Vanadate Stimulates Oxygen Consumption and Tyrosine Phosphorylation in Electropermeabilized Human Neutrophils*

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To determine the role of protein phosphorylation in neutrophil activation, electropermeabilized cells were treated with vanadate, a phosphatase inhibitor. Micromolar concentrations of vanadate elicited a NADPH-dependent burst of oxygen utilization in permeabilized, but not in intact cells, indicating an intracellular site of action. Stimulation of oxygen consumption by vanadate was reversible, concentration dependent and required the presence of ATP and Mg++. Generation of a respiratory burst by vanadate was associated with accumulation of phosphorylated proteins. Such accumulation was due, at least in part, to inhibition of phosphoprotein phosphatase activity, as indicated by pulse-chase experiments. No evidence for stimulation of protein kinases by vanadate was found. Phosphoamino acid analysis revealed that a large fraction of the vanadate-induced phosphorylation occurred on tyrosine residues. The pronounced accumulation of tyrosine-phosphorylated proteins was confirmed by immunoblotting with anti-phosphotyrosine antibodies. The data suggest that neutrophils possess one or more constitutively active tyrosine kinases and that phosphoprotein accumulation is normally prevented by vigorous concomitant phosphatase activity. Inhibition of the latter by vanadate leads to phosphoprotein accumulation and is accompanied by stimulation of oxygen consumption.

When stimulated, neutrophils undergo a large increase in their rate of oxygen consumption, known as the respiratory burst. Most of the oxygen consumed is converted to superoxide anions by the NADPH oxidase, a membrane-bound enzymatic complex. Superoxide can in turn generate a family of reduced oxygen metabolites, many of which are potent bactericidal agents (see Refs. 1 and 2 for reviews). The mechanism whereby the oxidase is activated when neutrophils are exposed to microorganism or their products is not completely understood. It is clear, however, that the respiratory burst is accompanied and perhaps preceded by marked changes in protein phosphorylation (2, 3). This association has led to the proposal that stimulation of protein kinases mediates the activation of the oxidase. In support of this notion is the finding that marked superoxide generation can be triggered by agents such as phorbol esters or diacylglycerol, whose main and perhaps sole target is protein kinase C (4). Circumstantial evidence for the involvement of protein kinases was also provided by ATP depletion experiments (5, 6). The respiratory burst was precluded in cells treated with metabolic inhibitors, although it is unclear whether inhibition was due to depletion of ATP or of NADPH, the substrate of the oxidase.

Recently, electrically permeabilized cells were used to more directly analyze the role of phosphorylation reactions in the activation of the NADPH oxidase (7). In such cells the presence of exogenously added ATP was found to be essential for receptor-mediated activation of the respiratory burst. The nucleotide was required for phosphotransferase reactions, inasmuch as the non-hydrolyzable analog, AMP-PNP was ineffective. As in intact cells, displacement of the stimulus from its receptor on the surface of permeabilized cells resulted in rapid termination of the respiratory burst. If the response is initiated and/or sustained by phosphorylation reactions, the rapid reversal observed would be suggestive of an active dephosphorylation process. Experiments using ATPγS are consistent with this conclusion. Like ATP, ATPγS serves as the substrate for several protein and lipid kinases. However, the resulting thio phosphorylated product is generally less susceptible to hydrolysis by cellular phosphatases (8, 9). For this reason, removal of the stimulus in cells stimulated in the presence of ATPγS was not followed by immediate termination of the burst (10).

In addition to supporting the oxidative response to exogenous stimuli, ATPγS (≥50 μM) was also found to induce a spontaneous activation in otherwise unstimulated permeabilized cells (10). This observation is suggestive that kinases are active in neutrophils even in the absence of stimuli, but that accumulation of phosphorylated intermediates is normally prevented by ongoing phosphatase activity.1 In cells exposed

1 The ATP requirement has also been studied in a cell-free reconstitution system (32-34). Although some disagreement exists (cf. Ref. 32 with 33 and 34), most groups have reported that, in this system, addition of exogenous ATP is not essential to elicit superoxide generation by treatment with sodium dodecyl sulfate or arachidonic acid.

2 The abbreviations used are: AMP-PNP, 5'-adenyl-imido diphosphate; EDTA, [ethylenediamine (cycloexylenediethyl)tetraacetic acid; TPA, 12-O-tetradecanoylphorbol 13-acetate; CDTA, trans-1,2-diaza-cyclohexane-N,N,N',N'-tetraacetic acid; GDPβS, guanosine 5'-(β-thio)diphosphate; GDPγS, guanosine 5'-(γ-thio)triphosphate; ATPγS, adenosine 5'-(γ-thio)triphosphate; FMLP, formyl-methionyl-leucyl-phenylalanine; BocPLPLP, N-t-butoxycarbonyl-phenylalanyl-D-ileucyl-D-phenylalanyl-L-leucyl-L-phenylalanine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

3 Stimulation by GDPβS, generated by a nucleotide diphosphokinase from GDP and ATPγS, cannot be strictly ruled out. However, several observations, summarized in Ref. 10 make this possibility unlikely.

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to ATPγS, dephosphorylation is prevented or at least retarded, with consequent accumulation of phosphorylated (active) proteins. If the extent of cellular activation is indeed proportional to the accumulation of specific phosphoproteins, it follows that stimulation could be obtained not only by activation of kinase activity, but also by inhibition of phosphatase activity. This model predicts that, using ATP as the substrate, inhibition of the relevant phosphatases should induce a spontaneous respiratory burst, in the absence of exogenous stimulation. This prediction was tested experimentally using vanadate, a potent inhibitor of phosphorytrosine-specific phosphatases (11, 12).

**EXPERIMENTAL PROCEDURES**

**Materials**—Ficoll 400 and dextran T-500 were purchased from Pharmacia LKB Biotechnology Inc. Sodium orthovanadate was from Aldrich. Sodium salicylate was obtained from Fisher. Molecular weight standards, phenylmethysulfonyl fluoride, medium RPMI 1640, AMP-PNP, ATP, EGTA, CDTA, GDP, GTP, GDPβS, GTPγS, ATPγS, IMLP, BocPLPLP, TPA, NADPH, 2-deoxy-D-glucose, Nonidet P-40, Ponceau S stain, phosphoserine, phosphothreonine, phosphotyrosine, ninhydrin, and Coomassie Blue were from Sigma. Perforamid grade cellulose was from List. Albumin was from Calbiochem. 125I-labeled protein A, [γ-32P]ATP, and the monoclonal anti-phosphotyrosine antibody PY20 IgC2B were obtained from ICN. [35S]ATPβS was from Amersham Corp. The polyclonal phosphorytrosine-specific antibody was the kind gift of Drs. M. P. Kamps and B. M. Sefton.

