingly, NDGA plus vanadate closely mimicked the A23187/vanadate response in WB cells and, like A23187/vanadate, was ineffective in GN4 cells. NDGA contains catechol nuclei (i.e., aromatic 1,2-diols) and therein resembles the flavonoid anti-oxidant quercetin, another compound found to increase tyrosine phosphorylation synergistically with vanadate. In our studies, flavonoids that contain 1,2-diols (quercetin, fisetin) were synergistic with vanadate in elevating P-Tyr (in WB but not GN4 cells) while those lacking 1,2-diols (apigenin, morin) were inactive. Overnight pretreatment with a glutathione precursor, *N*-acetylcysteine, inhibited the responses to A23187/vanadate and NDGA/vanadate. We postulate that combinations of catechols with vanadate, separately or as chelation complexes, may potentiate tyrosine phosphatase inhibition by vanadate or may delay the metabolic inactivation of vanadate [e.g., reduction to V(IV) by glutathione]. Similarly, the sustained elevation of calcium caused by A23187 may change intracellular redox status, perhaps by the depletion of intracellular glutathione. Since tyrosine phosphtases are exquisitely sensitive to sulfhydryl oxidation and potentially to the regulated oxidative state of *o*-vanadate, these results reinforce the concept that local regulation of intracellular redox state may be an important determinant of tyrosine phosphatase activities.

Phosphorylation of proteins on tyrosine residues is a regulatory mechanism frequently associated with the control of cell proliferation. Steady-state levels of tyrosine-phosphorylated proteins are determined by the opposing actions of tyrosine kinases and phosphatases (PTPases),[1] enzymes that have been found to occur in remarkably large and diverse families. For example, both tyrosine kinases and PTPases can occur as transmembrane, membrane-associated, or cytosolic isoforms (Hunter, 1991; Charbonneau & Tonks, 1992). Although much has been learned about the regulation of ligand-activated, transmembrane tyrosine kinases such as the receptors for epidermal growth factor (EGF), platelet-derived growth factor, and insulin (Schlessinger & Ullrich, 1992; Pawson, 1992), control of the nonreceptor kinases as well as that of the PTPases as a whole remains poorly understood. Available data suggest that nonreceptor tyrosine kinases may be controlled acutely by cycles of phosphorylation and dephosphorylation. Specifically, phosphorylation of a C-terminal Tyr residue (Tyr^{527}) in $pp60^{c\text{-}src}$ negatively regulates kinase activity, whereas (auto)phosphorylation of an internal residue (Tyr^{416}) is associated with kinase activation (Cooper & Howell, 1993). Recently, kinases capable of phosphorylating Tyr^{527} of $pp60^{c\text{-}src}$ have been identified (Thomas et al., 1991; Nada et al., 1991). In addition, studies of T-cell activation have suggested that activation of T cells via antigen receptor may involve regulated dephosphorylation of the src-family kinase $pp56^{lck}$ by the transmembrane lymphocyte PTPase CD45 (Mustelin et al., 1989; Ostergaard et al., 1989). PTPases may themselves be regulated by tyrosine phosphorylation (Stover et al., 1991; Yeung et al., 1992; Feng et al., 1993; Vogel et al., 1993), adding yet another layer of complexity to their regulatory pathways.

We have reported previously that hormones such as angiotensin II and the Ca^{2+}-ATPase inhibitor thapsigargin can stimulate increases in tyrosine phosphorylation in WB

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[1] Abbreviations: BAPTA/AM, bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NDGA, nordihydroguaiaretic acid; PAGE, polyacrylamide gel electrophoresis; PTPase, protein tyrosine phosphatase.

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liver epithelial cells (Huckle et al., 1990). This effect of angiotensin II is mediated in part by the activation of cellular tyrosine kinases (Huckle et al., 1992) and is dependent on a rise in intracellular Ca^{2+} (Huckle et al., 1990, 1992). The angiotensin II-stimulated increase in tyrosine phosphorylation was not blocked in the presence of EGTA, which would not alter intracellular Ca^{2+} levels but was blocked in cells preloaded with a form of the Ca^{2+} chelator bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) that can penetrate intact cells. Treatment of WB cells with the Ca^{2+} ionophore A23187 faithfully reproduced the effects of Ca^{2+}-mobilizing hormones on tyrosine phosphorylation; as anticipated, this effect of A23187 was blocked by EGTA. Preincubation of liver epithelial cells with the tyrosine phosphatase inhibitor sodium orthovanadate slowly increased protein tyrosine phosphorylation, and when combined with angiotensin II, the effects of the two agents were additive. In contrast, treatment with A23187 in the presence of vanadate produced increases in tyrosine phosphorylation that were much greater than additive (Huckle et al., 1990). In the present studies, we have characterized the markedly synergistic action of A23187 and vanadate on tyrosine phosphorylation. In these investigations, we have found that the combination of vanadate plus phenolic antioxidants with 1,2-diols closely reproduces the synergistic effects of A23187 plus vanadate. This parallel suggests that sustained, high, intracellular Ca^{2+} concentrations and aromatic 1,2-diols may have similar effects on the efficacy of vanadate as a modulator of tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Materials. Ionophore A23187 (Calbiochem) was prepared as a 50 mM stock solution in DMSO (Pierce Chemical) and stored in 5-μL single-use aliquots at −20 °C. Immediately prior to use, 100-fold concentrated working aqueous dilutions (≤1 mM A23187) were prepared. NDGA and flavonoids (obtained from either Sigma or Aldrich) were stored at −20 °C as 30 mM stock solutions in DMSO. BAPTA/AM was obtained from GIBCO-BRL.

