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Activation of permeabilized HL60 cells by vanadate

Evidence for divergent signalling pathways

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The possible role of tyrosine phosphorylation in the activation of granulocytic HL60 cells was examined using vanadate, a phosphotyrosine phosphatase inhibitor. Treatment of permeabilized cells with micromolar concentrations of vanadate resulted in a substantial accumulation of tyrosine-phosphorylated proteins, detected by immunoblotting. At comparable concentrations, vanadate was also found to elicit an NADPH-dependent burst of oxygen utilization. Actin assembly, studied using 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin, was similarly stimulated by vanadate, though considerably higher concentrations were required to observe this effect. In contrast with these responses, the secretion of lysozyme was not stimulated by vanadate, nor did vanadate affect calcium-induced secretion. Therefore, accumulation of tyrosine-phosphorylated proteins is associated with stimulation of some, but not all, of the responses characteristic of granulocytic cell activation. This indicates that the effects of vanadate are selective and suggests divergence of the signalling pathways leading to the individual effectors.

INTRODUCTION

When stimulated by a variety of soluble or particulate activators, neutrophils undergo diverse responses such as chemotaxis, degranulation and a respiratory burst [1,2]. Such chemotactic and bactericidal responses are believed to be mediated largely by the stimulation of protein kinase C (PKC) and/or by the increase in cytosolic Ca^{2+} that follow the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C [3–5]. This notion is supported by the marked increase in protein phosphorylation that accompanies chemotactic stimulation, which has been attributed primarily to activation of PKC, and by the stimulatory effects of exogenous diacylglycerol and phorbol esters [4,5]. On the other hand, other observations are inconsistent with this model. For some stimuli, neutrophil activation is not prevented by inhibitors of PKC [6–8], suggesting the existence of additional activation pathways and possibly the involvement of other kinases.

Some evidence has been presented which suggests that protein tyrosine phosphorylation may be involved in the activation of neutrophils by chemoattractants. Firstly, neutrophils have been shown to possess both the tyrosine and the phosphotyrosine phosphatase activities necessary to modulate the cellular levels of tyrosine-phosphorylated proteins [9]. Moreover, increased tyrosine phosphorylation has been demonstrated in both fMet-Leu-Phe- [10] and granulocyte-macrophage-colony-stimulating-factor [11]-stimulated cells. Treatment of permeabilized neutrophils with guanosine 5′-[γ-thio]triphosphate (GTP[S]), a direct activator of GTP-binding proteins (G-proteins), also induced accumulation of tyrosine-phosphorylated proteins [12]. Finally, two groups have reported inhibition of the chemoattractant-induced respiratory burst by ST 638, a tyrosine kinase inhibitor [13,14]. Together, these observations provide circumstantial evidence for the functional involvement of tyrosine-phosphorylated proteins in neutrophil activation.

Vanadate is known to mimic the effects of hormones such as insulin, which are believed to operate primarily by activation of receptor-associated tyrosine kinase activity [15]. Functional stimulation by vanadate is accompanied by accumulation of tyrosine-phosphorylated proteins due to stimulation of a kinase or, more likely, to the inhibition of tyrosine phosphatase activity. Preliminary results from our laboratory indicated that vanadate also increases tyrosine phosphorylation in phagocytic cells [16]. In the present report we used vanadate to assess the potential role of tyrosine phosphorylation in the activation of the promyelocytic leukaemia HL60 cell line, differentiated to the neutrophil-like phenotype using dimethyl sulphoxide (Me_2SO). In intact cells vanadate traverses the membrane poorly and can be rapidly converted to its reduced and generally inactive vanadyl form [17,18]. To circumvent these difficulties, HL60 cells were permeabilized with the bacterial toxin streptolysin O (SLO) or by electroporation, allowing rapid entry of vanadate. The results indicate that low (micromolar) concentrations of vanadate can induce a large increase in the cellular level of phosphotyrosine. Furthermore, vanadate stimulated the respiratory burst and induced actin assembly, but had no effect on lysozyme secretion from these cells. Thus tyrosine phosphorylation may represent an alternative pathway for the activation of specific functions in phagocytes.