**Solutions**—Bicarbonate-free medium RPMI 1640 was buffered to pH 7.3 with 25 mM NaHepes. Permeabilization medium contained (in mM): 140 KCl, 1 MgCl₂, 1 EGTA, 10 KHepes (pH 7.0), and sufficient CaCl₂ to give a final free Ca²⁺ concentration of 100 nM, calculated as described in Ref. 13. Where indicated, Ca⁴⁺ or Mg⁴⁺ were omitted. Except for experiments in ATP-depleted cells, the permeabilization medium contained 100 μM ATP and 10 mM glucose. Unless otherwise specified, 100 μM GTP and 2 mM NADPH were added to the permeabilization medium immediately before the O₂ consumption or phosphorylation assays. Na⁺ solution contained (in mM): 140 NaCl, 5 KCl, 10 glucose, 1 CaCl₂, 1 MgCl₂, 10 NaHepes (pH 7.3). Glucose was omitted from this medium for ATP depletion experiments. All media were adjusted to 290 ± 5 mOsM with the major salt.

**Cell Isolation and Permeabilization**—Neutrophils were isolated from fresh heparinized human blood by dextran sedimentation followed by Ficoll Hypaque gradient centrifugation (14). Contaminating red cells were removed by NH₄Cl lysis. Cells were washed, counted using a Model 1C Cell Counter and resuspended at 10⁷ cells/ml in Hepes-buffered RPMI.

Where indicated, the cells were ATP-depleted prior to permeabilization by incubation for 5 min at 37°C in a medium containing (in mM): 140 NaCl, 5 KCl, 10 Hepes, 1 MgCl₂, 1 CaCl₂ and 5 2-deoxy-D-glucose. For electroporation, intact or ATP-depleted cells were sediemented and resuspended in ice-cold permeabilization solution at 10⁷ cells/ml. Aliquots (0.8 ml) of this suspension were then transferred to a Bio-Rad Pulsed cuvette and permeabilized using two discharges as described earlier (10). The cells were sedimented using an Eppendorf 5415 microcentrifuge and resuspended in fresh, ice-cold permeabilization solution between pulses. The electroporated cells were either used immediately or transferred to a plastic tube and stored on ice for up to 15 min.

**Oxygen Consumption**—O₂ consumption was measured polarographically with a model 53 or a model 5300 biological oxygen monitor (Yellow Springs Instrument Co.), as described (7) using a cell density of 2 × 10⁶/ml. O₂ consumption was calculated using a solubility coefficient of 0.024 ml O₂/ml at 37°C, the temperature used for all experiments.

**Phosphoprotein Analysis**—For 32P labeling, 4 × 10⁶ cells were permeabilized as described above and then suspended in 1 ml of modified permeabilization medium containing only 100 μM ATP at 37°C. After 1.5 min, 25–30 μCi of [γ-32P]ATP was added and 0.5 min later the cells were treated with or without the indicated concentration of vanadate for 5 min at 37°C. Phenylmethysulfonyl fluoride (100 μM) was then added and the cells were sedimented, resuspended in 300 μl of boiling 2% SDS sample buffer containing mercaptoethanol and immediately boiled for 5 min. Polycrylamide gel electrophoresis of the samples and of molecular weight standards was performed in the presence of SDS on 10% gels by the method of Laemmli (15), using the Bio-Rad MiniProtein II system. The gels were then stained using Coomassie Blue, dried overnight at room temperature using Bio-Gel Wrap (BioDesign Inc.), and used for autoradiography with intensifying screens at -70°C.

For 35S labeling, 4 × 10⁶ ATP-depleted and permeabilized cells were suspended in 1 ml of permeabilization medium without ATP. The cells were then incubated at 37°C with 30 μCi of [35S]ATPβS for 5 min in the presence or absence of 10 μM vanadate. The reaction was then stopped and the peptide composition analyzed by gel electrophoresis as above. The gels were equilibrated for 30 min with 1 mM sodium salicylate before drying for fluorography.

**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was performed by a method similar to that of Cooper et al. (16). Cells were permeabilized and labeled with [γ-32P]ATP as described above. The samples were then subjected to gel electrophoresis and the phosphoproteins transferred onto Immobilon (Millipore). The blotted samples were stained with Ponceau S (0.2% in 3% trichloroacetic acid) and individual lanes were identified and marked with a pencil. The sections of the Immobilon containing the stained phosphoproteins were cut into small pieces and transferred to a microcentrifuge tube. After washing the Immobilon with methanol and Tris-buffered saline (150 mM NaCl and 50 mM Tris, pH 7.4), some of the samples were subjected to alkaline hydrolysis by incubation in 1 N KOH for 2 h at 55°C. The KOH solution was then aspirated and the Immobilon membranes neutralized by washing briefly with Tris-buffered saline, incubating for 5 min in 1 mM Tris chloride, pH 7.0, and finally washing with water. The proteins were then hydrolyzed under nitrogen in 0.5 ml of 5.7 N HCl for 1 h at 110°C. The hydrolyzed samples were dried using a Speed-Vac concentrator (Savant) and then resuspended in 5 μl of a solution composed of 100:10:1890 (v/v/o) acetic acid:pyridine:water containing phosphoserine, phosphothreonine, and phosphotyrosine (0.3 mg/ml each). The resuspended samples were spotted on thin layer cellulose plates (EM Chemicals) and subjected to electrophoresis at 1 kV for 75 min with water cooling. The plates were then dried, sprayed with ninhydrin to localize the phosphoamino acid standards and used for autoradiography. In some experiments the individual phosphoamino acid spots were scraped off the plate and counted by liquid scintillation.