Vanadate is reported to form oligomers when stored in nonalkaline aqueous solution at concentrations greater than 1 mM (Gordon, 1991). However, in our experiments, sodum *o*-vanadate (Sigma) was equally effective when used either from 100 mM aqueous stock solutions or from freshly-prepared solutions at lower concentrations.

Cell Cultures. WB and GN4 cells were maintained at 37 °C in Richter's improved minimal essential medium containing 10% fetal bovine serum and 0.1 μM insulin in a humidified 5% CO_2 atmosphere as described previously (Hepler et al., 1988). Seven to 10 days prior to each experiment, cells of passage number 19–26 were seeded onto plastic culture dishes (Costar). Cells were used for experiments 1–2 days after reaching confluence. Prior to the addition of experimental agents, cells were washed once with Eagle's minimal essential medium containing 20 mM 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 by a modification of procedures described previously (McCune & Earp, 1989). Briefly, treatment incubations were terminated by rapid aspiration of the medium and addition of 250 μL of ice-cold radioimmunoprecipitation assay buffer (McCune & Earp, 1989). Cell lysates were scraped from culture dishes and further solubilized by the addition of 125 μL of 3-fold concentrated SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer and heating to 100 °C for 3 min. Proteins were separated by electrophoresis on 8% polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed by sequential incubation with monoclonal anti-P-Tyr antibody PT66 (Sigma; 1:2000 dilution), rabbit anti-mouse IgG (1 μg/mL; Jackson ImmunoResearch), and ^{125}I labeled protein A (200 000 dpm/mL, 2–10 μCi/μg, DuPont-New England Nuclear), followed by autoradiography. Molecular weights of [P-Tyr] proteins were estimated using prestained standards calibrated by comparison to the migration of non-prestained standards (Sigma).

Immune Complex Tyrosine Kinase Assay. Confluent WB and GN4 cell cultures in 60-mm dishes were treated as described above. Tyrosine-phosphorylated proteins were immunoprecipitated from Triton X-100 cell lysates, and immunoprecipitates were resuspended in 2-fold concentrated tyrosine kinase assay buffer as described previously (Huckle et al., 1992). For kinase assay, 10-μL aliquots of immune complex suspension were preincubated for 5 min at 4 °C with 160 μg of the synthetic tyrosine kinase substrate polyGluTyr (4:1) (Braun et al., 1984) or the control substrate polyGlu. Reactions (80 μL total) were initiated by the addition of 5 μM [γ-^{32}P]ATP (5 μCi/reaction). 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 counted by liquid scintillation for acid-insoluble ^{32}P (Corbin & Reimann, 1974). Tyrosine kinase activity was defined as cpm ^{32}P incorporation occurring in the presence of polyGluTyr minus that occurring in the presence of polyGlu. This procedure allows the estimation of polyGluTyr 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.

Autophosphorylation and Phosphoamino Acid Analysis. For assay of autophosphorylation, 25-μL aliquots of washed immune complex suspensions were incubated for 10 min at 25 °C in the presence of 25 μCi of [$\gamma^{32}P$]ATP without polyGluTyr (4:1) or polyGlu 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 (Kamps & Sefton, 1989). [^{32}P]Phosphoserine, [^{32}P]phosphothreonine, and [^{32}P]phosphotyrosine were located by comigration with ninhydrin-stained standards.

RESULTS

Initial experiments sought to determine the time, concentration, and Ca^{2+} dependence of synergistic tyrosine phosphorylation in response to A23187 plus *o*-vanadate. Treatment of WB cells with either A23187 (10 μM) or *o*-vanadate (200 μM) alone provoked increases in tyrosine phosphorylation that were detectable after ≤30 s and continued for ≥5 min (Figure 1A). In extended time-course experiments (not shown), the tyrosine phosphorylation response to A23187 plus vanadate reached a plateau at 15–20 min that remained elevated for at least 60 min. This effect of A23187 is consonant with the existence of Ca^{2+}-activated pathways of tyrosine phosphorylation that we and others have characterized previously (Bading & Greenberg, 1991; Gomez-Cambronero et al., 1991; Gusovsky et al., 1993; Huang et al., 1990; Huckle et al., 1992, 1990; Offermanns et al., 1993; Takayama et al., 1991; Tsuda et al., 1991; Vostal et al., 1991; Yu et al., 1991).