MATERIALS AND METHODS

Materials

HL60 cells were purchased from the American Type Culture Collection. Fetal bovine serum was from Flow Laboratories Inc. L-Glutamine and penicillin/streptomycin were from Gibco. Molecular mass standards, phenylmethanesulphonyl fluoride, 5′-adenylylimido diphosphate (AMP-PNP), ATP (K^+ salt), EGTA, GTP, GTP[S], fMet-Leu-Phe, NADPH, Me_2SO, Nonidet P40, Ponceau S stain, phosphoserine, phosphotyrosine, Coomassie

Abbreviations used: AMP-PNP, 5′-adenylylimido diphosphate (adenosine 5′-[βγ-imido]triphosphate); Me_2SO, dimethyl sulphoxide; fMet-Leu-Phe, formyl-methionyl-leucyl-phenylalanine; G-protein, GTP-binding protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PKC, protein kinase C; RFI, relative fluorescence index; SLO, streptolysin O.

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Blue, Hepes and dried *Micrococcus lysodeikticus* were obtained from Sigma. Albumin was from Calbiochem. Monoclonal anti-phosphotyrosine antibody PY20 IgG 2B was obtained from ICN. 7-Nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin was purchased from Molecular Probes. Lysophosphatidylcholine was from Avanti Polar Lipids. SLO was from Difco. Medium RPMI 1640 was prepared by the University of Toronto Media Preparation Service.

Solutions

Bicarbonate-free medium RPMI 1640 was buffered to pH 7.3 with 25 mM-NaHepes. KCl permeabilization medium contained (in mM): 140 KCl, 1 $MgCl_2$, 2 NADPH, 1 EGTA, 10 KHepes (pH 7.0), 1 ATP and 10 glucose, as well as sufficient $CaCl_2$ to give a final free Ca^{2+} concentration of 100 mM, calculated as described in [19]. Potassium glutamate permeabilization medium contained (in mM): 138.7 potassium glutamate, 7 magnesium acetate, 2 NADPH, 1 EGTA, 10 KHepes (pH 7.0), 5 ATP and 10 glucose, and sufficient $CaCl_2$ to give a final free Ca^{2+} concentration of 100 nM, verified fluorimetrically using indo-1.

HL60 cell culture

HL60 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), streptomycin (100 units/ml) and penicillin (100 mg/ml). The cells were passaged at starting densities of $(2.5–3.5) \times 10^5$ cells/ml and maintained in suspension culture at 37 °C in an air atmosphere containing 5% CO_2. The cell cultures were diluted every 3–4 days so that the cell density did not exceed $(1–2) \times 10^6$ cells/ml. To induce granulocytic differentiation, the cells were inoculated at 3.5×10^5 cells/ml of medium in spinner flasks and treated with 1.3% (v/v) Me_2SO for 6–7 days. The cells were harvested by centrifugation and resuspended at a density of 10^7 cells/ml in Hepes-buffered RPMI 1640 for use in experiments.

Cell permeabilization procedures

For electroporation, intact cells were sedimented and resuspended in ice-cold permeabilization solution (KCl or potassium glutamate) at 10^7 cells/ml. Aliquots (0.8 ml) of this suspension were then transferred to a Bio-Rad Pulser cuvette and permeabilized using two discharges of 1.0 kV/cm from a 25 μF capacitor. The cells were sedimented using an Eppendorf 5415 Microfuge and resuspended in fresh ice-cold permeabilization solution between pulses. The cells were kept on ice and used within 15 min of permeabilization.

For permeabilization with SLO, the toxin was first activated with 10 mM-dithiothreitol when not freshly prepared. HL60 cells (10^7 cells) were permeabilized at 37 °C in 1 ml of KCl medium containing 0.5 units of SLO for 1 min (1 unit causes 50% lysis of 2% red blood cell suspensions in phosphate-buffered saline, pH 7.4, on incubation at 37 °C for 30 min). The percentage of permeabilized cells was assayed by their ability to exclude Trypan Blue and by loss of lactate dehydrogenase. Under the conditions used, approx. 70% of the cells took up Trypan Blue, and 45–70% of the total lactate dehydrogenase, determined as described [20], was released during a 10 min incubation at 37 °C. Cells were used immediately after permeabilization.