**Immunoblotting with Anti-phosphotyrosine Antibodies**—Where indicated, cells were ATP-depleted using 2-deoxy-D-glucose as above. The cells were then electrophoresed and resuspended in permeabilization medium with or without the indicated concentration of ATP. After 2 min at 37°C, vanadate was added where indicated and the suspension was incubated for a further 5 min. Phenylmethysulfonyl fluoride was added, the samples were sedimented and the pellets solubilized in Laemml sample buffer as described above. Following electrophoresis on 10% polyacrylamide gels, the samples, together with pre-stained molecular weight markers (Bio-Rad), were blotted onto nitrocellulose using the Bio-Rad Mini Trans-Blot system for 1 h at 100 V. The nitrocellulose blot was then stained with Ponceau S for 5 min and subsequently destained in distilled water. For blotting with alkaline phosphatase, the blot was incubated overnight at 4°C in a blocking solution containing 5% fatty acid-, nuclease-, and protease-free albumin and 0.04% Nonidet P-40 in Tris-buffered saline. The blot was then incubated with 10 ml of blocking solution containing 2 μg of the polyclonal antibody or 6 μg of the monoclonal antibody for 2 h while shaking at room temperature. The blot was next washed three times with blotting solution and incubated with goat anti-rabbit or goat anti-mouse antibodies covalently linked to alkaline phosphatase (Bio-Rad), for the polyclonal and monclonal antibodies, respectively. Staining was then performed as recommended by the kit manufacturer (Bio-Rad).

For labeling with 3H-labeled protein A, the blots were incubated with the polyclonal primary antibody and then washed as described above. Two μCi of 3H-labeled protein A in blocking solution was added next, followed by incubation for 1 h at room temperature. The blots were then washed three times and dried. Autoradiography was performed as above.

All experiments were performed at least three times with blood from different donors and are presented as representative traces or autoradiograms or as the mean ± S.E. of the indicated number of determinations (n).
RESULTS

Effect of Vanadate on Oxygen Consumption—The effect of vanadate on oxygen consumption in intact and permeabilized human neutrophils is illustrated in Fig. 1. Vanadate, at concentrations as high as 1 mM, did not significantly affect the rate of oxygen utilization by intact cells. The viability and responsiveness of these cells was ascertained by subsequent stimulation with TPA, a well-established activator (2, 4). This result is consistent with earlier findings in murine macrophages, where vanadate was similarly unable to alter the activity of the NADPH oxidase (17). In contrast to the intact cells, electroporabilized neutrophils responded to the addition of 10 μM vanadate with a pronounced increase in the rate of oxygen consumption. This respiratory burst is apparently due to activation of the oxidase, inasmuch as the absence of exogenously added NADPH eliminated the response (Fig. 1B). Readdition of NADPH following vanadate restored oxygen consumption after a delay of 2–4 min. The delayed response to NADPH possibly reflects the time required for the nucleotide to attain a critical concentration in the cytoplasm.

Two other pieces of evidence confirmed that oxygen consumption was due, at least in part, to activation of the NADPH oxidase: first, the oxygen consumption burst was substantially inhibited by addition of 5 μg/ml superoxide dismutase. Second, vanadate stimulated superoxide production, as measured by the superoxide dismutase-catalyzed reduction of cytochrome c (not illustrated). Together, these findings suggest that vanadate acts at an intracellular site to stimulate the NADPH oxidase.

A detailed analysis of the concentration dependence of the effect of vanadate on oxygen consumption is shown in Fig. 2. At low concentrations, a substantial lag preceded the appearance of the respiratory burst. The lag time was inversely proportional to the concentration of vanadate. Conversely, the magnitude of the response varied in direct proportion to the concentration of the activator. Notice that the concentration dependence appears to be biphasic: an initial component is apparent at doses ≤25 μM and a second, larger component which saturates at near millimolar concentrations. It is important to note that, in the absence of cells, vanadate induced a reproducible, NADPH-dependent decrease in the oxygen content of the medium (inset, Fig. 2A). The mechanism underlying this phenomenon is not clear, but it may represent catalysis of the non-enzymatic oxidation of NADPH (18, 19). In any event, this “spontaneous” effect was considerably smaller than the burst displayed by the cells and was readily subtracted from the total oxygen consumption rate (Fig. 2B).

The induction of a respiratory burst by vanadate was found to be reversible (Fig. 3). Unlike the relatively sustained response observed in the continued presence of vanadate (Figs. 1–3), a return to the basal rate of oxygen consumption was recorded within minutes of removal of extracellular vanadate (Fig. 3), despite the continued presence of NADPH. Diffusional exit of intracellular vanadate through the pores on the cell membrane is likely to account for at least part of the time needed for reversal. Re-addition of vanadate following reversal of the initial response resulted in a second stimulation of oxygen utilization. For reasons that are not apparent, the latter response was consistently smaller than the original one, even when comparable or larger concentrations of vanadate were used.

It has been reported earlier that the oxidative response of permeabilized cells to fMLP (10), TPA, or to exogenous diacylglycerol4 is strictly dependent on the availability of ATP. If, as postulated in the introduction, the effect of vanadate is attributable to accumulation of phosphoproteins due to inhibition of phosphatases, a similar ATP dependence would be expected. To test the ATP dependence of the vanadate-induced burst, intact cells were ATP-depleted with 2-deoxy-D-glucose and then permeabilized and suspended in

4 W. Furuya, unpublished observations.
medium devoid of ATP. Typical results are illustrated in Fig. 4A. In contrast to the stimulation obtained in ATP containing media, omission of ATP greatly inhibited the respiratory burst. In four experiments using 10 $\mu$M vanadate the maximal rate of oxygen utilization was 11.8 $\pm$ 1.6 nmol of O$_2$/10$^6$ cells/min in the presence of 1 mM ATP and 0.54 $\pm$ 0.01 nmol of O$_2$/10$^6$ cells/min in its absence, a 95.4% inhibition. In cells depleted of ATP and treated with vanadate, reintroduction of the nucleotide restored the oxidative response (Fig. 4A), indicating that the depletion procedure did not result in irreversible cell damage.