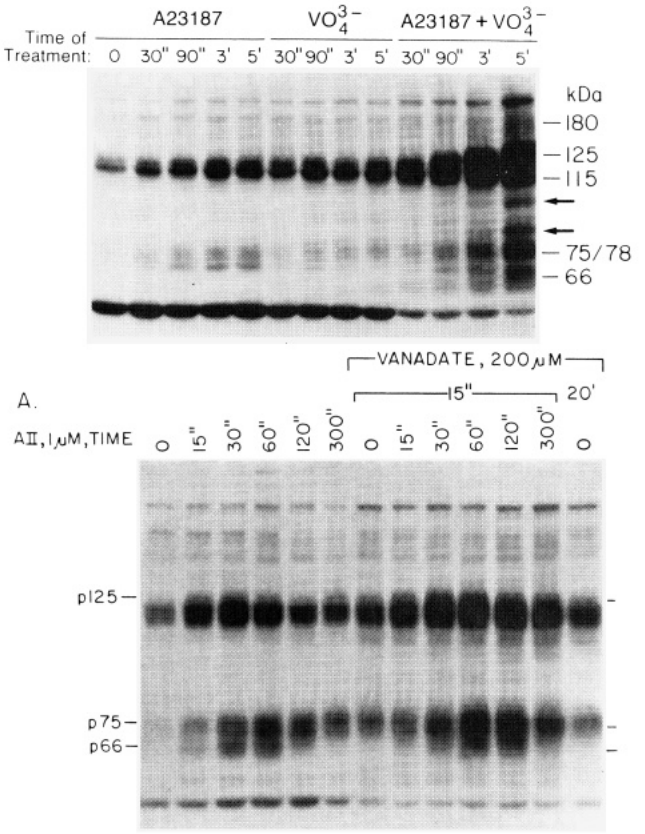

FIGURE 1: Ca^{2+} ionophore A23187 and vanadate have synergistic effects on tyrosine phosphorylation. (A, top) Confluent cultures of WB cells were treated with A23187 (10 μM), sodium vanadate (VO_4^{3-}; 200 μM), or both agents for the times indicated at 37 °C. Incubations were terminated, and cellular proteins were analyzed by anti-phosphotyrosine immunoblotting as described under Experimental Procedures. Apparent molecular weights of stimulated phosphoproteins are indicated on the right margin; phosphoproteins appearing in response to A23187 plus vanadate include at least two species (100 and 83 kDa, indicated by arrows) not detectable upon treatment with either agent alone. (B, bottom) WB cells were treated with angiotensin II (AII, 1 μM) for the indicated periods with or without a 15-min pretreatment with 200 μM vanadate. The righthand-most lane represents cells treated with vanadate for 20 min without angiotensin II.

The ability of *o*-vanadate to allow the accumulation of tyrosine-phosphorylated proteins is consistent with the inhibition of cellular PTPases in the presence of basal tyrosine kinase activities. Together, however, A23187 and *o*-vanadate stimulated increases in tyrosine phosphorylation that clearly were greater than additive. A similar effect is seen with another calcium ionophore, ionomycin, and therefore, the effect is not A23187-specific. Phosphoproteins appearing in response to A23187 plus *o*-vanadate include species (indicated by the lower arrow in Figure 1A) not readily detectable upon treatment with either agent alone or with angiotensin II. The dramatic potentiating effect of vanadate on phosphorylation in response to A23187 stands in contrast to the combined effects of vanadate and angiotensin II. As shown previously (Huckle et al., 1990), treatment of WB cells with angiotensin II stimulates a rapid and transient increase in tyrosine phosphorylation (Figure 1B), peaking at 30–60 s and returning to near-basal levels after 5 min. In cells pretreated with 200 μM vanadate for 15 min, peak levels of tyrosine phosphorylation in response to angiotensin II were only moderately potentiated, and the return toward basal phosphorylation levels was delayed but not prevented.

In the presence of 200 μM vanadate, A23187 produced increases in tyrosine phosphorylation over the concentration

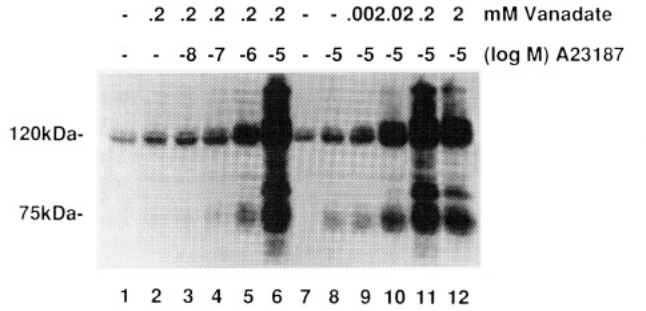

FIGURE 2: Effects of A23187 and vanadate on tyrosine phosphorylation are dose-dependent. (Lanes 1–6) WB cells were treated for 10 min with the indicated concentrations of A23187 in the presence of 200 μM vanadate. (Lanes 7–12) WB cells were treated for 10 min with the indicated concentrations of vanadate in the presence of 10 μM A23187. Incubations were terminated, and cellular proteins were analyzed by anti-phosphotyrosine immunoblotting as described under Experimental Procedures.

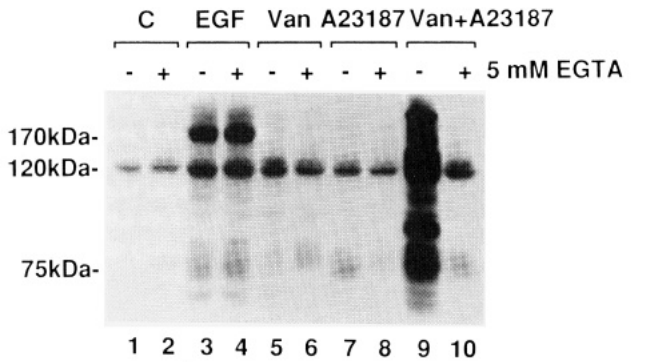

FIGURE 3: Synergistic response to A23187 and vanadate is calcium-dependent. WB cells were treated with either EGF (300 ng/mL, 1 min), vanadate (200 μM, 5 min), A23187 (10 μM, 5 min), or vanadate plus A23187 with (even lanes) or without (odd lanes) a 5-min pretreatment with 5 mM EGTA. Control cells ("C") received no treatment but were incubated with or without EGTA for a total of 10 min. Incubations were terminated, and cellular proteins were analyzed by anti-phosphotyrosine immunoblotting as described under Experimental Procedures.

range 0.1–10 μM in a 10-min treatment (Figure 2, lanes 1–6); concentrations above 10 μM were not examined. In the presence of 10 μM A23187, vanadate had biphasic concentration-dependent effects on tyrosine phosphorylation, with a maximal response appearing at 200 μM vanadate (lanes 7–12). The effects of vanadate alone were monophasic, with no decrease in tyrosine phosphorylation above 200 μM (not shown).