Phosphotyrosine detection

Cells were permeabilized by the indicated procedure and suspended in the KCl permeabilization medium. After 2 min at 37 °C, vanadate was added where indicated and the suspension was incubated for a further 5 min. Phenylmethanesulphonyl fluoride (100 μM) was added, the samples were sedimented and the pellets were solubilized in boiling Laemmli [21] sample buffer. Immunoblotting was performing as outlined in [22]. Briefly, following electrophoresis and transfer to nitrocellulose, the proteins were revealed by staining with Ponceau S (0.2% w/v) in 3% (w/v) trichloroacetic acid, and destained in water. The blot was then incubated with phosphotyrosine-specific antibodies and washed. The monoclonal anti-phosphotyrosine antibody was detected by goat anti-mouse antibody conjugated to alkaline phosphatase. Alkaline phosphatase staining was then performed as recommended by the kit manufacturer (Bio-Rad).

Oxygen consumption

O_2 consumption was measured polarographically with a model 53 biological monitor (Yellow Springs Instrument Co.); 8×10^6 cells were permeabilized as indicated and resuspended in 2 ml of KCl permeabilization medium at 37 °C with stirring. O_2 consumption was calculated using a solubility coefficient of 0.024 ml of O_2/ml at 37 °C.

Actin polymerization

HL60 cells were permeabilized by electroporation in potassium glutamate medium. The cells were then equilibrated for 1 min in this medium at 37 °C before stimulation. Where indicated, vanadate was added and the cell suspension was incubated for a further 2 min at 37 °C. Cells were then fixed and permeabilized by the two-step method of Howard & Meyer [23], except that the formalin/lysophosphatidylcholine mixture was sonicated for 10 min to facilitate the formation of a micellar suspension. The cellular content of polymerized actin (F-actin) was then determined by NBD-phallacidin staining and flow cytometry. The cells were analysed on an Epics Profile fluorescence-activated cell sorter, with excitation from an argon laser at 488 nm and emission recorded at 520 nm with band pass and short pass filters. Gating was done on the forward and right-angle light scatter only to exclude debris and cell clumps. In each experiment, 2×10^4 cells were measured per condition and all values are expressed as relative fluorescence index (RFI). The RFI was calculated using the ratio of the linearized mean fluorescence of the cell population in question with the Epics data processing software.

Lysozyme release

SLO-permeabilized cells were sedimented and resuspended in KCl permeabilization medium containing the indicated concentration of Ca^{2+}, GTP[S], vanadate or vanadate plus Ca^{2+}, and incubated at 37 °C for 10 min. Cells were sedimented, the supernatant was saved and the pellet was solubilized in KCl medium containing 0.1% Triton X-100. Aliquots of the supernatant and cell lysate were used for measurement of lysozyme activity, determined as the rate of lysis of *M. lysodeikticus* as measured by the decrease of absorbance at 450 nm [24].

Statistical analysis

All data are reported as means ± S.E.M. of the numbers of experiments indicated. Data in Figs. 2 and 3 were analysed by the paired *t* test. Data in Table 1 were analysed by analysis of variance for repeated measures with correction for multiple comparison (Sheffe).

RESULTS

Effects of vanadate on tyrosine phosphorylation

The effects of vanadate on phosphotyrosine accumulation were studied in intact and permeabilized HL60 cells. The presence of tyrosine-phosphorylated polypeptides was established by immunoblotting using monoclonal antibodies. As shown in Fig. 1, only marginal levels of phosphotyrosine were detectable in the absence of vanadate. In contrast, substantial phosphotyrosine