As illustrated in Fig. 4B, the response to vanadate was not supported by AMP-PNP, a non-hydrolyzable analog of ATP. This observation implies that hydrolysis of the $\gamma$-phosphate of the nucleotide is necessary for vanadate to generate the respiratory burst, consistent with the involvement of phosphorylation/dephosphorylation reactions in the control of the NADPH oxidase. Because the ATP-Mg complex is the substrate utilized by most kinases, the requirement for the divalent cation was also analyzed. As shown in Fig. 4C, suspension of permeabilized cells in medium devoid of Mg$^{2+}$ and containing 100 $\mu$M CDTA, a Mg$^{2+}$ chelator, resulted in obliteration of the response to vanadate. In 3 experiments, the rate of O$_2$ consumption recorded in the absence of Mg$^{2+}$ was only 3.6% of the control rate. As was the case with ATP, reintroduction of Mg$^{2+}$ at concentrations in excess over CDTA restored the oxidative burst, ruling out non-specific inhibition due to irreversible cell damage. In contrast to the dependence on Mg$^{2+}$, omission of Ca$^{2+}$ in the continued presence of 1 mM EGTA had little effect on the burst elicited by vanadate (Fig. 4D). This observation differs from the Ca$^{2+}$ dependence recorded using fMLP as the stimulus (7), indicating that, if the tripeptide and vanadate act via the same pathway, the Ca$^{2+}$-sensitive step must precede the site of action of vanadate.

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**Neutrophil Activation by Vanadate**

**FIG. 3. Reversibility of the stimulation of oxygen consumption by vanadate.** Left trace, permeabilized but otherwise untreated cells stimulated with 10 $\mu$M vanadate where indicated, as in Fig. 1B. Right trace, permeabilized cells were preincubated in permeabilization medium containing 100 $\mu$M GTP, 1 mM ATP, and 2 mM NADPH, and 10 $\mu$M vanadate for 5 min at 37 °C. The cells were then sedimented and resuspended for measurement of oxygen utilization in permeabilization medium with 100 $\mu$M ATP, 2 mM NADPH but devoid of vanadate. The trace starts immediately after resuspension of the cells in the vanadate-free medium. Where indicated, 50 $\mu$M vanadate was added. The traces are representative of at least three similar experiments.

**FIG. 4. Adenine nucleotide and divalent cation dependence of the stimulation of oxygen consumption by vanadate.** A, intact neutrophils were depleted of ATP by incubation for 5 min in glucose-free Na$^+$ solution containing 5 mM 2-deoxy-d-glucose, followed by electroporation in glucose and ATP-free permeabilization medium. The cells were then suspended in medium containing 100 $\mu$M GTP and 2 mM NADPH, with (bottom trace) or without (top trace) 1 mM ATP. Where indicated, 10 $\mu$M vanadate was added to both samples and later 1 mM ATP to the top trace only. B, cells were ATP-depleted as in A and suspended in permeabilization medium with either 0.5 mM AMP-PNP or 1 mM ATP, as indicated. Vanadate (10 $\mu$M) was added to both samples at the arrow. The $K_a$ for ATP was 0.07 mM (W. Furuya, unpublished observations), so that comparable results are obtained in the 0.25-1 mM ATP range. C, electrically perforated cells were suspended in permeabilization solution containing 100 $\mu$M GTP, 2 mM NADPH, 1 mM ATP with (bottom trace) or without (top trace) 1 mM MgCl$_2$. Where indicated, 100 $\mu$M CDTA, 10 $\mu$M vanadate, and 1 mM MgCl$_2$ were added to the top trace. Only vanadate (10 $\mu$M) was added to the bottom control trace. D, cells suspended in permeabilization medium containing 100 $\mu$M GTP, 2 mM NADPH, 1 mM ATP, and 1 mM MgCl$_2$. The free calcium concentration was 100 nM (top trace) or <10 nM (nominally calcium free plus 1 mM EGTA; bottom trace). The traces are representative of at least three experiments.

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$^6$A relatively low concentration (100 $\mu$M) of CDTA was used to chelate trace amounts of Mg$^{2+}$ present in the medium or inside the cells to alter minimally the free Ca$^{2+}$ concentration, which was buffered using 1 mM EGTA (see "Experimental Procedures").
or GDP/$S$ prior to treatment with vanadate in fact inhibited the respiratory burst, contrary to what would be anticipated for the formation of a GDP-vanadate complex (not illustrated). The reason for this inhibition is presently not clear.

In some systems, the biological responses elicited by vanadate are obliterated when the cells are pretreated with pertussis toxin (21), indicating the involvement of $G$ proteins. Because a pertussis toxin-sensitive $G$ protein is known to mediate the activation of the NADPH oxidase by physiologic stimuli, we determined the effect of this toxin on the vanadate-induced response. Intact cells were preincubated for 2 h in the presence or absence of 500 ng/ml of pertussis toxin, followed by electroporation and challenge with 10 $\mu$M vanadate. Pretreatment with the toxin did not modify the rate of oxygen utilization induced by vanadate. Parallel measurements demonstrated that the treatment with pertussis toxin led to inactivation of $G$ proteins presumably through ADP-ribosylation. This was indicated by the pronounced inhibition of the respiratory burst elicited by fMLP in cells of the same batch. In three experiments, the response to the chemotactic stimulus was only 16.9 ± 1.3% of the control response, while the sample from the bottom trace was subsequently treated with 100 $\mu$M GDP/$S$ and finally with $10^{-4}$ M TPA. $B$, samples prepared as in $A$ were stimulated with 10 $\mu$M vanadate. Where indicated, the top sample only was treated with 100 $\mu$M GDP/$S$. Traces are representative of three or more similar experiments.

Further evidence against this mechanism was obtained comparing the effects of fluoroaluminate to those of vanadate. When added to permeabilized cells, fluoroaluminate was also found to activate oxygen consumption (Fig. 5A). As expected for an effect on $G$ proteins, the stimulation was reversed when GDP/$S$ was added to the medium.$^2$ Similar results were obtained with GDP (not illustrated). Reversal of the response does not result from a nonspecific effect of the nucleotide, since the respiratory burst could be reinstituted by addition of phorbol ester (Fig. 5A), which is thought to act at an intermediate stage between the $G$ protein and the oxidase (1, 2). When a stimulation of comparable magnitude was induced by vanadate, the subsequent addition of GDP/$S$ or GDP had little effect on the rate of oxygen utilization (Fig. 5B), suggesting that the effects of vanadate and fluoroaluminate occur by different mechanisms.