To confirm that the observed effects of A23187 were Ca^{2+}-dependent, WB cells were treated in the presence of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to chelate extracellular Ca^{2+}. Tyrosine phosphorylation responses in control, epidermal growth factor (EGF)-, and vanadate-treated cells were essentially unchanged by 5-min pretreatment with 5 mM EGTA (Figure 3). The response to A23187 alone was inhibited by EGTA, again consistent with the existence of Ca^{2+}-dependent tyrosine phosphorylation pathways. Moreover, the synergistic effects of A23187 plus vanadate were totally abolished by EGTA, whereas EGTA had little or no effect on the response to vanadate alone. Thus, the synergy between A23187 and vanadate appears to be Ca^{2+}-dependent. The response to A23187 did not appear to derive from spurious chemical properties of the ionophore, since two chemical constituents of A23187, 2-acetylpyrrole and 1,7-dioxaspiro[5.5]undecane, did not mimic the effects of A23187 on WB cells when

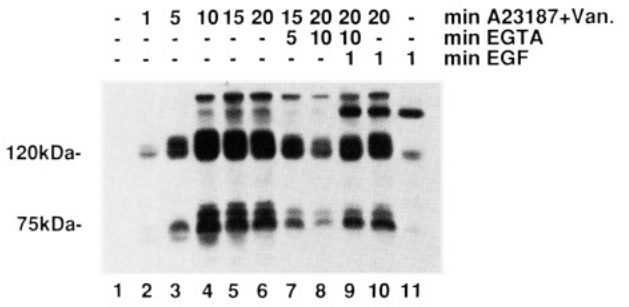

FIGURE 4: Synergistic response to A23187 and vanadate is reversible. (Lanes 1–6) WB cells were treated without (lane 1) or with (lanes 2–6) A23187 (10 μM) plus vanadate (200 μM) for the indicated periods in the absence of EGTA. (Lanes 7–9) After 10 min of A23187 treatment, EGTA (5 mM) was added, and incubations were continued for 5 or 10 min as indicated. (Lanes 9, 10) Cells treated with A23187/vanadate for 20 min, with or without EGTA for the final 10 min, were treated with EGF (300 ng/mL) for 1 min. (Lane 11) Cells were treated with EGF for 1 min. Incubations were terminated, and cellular proteins were analyzed by anti-phosphotyrosine immunoblotting as described under Experimental Procedures.

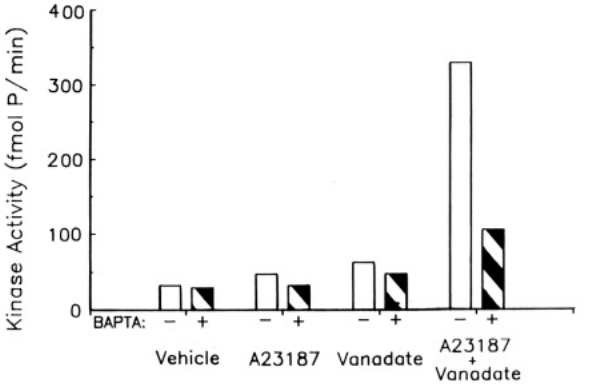

FIGURE 5: Anti-P-Tyr immunoprecipitates from A23187/vanadate-treated cells contain elevated tyrosine kinase activities. Tyrosine phosphorylated proteins from WB cells treated for 10 min with either vehicle (0.03% DMSO), 10 μM A23187, 200 μM vanadate, or A23187 plus vanadate were recovered by anti-phosphotyrosine immunoprecipitation. The cultures were (+) or were not (–) pretreated with 25 μM BAPTA/AM for 10 min prior to the start of the test incubation. Immune complex tyrosine kinase activity was assayed using poly-GluTyr and [γ-^{32}P]ATP in the presence of 30 mM Mg^{2+} and 1 mM Mn^{2+} as described previously (Huckle et al., 1990) and under Experimental Procedures.

combined, individually or together (10 μM each), with vanadate (data not shown).

The reversibility of tyrosine phosphorylation stimulated by A23187 plus vanadate also was examined. In these experiments, cells were treated with A23187 plus vanadate for 10 min prior to the addition of EGTA. As shown in Figure 4, the addition of EGTA at 10 min provoked a progressive loss of tyrosine phosphorylation subsequently measured at 15 or 20 min of A23187/vanadate treatment (lanes 7 and 8) compared to EGTA-untreated cultures (lanes 5 and 6). After treatment with A23187/vanadate with or without EGTA, cells responded normally to a final 1-min exposure to EGF, based on the appearance of the 170-kDa autophosphorylated EGF receptor (lanes 9–11). These findings indicate that treatment with ionophore, EGTA, or both does not adversely influence ligand-activated tyrosine phosphorylation. These results also indicate that the synergistic response to A23187/vanadate is a dynamic process requiring continual access to Ca^{2+} and that the intracellular actions of Ca^{2+} in this context can be rapidly reversed. Moreover, since A23187/vanadate treatment is reversible and does not produce detectable loss of a calpain-sensitive protein (EGF receptor), the amplified tyrosine phosphorylation response probably does not result from Ca^{2+}-dependent proteolytic activation of tyrosine kinases (Taniguchi et al., 1991).