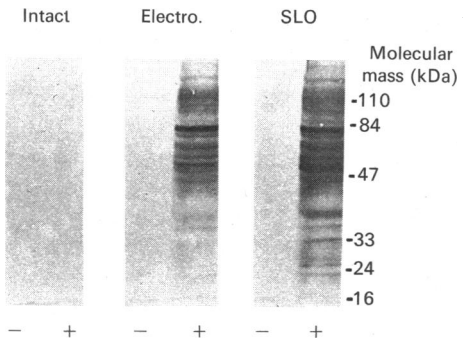

Fig. 1. Detection of vanadate-induced tyrosine phosphorylation by immunoblotting

Intact, electroporated and SLO-permeabilized cells were incubated in the absence (−) or presence (+) of 100 μM-vanadate for 5 min at 37 °C. Following electrophoresis and blotting, polypeptides containing phosphotyrosine were detected using a monoclonal anti-phosphotyrosine antibody and alkaline phosphatase staining.

accumulation was detected in both electroporated and SLO-permeabilized cells treated with vanadate. Several polypeptides, particularly in the molecular mass range 45–130 kDa, were phosphorylated. The response to vanadate was evident at concentrations as low as 10 μM and increased with increasing vanadate concentration in the range explored, i.e. 0–500 μM (results not shown). Vanadate did not cause changes in the polypeptide composition of the cells, as detected by Ponceau S staining of proteins transferred to nitrocellulose paper.

The specificity of labelling was tested by equilibrating the blots with antibody in the presence or absence of 2 mM-phosphoserine or -phosphotyrosine. Free phosphotyrosine completely eliminated binding of the antibody, whereas labelling was unaffected by an equivalent concentration of phosphoserine. No labelling was detected when anti-phosphotyrosine antibody was omitted during the first incubation (results not shown).

At equivalent vanadate concentrations, greater levels of phosphotyrosine were observed in SLO-permeabilized cells than in electroporated cells. This effect may be attributed to greater accessibility of vanadate to the interior of SLO-treated cells, due to the larger pore size produced by this permeabilization method [25]. Vanadate failed to increase phosphotyrosine accumulation in intact cells, suggesting an intracellular site of action.

Effects of vanadate on O_2 consumption

We next tested whether vanadate induced functional responses in HL60 cells. Fig. 2 illustrates the effect of vanadate on O_2 consumption in intact and permeabilized (SLO-treated and electropermeabilized) HL60 cells. Vanadate, at concentrations as high as 500 μM, did not significantly affect the rate of O_2 utilization in intact cells. The viability and responsiveness of the cells was established by their response to subsequent stimulation with fMet-Leu-Phe (results not shown). The chemoattractant also stimulated the respiratory burst in both electropermeabilized and SLO-treated cells, demonstrating that the receptor-mediated response remained functional following permeabilization. Both SLO- and electro-permeabilized cells responded to the addition of 50 μM-vanadate with a pronounced increase in the rate of oxygen consumption. The presence of exogenously added NADPH was essential for the response (results not shown), suggesting that O_2 consumption was due to the activation of the NADPH oxidase. Vanadate did not potentiate the stimulatory effect of fMet-Leu-Phe on the NADPH oxidase. However, when present together at subsaturating concentrations, the effects of vanadate and fMet-Leu-Phe were additive (results not shown).