**Effects of Vanadate on Phosphoprotein Accumulation.—** Vanadate is known to be a potent inhibitor of some types of phosphoprotein phosphatases (11, 12). If this mechanism underlies the stimulation of oxygen consumption, a concomitant accumulation of phosphoproteins would be anticipated. To test this prediction, permeabilized neutrophils were exposed to $\gamma$-$^{32}$P$|$ATP in the presence and absence of vanadate for the length of time required for development of the respiratory burst. Results of one such experiment are shown in Fig. 6A. The presence of vanadate induced a marked accumulation of phosphoproteins. The increase was apparent throughout the range of molecular mass resolved by 10% polyacrylamide gels, but was particularly noticeable in the 40–100-kDa range. Similar results were obtained in five experiments.

Although some serine/threonine phosphatases are blocked at high concentrations of vanadate, lower concentrations of this agent preferentially block phosphotyrosine phosphatases (11, 12). On the other hand, phosphotyrosine usually represents only a very small ($\leq$1%) fraction of the total phosphoamino acid content of most cells. Such a small fraction would be hardly detectable when total phosphoproteins are analyzed, as in Fig. 6A. To elucidate the nature of the residues phosphorylated in the presence of vanadate, the proteins were hydrolyzed and phosphoamino acid analysis was performed. To preserve phosphotyrosine, which is relatively acid-labile (15), the protein hydrolysis procedure was comparatively mild (1 h at 110 °C), yielding incomplete degradation of the phosphoproteins, evidenced by the presence of low mobility oligopeptides near the origin (Fig. 6B). In permeabilized but otherwise untreated cells, the primary phosphoamino acid detected was phosphoserine (Fig. 6B). Small amounts of phosphothreonine were sometimes observed, but phosphotyrosine was undetectable. In contrast, substantial amounts of phosphothreonine were apparent after exposure to vanadate. In addition, the amounts of phosphoserine and phosphothreonine were also elevated. It is important to point out that, under the conditions used, the content of phosphotyrosine is underestimated, due to partial loss during acid proteolysis (16). The appearance of phosphotyrosine was even more evident when protein hydrolysis was preceded by treatment with alkaline hydrolysis. Individual lanes were then cut and hydrolyzed in 6N HCl (17). The appearance of phosphotyrosine was even more evident when protein hydrolysis was preceded by treatment with high voltage electrophoresis. A typical autoradiogram is illustrated. $B$, samples were phosphorylated in the presence or absence of vanadate as described for $A$. Following electrophoresis the samples were blotted onto Immobilon and, where indicated, subjected to alkaline hydrolysis. Individual lanes were then cut and hydrolyzed in acid. The resulting phosphoamino acids were collected and analyzed by high voltage electrophoresis. The position of individual phosphoamino acids was determined using standards, detected with ninhydrin. $PS$, phosphoserine; $PT$, phosphothreonine; $PY$, phosphotyrosine. The four samples shown were obtained from the same experiment and were simultaneously subjected to gel and high voltage electrophoresis. Representative of three experiments.

[Diagram and figure captions are included as text for better readability.]
alkali, a procedure known to preferentially hydrolyze phosphoserine and phosphothreonine, while affecting phosphotyrosine only marginally (16). As shown in Fig. 6B, little phosphorylation was visible in alkali pretreated samples obtained from unstimulated cells. By comparison, a substantial amount of phosphotyrosine was present in alkali pretreated samples obtained from vanadate-treated cells, while phosphoserine and phosphothreonine were barely detectable.

The above observations suggest that, in permeabilized neutrophils, vanadate induces accumulation of tyrosine-phosphorylated proteins, likely by inhibition of phosphotyrosine phosphatase activity. This conclusion was confirmed by immunological means, using antibodies raised against phosphotyrosine. The specificity of the antibodies is demonstrated in Fig. 7A. Samples containing tyrosine-phosphorylated polypeptides were analyzed by gel electrophoresis and blotted onto nitrocellulose. The blots were then equilibrated with the antibody in the presence or absence of 2 mM of phosphoserine, phosphothreonine, or phosphotyrosine. As expected, free phosphotyrosine precluded binding of the antibody to phosphotyrosine residues on polypeptides. In contrast, labeling was virtually unaffected by equivalent concentrations of free phosphoserine or phosphothreonine. Having established the specificity of the antibody, we proceeded to assess the effect of vanadate on phosphotyrosine accumulation. Little phosphotyrosine was present in control permeabilized cells, but substantial amounts were evident at vanadate concentrations as low as 1 mM (Fig. 7B). The accumulation of phosphotyrosine increased in proportion to the concentration of vanadate, in the range explored (0–100 mM). Results of three similar experiments, quantified using 125I-labeled protein A, are summarized and discussed below in Fig. 11. Comparable results were obtained using the monoclonal and monocular anti-phosphotyrosine antibodies and detecting binding with either radiolabeled protein A or with alkaline phosphatase coupled to a secondary antibody (see “Experimental Procedures”).

Vanadate did not affect the polypeptide composition of the cells, as revealed by staining of gels with either silver or Coomassie Blue or staining of blots with Ponceau S. Further evidence of the specificity of the antibodies was obtained studying the ATP and Mg2+ dependence of the phosphotyrosine accumulation triggered by vanadate. Fig. 8 demonstrates that addition of exogenous ATP was necessary for optimal phosphorylation; only a small amount of phosphotyrosine was detected in cells treated with vanadate in the nominal absence of ATP. This residual phosphorylation could be attributed to a small amount of ATP remaining in the cells at the time of stimulation, or to the limited ability of GTP (which was present in all samples at 100 mM) to serve as a substrate to the kinase(s). The addition of exogenous ATP increased markedly the formation of tyrosine-phosphorylated proteins. The enhancement was noticeable at concentrations as low as 25 μM, setting an upper limit to the amount of residual endogenous ATP present in permeabilized cells. In the presence of ATP, tyrosine phosphorylation was partially inhibited by the non-hydrolyzable analog AMP-PNP (Fig. 8), presumably through competition at the binding site of the kinase(s). Finally, omission of Mg2+ and addition of the chelator CDTA resulted in pronounced inhibition of phosphotyrosine accumulation (not illustrated). Neither removal of ATP nor chelation of Mg2+ altered the polypeptide composition of the samples.