In principle, A23187/vanadate-stimulated tyrosine phosphorylation could stem from activation of tyrosine kinases, inhibition of PTPases, or both. To investigate the first possibility, tyrosine-phosphorylated proteins were recovered from lysates of control or treated cells by anti-P-Tyr immunoprecipitation, and tyrosine kinase activities in the immunoprecipitates were assayed using the synthetic phosphate acceptor polyGluTyr (4:1). This assay is designed to detect activated tyrosine kinases which, if similar to numerous known receptor and nonreceptor tyrosine kinases, autophosphorylate when activated. Using this approach, we have found that elevation of tyrosine phosphorylation in response to angiotensin II involves the activation of at least one tyrosine kinase whose activity is phosphorylation-dependent (Huckle et al., 1992). In the present studies, A23187 and vanadate synergistically stimulated increases in immune complex tyrosine kinase activity (Figure 5). Tyrosine kinase activity in immunoprecipitates from A23187/vanadate-treated cells were increased to 990% of the activity recovered from vehicle-treated control cells, while A23187 and vanadate separately increased activity to 150 and 190%, respectively. Thus, the results of immune complex tyrosine kinase assays parallel those of tyrosine phosphorylation in intact cells. Moreover, immune complex tyrosine kinase activity recovered from A23187/vanadate-treated cells was reduced when cells had been preloaded with the intracellular Ca^{2+} chelator BAPTA.

As a preliminary step in the identification of protein kinases present in anti-P-Tyr immunoprecipitates from A23187/vanadate-treated cells, immunoprecipitated proteins were incubated with [γ-^{32}P]ATP in the absence of polyGluTyr (4:1). In these "autokinase" experiments, the pattern of endogenous WB cell protein phosphorylation resembled that occurring in intact cells, with major phosphoproteins detectable at 66–78, 84, and 115–130 kDa (Figure 6A). Phosphoamino acid analysis of the major phosphoprotein species revealed that, as expected, the great majority of the kinase activity in the cell-free preparations was directed toward tyrosine residues (Figure 6B); interestingly, phosphoamino acid analysis also revealed evidence of Ser/Thr kinase activity in immunoprecipitates from A23187/vanadate-treated cells. The high specificity of the immunoprecipitation procedure is demonstrated here by the virtual absence of autokinase activity recovered in the presence of nonimmune mouse IgG (Figure 6A, lanes 1 and 3).

Since the combined effects of A23187 and vanadate on tyrosine phosphorylation were both Ca^{2+}-dependent and reversible in WB cells, we anticipated that these agents would be at least as effective in GN4 cells. We have shown that GN4 cells, a line derived from WB cells by treatment with a chemical carcinogen, are 2–3-fold more responsive than WB cells to the Ca^{2+}-dependent effects of angiotensin II on tyrosine phosphorylation and tyrosine kinase activation (Huckle et al., 1992). Although GN4 cells were similar to WB cells in their responses to EGF, vanadate, or A23187 alone, the synergistic response to vanadate plus A23187 was absent in GN4 cells (Figure 7). This unexpected result suggests that the synergistic effects of A23187 plus vanadate, while Ca^{2+}-requiring, must be qualitatively distinct from the Ca^{2+}-dependent events activated by angiotensin II.

In an effort to elucidate the nature of this distinction, we investigated the possible involvement of arachidonic acid metabolites that could be generated by Ca^{2+}-stimulated

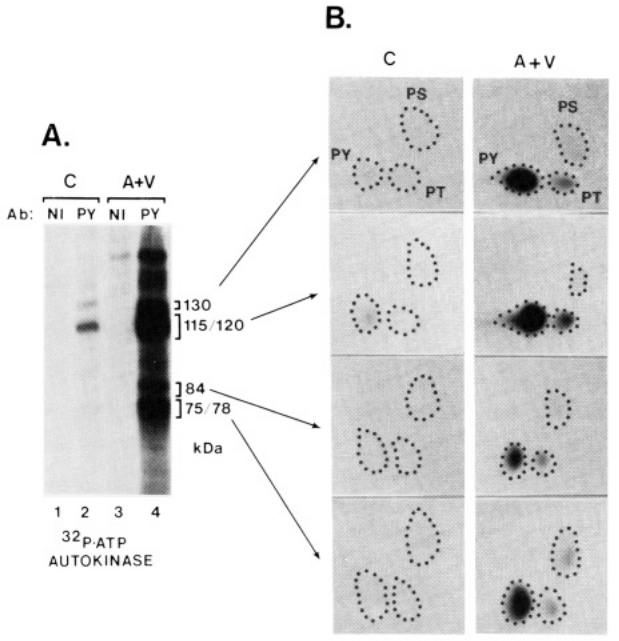

FIGURE 6: Anti-P-Tyr immunoprecipitates from A23187/vanadate-treated cells contain tyrosine and ser/thr kinases that phosphorylate endogenous proteins. (A) Clarified lysates of cells treated for 5 min with vehicle ("C") or A23187 plus vanadate ("A+V", 10 and 200 μM, respectively) were immunoprecipitated using either nonimmune mouse IgG ("NI", odd lanes) or monoclonal anti-P-tyr antibodies ("PY", even lanes). Immunoprecipitates were subjected to autophosphorylation assay, electrophoresis on an 8% polyacrylamide gel, transfer to Immobilon, and autoradiography as described under Experimental Procedures. (B) Portions of the Immobilon membrane containing the molecular weight species indicated were excised and subjected to phosphoamino acid analysis as described under Experimental Procedures. Migration of phosphoserine ("PS"), phosphothreonine ("PT"), and phosphotyrosine ("PY") standards are indicated by the dotted lines.