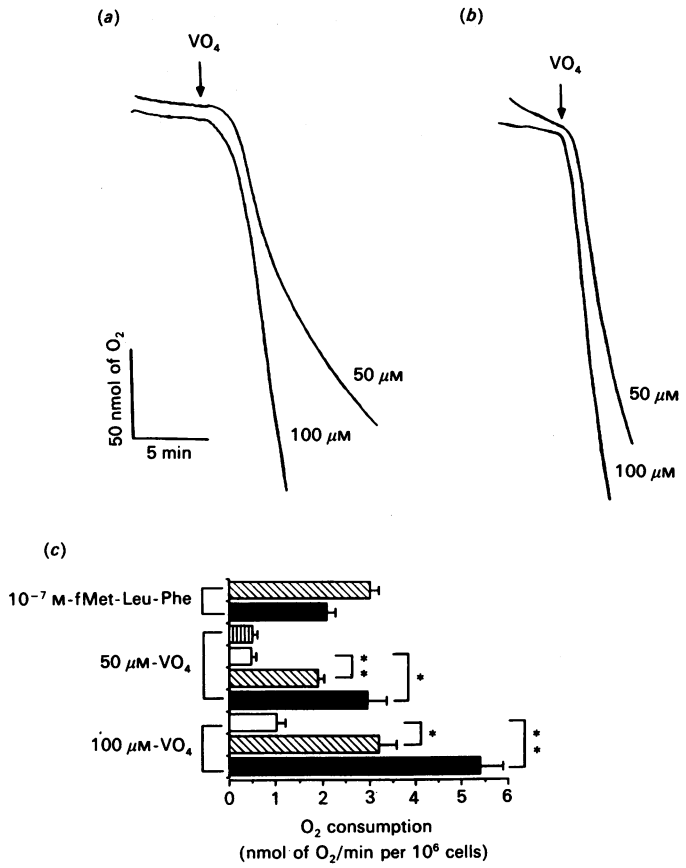

Fig. 2. Effect of vanadate on O_2 consumption in electropermeabilized (*a*) and SLO-permeabilized (*b*) HL60 cells

Cells were permeabilized and immediately resuspended in KCl permeabilization medium. At the arrow, the indicated final concentration of vanadate was added. The time and oxygen consumption scales apply to both (*a*) and (*b*). The tracings, obtained from the same batch of cells, are representative of at least three similar experiments. (*c*) Rate of O_2 consumption in the absence or presence of intact, electroporated or SLO-permeabilized cells. ▥, Intact cells; □, no cells; ▧, electropermeabilized cells; ■, SLO-treated cells. The rate of O_2 consumption in the absence of stimuli was negligible and is therefore not illustrated. Each bar represents the mean ± S.E.M. of three experiments, each carried out in duplicate. * and ** indicate significant differences ($P < 0.005$ and $P < 0.001$ respectively) between the bars specified, as determined by Student's *t* test for paired data. In this Figure and Fig. 3 the presence of vanadate is indicated by VO_4. This abbreviation was used for simplicity but does not imply that this is the only chemical form of vanadate present or the one responsible for the biological effects.

The magnitude of the O_2 consumption response was proportional to the concentration of vanadate. In addition, the lag time that preceded the appearance of the respiratory burst at lower vanadate concentrations was progressively shortened at higher concentrations (e.g. Fig. 2). At equivalent concentrations, vanadate elicited a greater rate of O_2 consumption in SLO-permeabilized than in electrically permeabilized cells. This finding parallels the greater extent of phosphotyrosine accumulation detected in the former cells.

It must be noted that, in the absence of cells, vanadate induced an NADPH-dependent uptake of O_2 from the medium. The mechanism underlying this phenomenon is not clear, but it may represent the non-enzymic oxidation of NADPH. This spontaneous effect was however considerably smaller than the response seen in the presence of cells (Fig. 2) and could be readily subtracted from the total rate of O_2 utilization.

Table 1. Effect of vanadate on actin polymerization in HL60 cells

Cells were electroporated as described in the Materials and methods section and stimulated with the indicated final concentration of vanadate for 2 min at 37 °C. Actin polymerization was measured by flow cytometry using NBD-phallacidin as described. Data are the means ± S.E.M. of three experiments, each carried out in duplicate. Statistical significance was determined by analysis of variance for repeated measures with correction for multiple comparison (Sheffe): * $P < 0.05$ compared with control.