The preceding observations are consistent with the notion that tyrosine-phosphorylated proteins accumulate in vanadate-treated permeabilized neutrophils as a result of inhibition of phosphotyrosine phosphatase activity. However, net accumulation of phosphotyrosine could equally result from stimulation of tyrosine kinase activity, without modification of phosphatase activity. Two types of experiments were performed to differentiate these two mechanisms. First, pulse-chase experiments were performed to study phosphatase activity in isolation. For these studies, tyrosine phosphorylation of proteins was first induced by exposure of permeabilized cells to 10 μM vanadate in the presence of 1 mM ATP. The nucleotide was then removed from the medium and the rate of disappearance of the accumulated phosphotyrosine was monitored in the presence and absence of vanadate. As shown in Fig. 9A, removal of vanadate and ATP led to the rapid disappearance of tyrosine-phosphorylated polypeptides, indicative of vigorous phosphotyrosine phosphatase activity. Phosphotyrosine was virtually undetectable after 10 min. In contrast, the continued presence of vanadate following removal of ATP markedly prolonged the life of the tyrosine phosphor-

**Fig. 7.** Detection of vanadate induced tyrosine-phosphorylation by immunoblotting. A, electroporated cells were suspended in permeabilization medium containing 100 μM GTP, 1 mM ATP, and 2 mM NADPH for 2 min at 37 °C. The sample was then stimulated for 5 min with 10 μM vanadate. After stopping the reaction, the sample was divided into 4 identical aliquots, which were analyzed by polyacrylamide gel electrophoresis, followed by blotting onto nitrocellulose. Individual lanes were cut and exposed to the primary, anti-phosphotyrosine antibodies in the absence (C) or presence of 2 mM of either phosphoserine (PS), phosphothreonine (PT), or phosphotyrosine (PY). Staining was with alkaline phosphatase. B, samples were permeabilized and treated with the indicated concentration of vanadate (in μM) for 5 min at 37 °C in complete permeabilization medium, as in A. Following electrophoresis and blotting, polypeptides containing phosphotyrosine were detected using the monoclonal anti-phosphotyrosine antibody and 125I-labeled protein A. A typical autoradiogram is shown. Results are representative of three experiments. Similar results were obtained using the monoclonal antibody (not illustrated).

**Fig. 8.** ATP dependence of vanadate-stimulated phosphotyrosine formation. Porated cells were suspended in permeabilization medium containing the indicated concentration (in mM) of ATP or AMP-PNP. Where indicated, the indicated samples were stimulated with 10 μM vanadate for 5 min at 37 °C. Electrophoresis, phosphotyrosine immunoblotting and alkaline phosphatase staining as in Fig. 7.
Fig. 7. A typical autoradiogram of samples developed with 32P-labeled protein A is shown. B, porated cells were prelabeled for 5 min with ATP in the presence or absence of 10 μM vanadate. After incubation for the indicated times (in minutes), the reaction was stopped and immunoblotting was performed as described for Fig. 6A. Following sedimentation, the samples were suspended in medium containing 1 mM nonradioactive ATP (without [γ-32P]ATP), 2 mM NADPH, and 100 μM GTP in the presence or absence of 10 μM vanadate and incubated for the indicated times. The reaction was terminated as described under “Experimental Procedures” and the samples subjected to electrophoresis and autoradiography. A typical radiogram is shown.

The inhibitory effect of vanadate on phosphoprotein phosphatase activity was also discernible measuring total phosphoprotein accumulation using radiolabeled ATP (Fig. 7B). For these experiments the cells were prelabeled with [γ-32P]ATP in the presence of vanadate, followed by removal of the isotope and addition of nonradioactive ATP in the presence or absence of vanadate. As was the case for phosphotyrosine, the total phosphoprotein content decreased rapidly after removal of vanadate, with little labeling remaining after 10 min. The continued presence of vanadate after replacement of [γ-32P]ATP with nonradioactive ATP lengthened the life of the radiolabeled phosphoproteins. The effect of vanadate on total phosphorylation was not as pronounced as that on phosphotyrosine accumulation (cf. Fig. 9, A and B). If vanadate selectively inhibits tyrosine phosphatases, this could result from exchange of 32P for nonradioactive phosphate on serine and threonine residues, conceivably due to ongoing dephosphorylation and rephosphorylation.

Together, the results described above are consistent with inhibition of tyrosine phosphatase activity by vanadate. However, they do not rule out the possibility that protein kinases are stimulated concomitantly. Possible effects of vanadate on protein kinase activity were investigated using ATPγS. This analog of ATP is utilized as substrate by several cellular kinases, but the resulting thio-phosphorylated residues are relatively refractory to hydrolysis by phosphatases (8, 9). Hence, the rate of accumulation of thiophosphoproteins is dictated mainly by the activity of the kinase(s), with little contribution of phosphatases. This strategy was used to assess the effect of vanadate on protein kinases. Electroporated cells were incubated in a medium devoid of ATP but containing [35S]ATPγS, in the presence or absence of 10 μM vanadate. After 5 min, the reaction was stopped and the radiolabel distribution was determined by gel electrophoresis followed by autoradiography. Typical results are illustrated in Fig. 10. Contrary to the results obtained with [γ-32P]ATP, thio-phosphorylation with [35S]ATPγS was not significantly affected by the presence of vanadate. This finding suggests that at least those kinases that utilize ATPγS as their substrate are not stimulated, pointing to inhibition of phosphatase activity as the likely mode of action of vanadate.

Correlation between Oxygen Consumption and Phosphotyrosine Accumulation—Because vanadate stimulates oxygen utilization as well as phosphoprotein accumulation, the possible relationship between these two events was analyzed. Fig. 11A compares the vanadate concentration dependence of the oxygen consumption (maximal rate; open symbols) and phosphotyrosine accumulation (solid symbols). The latter was quantified by immunoblotting and 125I-protein A labeling, as in Fig. 7B. Individual lanes were cut and counted in a γ counter. To enable comparison between independent experiments, the data were normalized to the value obtained at 100 μM vanadate, the highest concentration tested. The data are the mean ± S.E. of three or more experiments. B, time course of reversal of the effects of vanadate. The rate of oxygen consumption (open symbols) was determined from the slopes of traces obtained in experiments like that in Fig. 3. Phosphotyrosine content (solid symbols) was determined in experiments like that in Fig. 9A and quantified as described above. Data are the mean ± S.E. of three experiments.