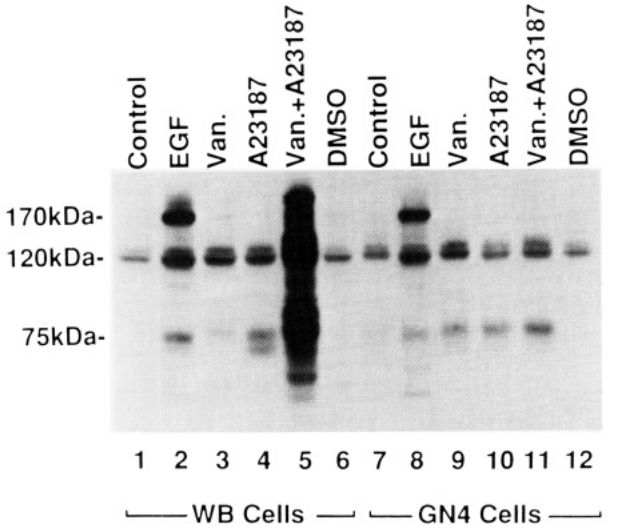

FIGURE 7: GN4 cells do not respond synergistically to A23187 and vanadate. WB cells (lanes 1–6) or GN4 cells (lanes 7–12) were treated with EGF (300 ng/mL, 1 min), A23187 (10 μM, 10 min), vanadate (200 μM, 10 min), or A23187 plus vanadate. Incubations were terminated, and cellular proteins were analyzed by anti-phosphotyrosine immunoblotting as described under Experimental Procedures. Both untreated ("C") and DMSO vehicle-treated controls are shown.

phospholipase A_2 enzymes stimulated by the ionophore's prolonged calcium signal. We have observed (unpublished data) that the effects of angiotensin II on tyrosine phosphorylation in GN4 cells are not inhibited by the cyclooxygenase inhibitor indomethacin (Bailey, 1989) or the lipoxygenase inhibitor nordihydrogiuiaretic acid (NDGA) (Yasumoto et al., 1970). In the present experiments, WB cells were treated with 30 μM indomethacin or NDGA for 10 min prior to the addition of vanadate or A23187 for 10 min. As shown in Figure 8, indomethacin had no detectable effect on tyrosine phosphorylation in the presence or absence of vanadate or A23187. However, NDGA had a small effect in the absence of other agents and, surprisingly, was synergistic with vanadate in a manner that was indistinguishable from A23187 (lane 10 *versus* lane 4). The lack of inhibition by indomethacin or NDGA suggests that arachidonic acid metabolites are not involved in synergistic tyrosine phosphorylation by A23187/vanadate. It might be argued that the stimulatory effects of NDGA could mask any inhibitory effects of this agent on the A23187/vanadate response. However, we have been unable to mimic the A23187/vanadate effects by adding arachidonic acid at concentrations up to 100 μM in the presence of vanadate (not shown).

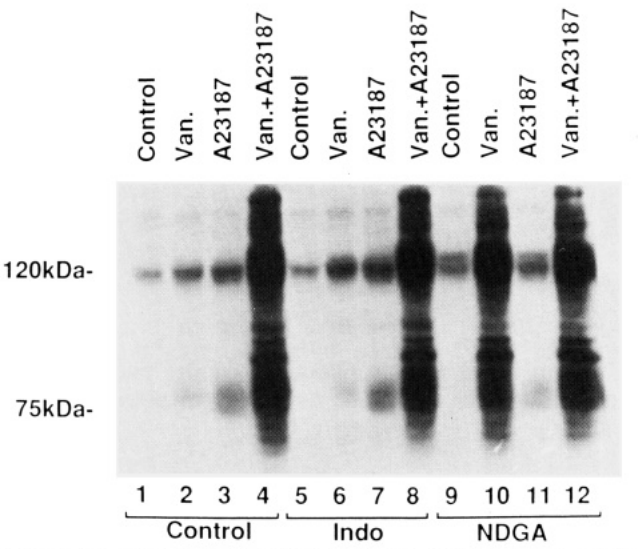

FIGURE 8: Effects of indomethacin and NDGA on tyrosine phosphorylation in WB cells. (Lanes 1–4) WB cells were treated with A23187 or vanadate as in Figure 7. (Lanes 5–12) WB cells were pretreated with 30 μM indomethacin or NDGA for 10 min prior to the addition of vanadate or A23187 for 10 additional min. Incubations were terminated, and cellular proteins were analyzed by anti-phosphotyrosine immunoblotting as described under Experimental Procedures.

In considering potential mechanisms of NDGA action, we noted that NDGA contains catechol (1,2-dihydroxyphenyl) residues and thus resembles the flavonoid antioxidant quercetin, a compound shown by other investigators to stimulate tyrosine phosphorylation synergistically with vanadate in chicken embryo fibroblasts (Van Wart-Hood et al., 1989). To investigate the possible importance of the catechol moiety in the synergy with vanadate, a structure–function study was performed using a series of flavonoids. In WB cells, NDGA, quercetin, and fisetin (30 μM), all of which contain catechol residues, each were synergistic with vanadate, whereas the analogs with 1,3-diols (apigenin and morin) were relatively inactive at the same concentrations. Moreover, none of the flavonoids exhibited substantial activity in GN4 cells, suggesting that similar factors may dictate the resistance of these cells to A23187/vanadate and to NDGA/vanadate.