Stimulus	Actin polymerization (RFI)
None	1.0
50 μM-Vanadate	1.11 ± 0.03
100 μM-Vanadate	1.28 ± 0.06*
500 μM-Vanadate	1.43 ± 0.05*

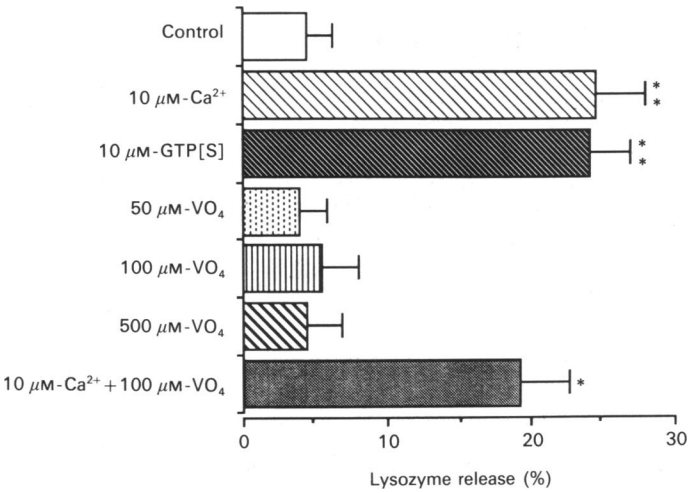

Fig. 3. Effect of vanadate on lysozyme release from SLO-permeabilized HL60 cells

Result are percentage of total cell lysozyme released in 10 min under the conditions specified. Each bar represents the mean ± S.E.M. of three experiments, each carried out in duplicate. * and ** indicate significant differences from control ($P < 0.005$ and $P < 0.001$ respectively) as determined by Student's t test for paired data. The difference between cells incubated with Ca^{2+} with and without vanadate was not statistically significant.

Effects of vanadate on actin polymerization

We next examined the effects of vanadate on actin assembly, measuring NBD-phallacidin fluorescence using a flow cytometer. The cells were electrically permeabilized to render the cell interior accessible to vanadate. Permeabilization by SLO was not employed as it was found to cause spontaneous actin assembly. As previously demonstrated for neutrophils by both Therrier & Naccache [26] and Downey & Grinstein [27], actin was found to polymerize in electropermeabilized HL60 cells in response to fMet-Leu-Phe (results not shown). Vanadate, at concentrations of 100 and 500 μM, significantly increased the amount of F-actin (Table 1). Flow cytometric analysis indicated that the cell population behaved homogeneously in response to vanadate. No significant effect was observed at 50 μM-vanadate. To verify that the increase in fluorescence detected was not due to an artifact induced by vanadate, the cells were first fixed and permeabilized with formalin/lysophosphatidylcholine and subsequently treated with various concentrations of vanadate. The fixed vanadate-treated cells showed no significant increase in fluorescence compared with untreated cells (results not shown).

Effects of vanadate on lysozyme release

For the study of exocytosis, HL60 cells were permeabilized with SLO, as described by Stutchfield & Cockcroft [28]. SLO-permeabilized cells exhibited both Ca^{2+}- and GTP[S]-induced release of lysozyme, an enzyme contained in both primary and specific granules (Fig. 3). The maximal Ca^{2+}-induced release was found to be approx. 24% of the total lysozyme present in the cells. Ca^{2+}-induced lysozyme release was found to be ATP-dependent; resuspension of SLO-permeabilized cells in a medium lacking ATP inhibited release by 56–90%. Ca^{2+}-dependent secretion could not be supported by AMP-PNP, a non-hydrolysable analogue of ATP. These observations are similar to those reported earlier by Stutchfield & Cockcroft [28] and are consistent with the notion that lysozyme is being released from permeabilized cells by exocytosis, as opposed to SLO-induced damage to cellular membranes.

In contrast to the stimulation obtained by micromolar concentrations of free Ca^{2+} and GTP[S], vanadate, at the concentrations explored, did not cause significant release of lysozyme from permeabilized HL60 (Fig. 3). Nor did vanadate significantly inhibit Ca^{2+}-dependent exocytosis (Fig. 3), ruling out the possibility that vanadate has a dual effect, acting both as an inhibitor and as an activator of secretion. In addition, vanadate did not affect the stimulation of lysozyme release induced by fMet-Leu-Phe (results not shown).