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**Fig. 9. Vanadate reduces the rate of dephosphorylation.** A, electroporated cells were suspended in permeabilization medium containing 100 μM GTP, 1 mM ATP, and 2 mM NADPH and preincubated without (left-most lane) or with (all other lanes) 10 μM vanadate for 5 min at 37°C. The samples were sedimented and resuspended in medium without ATP, in the presence or absence of 50 μM vanadate. After incubation for the indicated times (in minutes), the reaction was stopped and immunoblotting was performed as described for Fig. 7. A typical autoradiogram of samples developed with 32P-labeled ATP is shown. B, porated cells were prelabeled for 5 min with [γ-32P]ATP in the presence of 10 μM vanadate as described for Fig. 6A. Following sedimentation, the samples were suspended in medium containing 1 mM nonradioactive ATP (without [γ-32P]ATP), 2 mM NADPH, and 100 μM GTP in the presence or absence of 10 μM vanadate and incubated for the indicated times. The reaction was terminated as described under “Experimental Procedures” and the samples subjected to electrophoresis and autoradiography. A typical radiogram is shown.

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**Fig. 10. Labeling of permeabilized neutrophils with 35S-labeled ATPγS.** Intact cells were ATP depleted in glucose-free Na+ solution as described. The cells were then electroporated in permeabilization medium devoid of ATP and suspended in the same medium containing 100 μM GTP, 2 mM NADPH, plus 20 μCi/ml [35S]ATPγS, in the presence or absence of 10 μM vanadate. After 5 min, the reaction was stopped and the samples were used for electrophoresis and fluorography, as described under “Experimental Procedures.” One of three similar experiments is illustrated.

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**Fig. 11. Correlation between oxygen consumption and tyrosine phosphorylation.** A, concentration dependence of the effects of vanadate on oxygen consumption (maximal rate; open symbols) and phosphotyrosine accumulation (solid symbols). The latter was quantified by immunoblotting and 125I-protein A labeling, as in Fig. 7B. Individual lanes were cut and counted in a γ counter. To enable comparison between independent experiments, the data were normalized to the value obtained at 100 μM vanadate, the highest concentration tested. The data are the mean ± S.E. of three or more experiments. B, time course of reversal of the effects of vanadate. The rate of oxygen consumption (open symbols) was determined from the slopes of traces obtained in experiments like that in Fig. 3. Phosphotyrosine content (solid symbols) was determined in experiments like that in Fig. 9A and quantified as described above. Data are the mean ± S.E. of three experiments.
Neutrophil Activation by Vanadate

At micromolar concentrations, vanadate was found to be a potent activator of oxygen consumption in electroporated cells. This effect was not observed in intact cells at comparable or even higher concentrations, pointing to an intracellular site of action. In the permeabilized cells, the respiratory burst triggered by vanadate was strictly dependent on the presence of exogenous NADPH and was inhibited by superoxide dismutase, suggesting mediation by the NADP II oxidase and confirming the effectiveness of the permeabilization protocol. It is noteworthy that the rates of oxygen utilization attained in vanadate-treated cells are substantially larger than those induced by maximally effective doses of fMLP or TPA. The unexpectedly large rate of oxygen disappearance may be in part due to a chain reaction whereby vanadate and superoxide catalyze the oxidation of NADPH (18, 19). Superoxide anions are known to react with vanadate producing peroxovanadyl, which can oxidize NADPH (18, 19). By this mechanism, the initial generation of superoxide by the NADPH oxidase is magnified through nonenzymatic oxidation catalyzed by vanadate. This combination of events could account for the seemingly biphasic nature of the vanadate concentration dependence (Fig. 2B).

To define the mechanism whereby vanadate activates oxygen consumption, its known biological effects must be considered. Vanadate is a very potent inhibitor of ion pumping ATPases of the $E_1/E_2$ type, which form a phosphorylated intermediate during their catalytic cycle (11, 23). It is conceivable that inhibition of the Na$^+/K^+$ or the Ca$^{2+}$ pumps underlies activation by, for example, sparing ATP. However, this appears unlikely, since under the Na$^+$-free conditions of our experiments the Na$^+/K^+$ pump would be inactive. In addition, inhibition of the Ca$^{2+}$ pumps by chelation of Ca$^{2+}$ did not elicit oxygen consumption (see Fig. 4 in Ref. 7).

Vanadate is also known to activate some G proteins, by forming a stable complex with GDP in the nucleotide binding site of the enzyme (20). This mechanism could in principle account for the observed activation, since (a) the respiratory burst elicited by many stimuli is pertussis toxin-sensitive and (b) nonhydrolyzable analogs of GTP stimulate oxygen utilization (22). Nevertheless, the accumulated evidence does not favor a direct role of G proteins because: (a) the concentration of vanadate required to stimulate GTP-binding proteins is orders of magnitude higher than the one used for the present studies (20); (b) pertussis toxin, which in some cases prevents the effect of vanadate on G proteins (21), had no effect in neutrophils; and (c) the stimulation induced by vanadate was not reversed by GDP$\beta$S, which effectively displaces the GDP-fluoroaluminate complex.