As discussed below, the sensitivity of WB cells to A23187/vanadate or NDGA/vanadate might stem from a relatively lower capacity to reduce intracellular vanadate. To test this possibility, WB cells were treated overnight with the glutathione-repleting agent *N*-acetylcysteine (Issels et al., 1988). *N*-Acetylcysteine-treated cells became less responsive to sub-

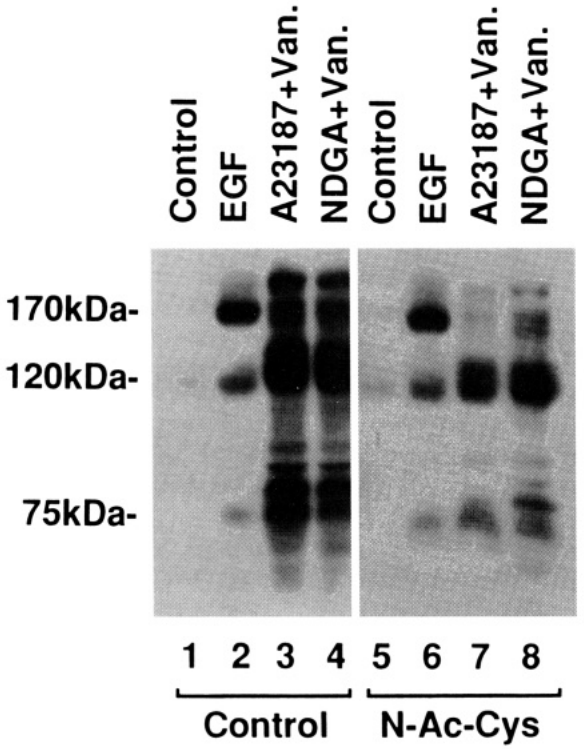

FIGURE 9: Effects of *N*-acetylcysteine on tyrosine phosphorylation. WB cells were pretreated for 18 h with or without 0.5 mM *N*-acetylcysteine and then were treated with EGF (300 ng/mL, 1 min), 10 μM A23187 plus 200 μM vanadate (10 min), or 30 μM NDGA plus vanadate (10 min). Incubations were terminated, and cellular proteins were analyzed by anti-phosphotyrosine immunoblotting as described under Experimental Procedures.

sequent treatment with A23187 or NDGA plus vanadate, while the response to EGF was unaffected (Figure 9).

DISCUSSION

The ability of intracellular Ca^{2+} to elevate protein–tyrosine phosphorylation in intact cells now has been demonstrated in several laboratories (Bading & Greenberg, 1991; Gomez-Cambronero et al., 1991; Gusovsky et al., 1993; Huang et al., 1990; Huckle et al., 1992, 1990; Offermanns et al., 1993; Takayama et al., 1991; Tsuda et al., 1991; Vostal et al., 1991; Yu et al., 1991). Previous studies from our laboratory have shown that the Ca^{2+}-mobilizing hormone angiotensin II increases tyrosine phosphorylation in part by activating one or more tyrosine kinases in WB and GN4 rat liver epithelial cells (Huckle et al., 1992). In the present studies, we have characterized a distinct process activated by the combination of A23187, a Ca^{2+} ionophore, and sodium vanadate, a tyrosine phosphatase inhibitor. In some respects, the synergistic activation of tyrosine phosphorylation by A23187 plus vanadate resembles the effects of angiotensin II. For example, both responses are Ca^{2+}-dependent, and both appear to involve activation of tyrosine kinases. While the A23187 plus vanadate response contains the angiotensin II component, an additional component can readily be distinguished: the A23187/vanadate response is greater in magnitude, provokes the appearance of unique phosphoprotein species, and is reversible by extracellular manipulation (e.g., addition of EGTA). Moreover, the pool of tyrosine-phosphorylated proteins recovered from A23187/vanadate-treated cells but not from angiotensin II-treated cells (Huckle et al., 1992) contains associated ser/thr kinase activities as well as tyrosine kinase activities. Finally, the synergistic effect of vanadate with A23187 is not detected in GN4 cells, a WB-derived line that is 2–3-fold more responsive to the Ca^{2+}-dependent effects of angiotensin II.

Although it is apparent that activation of WB cell tyrosine kinases occurs in response to A23187/vanadate treatment, events more proximal to the elevation of intracellular Ca^{2+} are less clear. One possibility is that the elevation of Ca^{2+} by A23187, in addition to activating Ca^{2+}-sensitive tyrosine kinases, potentiates the PTPase-inhibiting effects of vanadate [V(V)]. Vanadate is postulated to inhibit PTPases (in addition to other phosphoryl transferases that form covalent phosphoenzyme intermediates) by serving as an analog of the phosphate ester undergoing hydrolysis (Gresser & Tracey, 1990). Vanadate is believed to enter cells *via* plasma membrane anion transporters (Nechay et al., 1986) and rapidly be reduced to V(IV) by agents, principally glutathione, that govern the intracellular redox environment (Macara et al., 1980). One might predict, then, that diminishing the ability of cells to reduce vanadate would potentiate its PTPase-inhibiting activity.