DISCUSSION

Micromolar concentrations of vanadate promoted the rapid accumulation of tyrosine-phosphorylated proteins in permeabilized HL60 cells. Because vanadate is a potent and relatively specific inhibitor of phosphotyrosine phosphatases, this observation suggests that active tyrosine kinases are present in resting (unstimulated) cells, but that accumulation of tyrosine-phosphorylated proteins is normally precluded by vigorous phosphatase activity. In view of these findings, it is conceivable that the increased tyrosine phosphorylation reported in cells activated by chemoattractants [10,11] may result from inhibition of phosphatases instead of, or in addition to, the more commonly postulated stimulation of kinases.

Vanadate was also found to be a potent activator of O_2 consumption in permeabilized HL60 cells. The response occurred within the same range of vanadate concentrations required to induce accumulation of phosphotyrosine, suggesting a correlation between these two events. Though at present insufficient to establish a causal relationship, these observations are consistent with earlier reports that: (*a*) stimulation of the respiratory burst by chemoattractants is associated with increased tyrosine phosphorylation [10,11], and (*b*) ST 638 and erbstatin, two unrelated inhibitors of tyrosine kinases, block the respiratory burst generated by physiological stimuli ([13,14] and P. Naccache, personal communication). Together, these observations suggest that tyrosine phosphorylation may be involved in at least one of the pathways leading to the activation of phagocytes.

Actin assembly, determined by flow cytometry using NBD-phallacidin, was also found to be stimulated by addition of vanadate. However, the concentration of vanadate required to elicit this response (100–500 μM) was greater than that required to stimulate phosphotyrosine accumulation or the respiratory burst (detectable at concentrations as low as 10 μM). It is apparent that these responses are not tightly correlated, suggesting that initiation of actin assembly requires high levels of phosphorylation, or that polymerization is initiated by vanadate through a

mechanism other than tyrosine phosphorylation. In agreement with the latter option, Gomez-Cambronero *et al.* [13] and P. Naccache (personal communication) have reported that, under conditions where superoxide generation was inhibited, ST 638 and erbstatin had little effect on the fMet-Leu-Phe-induced polymerization of actin. In our experiments, induction of actin assembly may have resulted from a direct effect of vanadate on actin. Experiments *in vitro* using cell-free systems have shown that, together with ADP, vanadate can enter the nucleotide-binding site of actin, stabilizing its filamentous (F) form [29]. Further experiments are required to establish whether this is the mode of action of vanadate in permeabilized cells.

Unlike the other responses studied, lysozyme release was not stimulated by addition of vanadate, indicating that increased levels of phosphotyrosine are not sufficient to activate secretion in phagocytic cells. Accordingly, erbstatin failed to inhibit elastase release in fMet-Leu-Phe-stimulated neutrophils (P. Naccache, personal communication). Our results with HL60 cells differ from earlier data obtained in rat exocrine pancreas and mast cells, where vanadate was found to induce exocytosis [30,31]. In the latter studies, vanadate at millimolar concentrations was added to intact cells and its effects were attributed to increased cytosolic [Ca^{2+}], resulting from inhibition of the Ca^{2+}-ATPase. The mechanism of action of vanadate would be inoperative in permeabilized cells, where intracellular [Ca^{2+}] is stabilized by EGTA at a level identical to the extracellular [Ca^{2+}]. The reported effects of vanadate on secretion could also be a consequence of activation of G-proteins by GDP–vanadate. Such effects were not evident in our preparation, probably because the concentrations used were considerably lower than those required to activate G-proteins [32].

In summary, our results are consistent with the notion that phosphotyrosine accumulation is involved in some aspects of signal transduction in HL60 cells. The ability of vanadate to stimulate oxygen consumption without causing the release of lysozyme from the cells implies that the effects of tyrosine phosphorylation are selective and, more importantly, that the signalling pathways leading to these responses diverge. Similar conclusions can be derived from the selective inhibitory profiles of erbstatin and ST 638 described above. Divergence of signalling pathways could also account for the observation that some stimuli are excellent secretagogues, yet are poor activators of the respiratory burst, while the opposite is true for other stimuli [33]. Finally, the present observations raise the possibility that activation of HL60 cells may be exerted by inhibition of phosphatase activity in addition to, or instead of, stimulation of kinases.

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