The available evidence supports the contention that inhibition of phosphatase activity mediates the stimulation of the burst. Vanadate was shown to induce phosphoprotein accumulation, detectable by incorporation of radioactive $^{32}$P and by immunoblotting with anti-phosphotyrosine antibodies. A good correlation was found to exist between oxygen consumption and the formation of phosphoproteins: both parameters varied in parallel when vanadate was withdrawn or its concentration varied (Fig. 11). Two lines of evidence indicate that the primary and perhaps sole cause of phosphoprotein accumulation is phosphatase inhibition, as opposed to stimulation of kinase activity. First, vanadate had no effect on the formation of thiophosphorylated proteins. Because phosphatases hydrolyze the thiophosphate moiety poorly, if at all (8, 9), the rate of thiophosphorylation is dictated mainly by the activity of the kinases(s). Thus, vanadate seems to have little effect on the activity of those kinases that utilize ATP$\gamma$S as

Fig. 12. Vanadate prevents reversal of the effects of fMLP and GDP$\beta$S. A, permeabilized cells were suspended in medium containing 100, μM GTP and 2 mM NADPH. Where indicated, 1 μM vanadate was added to the top and bottom samples. No further additions were made to the top sample. Next, the middle and bottom samples were stimulated with 10$^{-7}$ M fMLP. Finally, where indicated, fMLP was displaced from its receptor by addition of the competitive antagonist Boc-PLPLP. B, cells were permeabilized and suspended in medium with 2 mM NADPH but devoid of GTP. Vanadate (1 μM) was added to the bottom sample only at the onset of the trace. Next, both samples were stimulated with 1 μM GDP$\beta$S. Where indicated, 200 μM GDP$\gamma$S was added to both samples.
substrate. Second, pulse-chase experiments revealed that vanadate markedly reduced the rate of dephosphorylation, consistent with inhibition of phosphoprotein phosphatases.

Because of the known selectivity of vanadate (11, 12), it is likely that its primary effect is exerted on phosphotyrosine phosphatases. Accordingly, a large accumulation of tyrosine phosphorylated proteins was detectable both by immunoblotting and by direct phosphoamino acid analysis. The latter method indicated that phosphotyrosine accounts for a substantial fraction of the total phosphorylation induced by vanadate. The abundance of phosphotyrosine is stressed further if the low recovery of this phosphoamino acid after acid hydrolysis is taken into consideration. That phosphotyrosine constitutes a large fraction of the phosphorylated sites is also suggested by the similarity of the phosphoprotein patterns revealed using [32P]ATP and by immunoblotting with a phosphotyrosine-specific antibody (cf. Figs. 6A and 7).

Clearly, the concentration of phosphoserine and phosphothreonine also increased substantially following treatment with vanadate (Fig. 6B). This may have resulted from direct inhibition of phosphoserine and phosphothreonine phosphatases by vanadate. However, it is also possible that the primary effect of vanadate is the accumulation of tyrosine phosphorylated proteins, which will secondarily lead to activation of serine/threonine specific kinases, to inhibition of the respective phosphatases, and/or to conformational changes of the substrate proteins, rendering them susceptible to phosphorylation at serine or threonine residues. Such a secondary accumulation of serine/threonine phosphorylated proteins has been observed in the case of insulin-stimulated cells (24). While the primary effect of the hormone is to activate the receptor tyrosine kinase, subsequent reactions induce serine/threonine phosphorylation, which will secondarily lead to activation of kinases (24).

If the assumption that vanadate primarily inhibits tyrosine phosphorylase is correct, the observed accumulation of tyrosine phosphorylated proteins implies that one or more tyrosine kinases are constitutively active in neutrophils. We cannot rule out that such kinase(s) become activated during handling and permeabilization of the cells. However, even in this case, the available endogenous phosphatases are sufficient to preclude the spontaneous accumulation of phosphorytrosine and the associated stimulation of oxygen consumption. Moreover, as discussed elsewhere (10), the electroporated cells resemble intact cells in that they remain susceptible to stimulation by physiological ligands. These observations, together with the well established turnover of phosphorylated proteins in unstimulated neutrophil (e.g. Refs. 25 and 26), are consistent with ongoing kinase and phosphatase activity in the quiescent (unstimulated) state.

Recently, tyrosine phosphorylation was reported to occur in chemoattractant-stimulated neutrophils (27). However, there is no direct evidence that the chemoattractant receptor itself is a tyrosine kinase, as is the case for polypeptide receptors in other systems (28). Unlike those receptors endowed with kinase activity, tyrosine phosphorylation stimulated by chemoattractants is obliterated by pretreatment of the cells with pertussis toxin (27). These findings suggest that tyrosine phosphorylation is performed not by the receptor itself, but rather by a downstream kinase and that the activation signal is conveyed by a G protein. This notion is supported by the finding that, in permeabilized human neutrophils, GTPγS induced the accumulation of tyrosine phosphorylated proteins (22). In addition, very active non-receptor tyrosine kinases such as hek (29) and c-fes (30) have been detected in phagocytic cells. It is presently not clear whether such kinases are stimulated by activation of GTP-binding proteins or if they are constitutively active. In the latter case, accumulation of tyrosine-phosphorylated proteins could result from inhibition of phosphatase activity, as suggested above for vanadate-treated cells. Conceivably, phosphatases could also be inhibited by GTP-binding proteins, accounting for both the receptor and GTPγS-induced phosphotyrosine accumulation in neutrophils. Consistent with this model, regulation of tyrosine phosphatase activity by chemotactic peptides was recently reported in neutrophils and HL60 cells (31).

It has recently been demonstrated that addition of H2O2 to hepatoma cells induces the accumulation of tyrosine-phosphorylated proteins (35). By analogy, it is conceivable that one of the reduced oxygen metabolites generated by the oxidase, rather than vanadate itself, is responsible for the tyrosine phosphorylation. This explanation is unlikely, since stimulation of similar or larger rates of superoxide by diacylglycerol or by TPA do not induce a comparable accumulation of tyrosine phosphorylated proteins (22).

In summary, oxygen consumption and phosphotyrosine accumulation were stimulated by vanadate in permeabilized neutrophils. Increased phosphorylation was found to be, at least in part, to inhibition of tyrosine phosphatase activity. The simultaneous occurrence of phosphorylation and of a respiratory burst may be entirely fortuitous and does not imply a causal relationship. However, in view of their parallelism and because the mechanism underlying activation of the oxidase remains incompletely understood, further study of the role of tyrosine phosphorylation in neutrophil activation is warranted.

REFERENCES

It is noteworthy, however, that omission of NADPH or the presence of exogenous superoxide dismutase reduced tyrosine phosphorylation (S. Trudel and S. Grinstein, unpublished observations). Thus, both vanadate and superoxide or a related oxygen metabolite are necessary for phosphorylation. This may indicate that peroxovanadyl or another reduced form of vanadate is responsible for inhibition of tyrosine phosphatase.
Neutrophil Activation by Vanadate

Vanadate stimulates oxygen consumption and tyrosine phosphorylation in electropermeabilized human neutrophils.
S Grinstein, W Furuya, D J Lu and G B Mills


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