In addition, the status of the intracellular reducing environment is directly relevant to the regulation of PTPases as a class. At least 25 cDNAs encoding distinct PTPases have been found (Charbonneau & Tonks, 1992). These proteins contain several regions of high sequence conservation, including a domain surrounding an essential cysteine residue that forms a thiophosphate intermediate during catalysis (Guan & Dixon, 1991). Studies of isolated PTPases have indicated that these enzymes are sensitive to sulfhydryl oxidation, resulting in the loss of enzymatic activity (Stover et al., 1991). This general feature of PTPases, then, suggests that impairment of cellular reducing pathways could promote the direct inactivation of PTPases by sulfhydryl oxidation as well as allow the intracellular accumulation of an exogenous PTPase inhibitor (vanadate). Indeed, it seems reasonable to postulate that the ability of oxidizing agents such as hydrogen peroxide and menadione to stimulate tyrosine phosphorylation and tyrosine kinase activation synergistically with vanadate (Kadota et al., 1987; Heffetz et al., 1990; Trudel et al., 1991) involves altering redox states, since peroxides are well-known to deplete cellular reduced glutathione pools (Bellomo & Orrenius, 1985). It should also be noted that vanadate itself has been linked to the catalytic generation of reactive oxygen intermediates in biological systems [reviewed in Liochev and Fridovich (1990)]. If, as has been reported in isolated hepatocytes (Olafsdottr et al., 1988), A23187 also decreases reduced glutathione levels in WB cells, then the ionophore's effects with vanadate may be mediated by a loss of reducing potential. The ability of the glutathione-repleting agent *N*-acetylcysteine to attenuate the increase in tyrosine phosphorylation stimulated by A23187/vanadate or NDGA/vanadate in WB cells supports this postulate.

Striking among our findings is the observation that the phenolic antioxidants NDGA and quercetin could effectively substitute for A23187 in combination with vanadate, producing similar synergistic increases in tyrosine phosphorylation of proteins with the same apparent molecular weights, susceptibility to inhibition by *N*-acetylcysteine, and cell-type selectivities (WB vs GN4 cells). Quercetin plus vanadate has been shown to synergistically increase tyrosine phosphorylation in chicken embryo fibroblasts (Van Wart-Hood et al., 1989), and we have found similar responses to NDGA plus vanadate in a human epithelial cell line, HEC18 (unpublished data). Thus, these findings are not confined to rat liver cells. These phenolic agents have not been shown to elevate intracellular Ca^{2+}. It is more likely that, if A23187/vanadate and NDGA/

vanadate have overlapping mechanisms of promoting tyrosine phosphorylation, the points in common lie beyond the elevation of Ca^{2+}.

The ability of oxidants (H_2O_2) and antioxidants (NDGA) to produce similar synergistic effects on tyrosine phosphorylation in combination with vanadate seems paradoxical. However, the finding that flavonoids with 1,2-diols, but not 1,3-diols, synergistically activate tyrosine phosphorylation with vanadate in WB cells suggests several possible explanations. Catechols are known to form chelation complexes with vanadate (Ferguson & Kustin, 1979), and the formation of such a complex is thought to underlie the ability of catecholamines to reverse Na^+–K^+–ATPase inhibition by vanadate (Cantley et al., 1978). In addition, vanadate and phenolic compounds have been shown to spontaneously form phenylvanadate esters under physiologic conditions (Tracey & Gresser, 1986). Thus, in the present case, a catechol/vanadate adduct might increase tyrosine phosphorylation by exerting unique effects *as a complex* on tyrosine kinases or PTPases. In preliminary tests, we have found that NDGA does not increase the potency of vanadate ($IC_{50} \sim 1\ \mu M$) as an inhibitor of total PTPase activity measured in WB cell lysates (unpublished data). Alternatively, a catechol–vanadate complex might effectively sequester vanadate, thereby preventing or delaying its intracellular metabolism. However, catechols are themselves capable of reducing vanadate in a cell-free system (Ferguson & Kustin, 1979); their actions in intact cells are unknown. It remains possible that catechols synergize with vanadate in a manner independent of complex formation, perhaps as inhibitors of or as alternate substrates for enzymes involved in vanadate metabolism.

We anticipate that the cell-type selectivity of the A23187/vanadate and catechol/vanadate tyrosine phosphorylation responses will facilitate testing of the hypotheses described above. In particular, if A23187 potentiates vanadate's effects on tyrosine phosphorylation by altering cellular redox status, then we would predict that these compounds might be more potent as glutathione depletors in WB cells than in GN4 cells. However, there remains the strong possibility that WB and GN4 cells differ in their capacities to respond to an ultimate phosphorylation-activating species, rather than in their abilities to generate such a species. For instance, GN4 cells might express relatively low levels of tyrosine kinases involved in a response to A23187/vanadate or might contain a relative abundance of PTPase isoforms that effectively confer resistance to A23187/vanadate. It has recently been reported that hydrogen peroxide induces the expression of a PTPase in human fibroblasts (Keyse & Emslie, 1992), which could be viewed as an adaptive response to a PTPase-inhibiting challenge. We believe that further study of these pathways will provide new insight into the regulation of tyrosine kinases and PTPases, and may be of particular relevance to the study of agents such as tumor necrosis factor α (Ishii et al., 1992) and transforming growth factor β (Shibanuma et al., 1991), whose signaling pathways are postulated to involve reactive oxygen species. Indeed, the ability of *N*-acetylcysteine to block transcription factor NF-κB activation by a variety of agents, including A23187 (Schreck et al., 1991), has suggested that regulation of intracellular redox state may be a common feature of numerous signaling mechanisms.

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