Activation of multiple pH-regulatory pathways in granulocytes by a phosphotyrosine phosphatase antagonist

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Activated phagocytes undergo a massive burst of metabolic acid generation, yet must be able to maintain their cytosolic pH (pH_i) within physiological limits. Peroxides of vanadate (V^{4+} -OOH), potent inhibitors of phosphotyrosine phosphatases, have recently been shown to produce activation of the respiratory burst in HL60 granulocytes. We therefore investigated the effects of V^{4+} -OOH on pH_i homeostasis in HL60 granulocytes, using a pH-sensitive fluorescent dye. V^{4+} -OOH stimulation induced a biphasic pH change: a transient cytosolic acidification followed by a significant alkalization. The initial acidification was prevented by inhibition of the NADPH oxidase and was absent in undif-

ferentiated cells lacking oxidase activity. Analysis of the alkalization phase demonstrated the involvement of the Na^+/H^+ antiporter, and also provided evidence for activation of two alternative H^+ -extrusion pathways: a bafilomycin-sensitive component, likely reflecting vacuolar-type H^+ -ATPase activity, and a Zn^{2+} -sensitive H^+ -conductive pathway. Our results indicate that V^{4+} -OOH stimulation not only activated the NADPH oxidase but concomitantly stimulated H^+ -extrusion pathways, enabling the cells to compensate for the massive production of intracellular H^+ associated with the respiratory burst.

INTRODUCTION

Neutrophils are itinerant cells that can migrate to sites of infection, where they destroy invading organisms by a variety of microbicidal mechanisms, including phagocytosis, degranulation and the production of toxic oxygen metabolites (Sha'afi and Molski, 1988). Activation of neutrophils is associated with a massive increase in their rate of oxygen consumption, known as the respiratory burst. Most of the O_2 consumed during this burst is converted by the NADPH oxidase into superoxide (O_2^-), which can in turn generate a variety of reduced metabolites that are potent bactericidal and tumoricidal agents (Rossi, 1986; Tauber, 1987).

Activation of the NADPH oxidase induces oxidation of NADPH to $NADP^+$, which is accompanied by release of H^+ into the cytosol. Regeneration of NADPH by the hexose monophosphate shunt also produces net acid equivalents. Activation of neutrophils is therefore associated with a large burst of intracellular H^+ generation. Despite this massive increase in net H^+ production, however, the cytosolic pH (pH_i) of activated cells remains very near resting levels, even in the nominal absence of HCO_3^- (Simchowitz, 1985; Grinstein and Furuya, 1986). Maintenance of pH_i within the narrow physiological range is accomplished by at least three independent H^+ -extrusion pathways: an Na^+/H^+ antiporter (Simchowitz, 1985; Grinstein and Furuya, 1986), an H^+ conductance (Henderson et al., 1987, 1988; Nanda and Grinstein, 1991) and a vacuolar- (or V-)type H^+ -ATPase (Nanda et al., 1992). The Na^+/H^+ antiporter mediates the electroneutral exchange of intracellular H^+ for extracellular Na^+ and is inhibited by amiloride and its analogues (Simchowitz, 1985; Grinstein and Furuya, 1986). Vacuolar H^+ -ATPases are electrogenic pumps that can be selectively blocked by the bafilomycins, a family of macrolide antibiotics (Bowman et al., 1988; Nanda et al., 1992). Flux through the H^+ -conductive

pathway is passive, driven by the electrochemical H^+ gradient. The conductance is insensitive to amiloride and bafilomycin, but is blocked by bivalent cations, most notably Cd^{2+} and Zn^{2+} (see, e.g., Henderson et al., 1987; Kapus et al., 1992).

The pathways leading to activation of the bactericidal responses remain poorly understood. The metabolic effects of chemoattractants can be mimicked by tyrosine phosphatase inhibitors, suggesting a role for tyrosine phosphorylation in the process. Thus micromolar concentrations of vanadate have been shown to elicit a burst of O_2 utilization in permeabilized HL60 granulocytes (Trudel et al., 1990). In intact cells, stimulation of the respiratory burst was obtained using a combination of vanadate and H_2O_2 , which generates vanadyl hydroperoxide (V^{4+} -OOH) (Trudel et al., 1991).

Although vanadate derivatives effectively activate the metabolic burst, it is not known whether the mechanisms responsible for pH_i homeostasis are also triggered by accumulation of tyrosine-phosphorylated proteins. The purpose of this study was therefore to investigate the effects of V^{4+} -OOH on pH_i in HL60 granulocytes. Specifically, we questioned whether the production of intracellular H^+ generated by activation of the oxidase was compensated by regulatory systems and, if so, whether the response was simply due to decreased pH_i or whether V^{4+} -OOH directly activated one or more of the known H^+ -extrusion systems present in the plasma membrane of phagocytic cells.

EXPERIMENTAL

Materials

HL60 cells were purchased from the American Type Culture Collection. Bafilomycin A_1 was generously given by Dr. K. Altendorf (Universitat Osnabruck, Germany). Diphenyleneiodonium (DPI) was synthesized by the method of Collette et al. (1956). Nigericin and the acetoxymethyl ester of 2',7'-bis(carboxyethyl)-

Abbreviations used: BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; DMSO, dimethyl sulphoxide; DPI, diphenyleneiodonium; EPA, 5-(*N*-ethyl-*N*-propyl)amiloride; NMG $^+$, *N*-methyl-D-glucammonium; pH_i , cytosolic pH; PMA, phorbol 12-myristate 13-acetate ('TPA'); V, vacuolar; V^{4+} -OOH, vanadyl hydroperoxide.

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5(6)-carboxyfluorescein (BCECF) were obtained from Molecular Probes. *N*-Methyl-D-glucamine (NMG) and sodium orthovanadate were from Aldrich. 5-(*N*-Ethyl-*N*-propyl)amiloride (EPA) was kindly given by Merck, Sharp and Dohme. Fetal-bovine serum was from Flow Laboratories. L-Glutamine and penicillin/streptomycin were from GIBCO. Catalase was from Boehringer Mannheim. H_2O_2 was purchased from Fisher Scientific. Complete medium RPMI 1640 was prepared by the University of Toronto Media Preparation Service. Bicarbonate-free medium RPMI 1640 (Hepes-buffered, containing L-glutamine) and dimethyl sulphoxide (DMSO) were from Sigma.

Solutions

Na^+ medium contained (in mM): 140 NaCl, 1 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 glucose, 20 Hepes titrated to pH 7.3 at 37 °C with concentrated NaOH. K^+ and NMG^+ media were made by iso-osmotic replacement of NaCl with the chloride salts of K^+ and NMG^+ respectively, and titrated to pH 7.3 with the corresponding bases. The osmolarity of all solutions was adjusted to 295 ± 5 mosM with the major salt. V^{4+} -OOH was prepared by mixing equimolar concentrations of sodium orthovanadate and H_2O_2 (10 mM). After incubation for 15 min at room temperature, catalase (final concn. 200 $\mu\text{g}/\text{ml}$) was added to remove residual H_2O_2 . Portions of V^{4+} -OOH were used shortly after addition of catalase. This procedure is similar to that described by Fantus et al. (1989) to generate 'pervanadate'. Throughout this paper, the product generated by this mixture is referred to as vanadyl hydroperoxide (V^{4+} -OOH), but the presence of other products cannot be ruled out.

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 $\mu\text{g}/\text{ml}$). The cells were passaged at starting densities of $(2-3) \times 10^5$ cells/ml and kept at 37 °C in an air atmosphere containing 5% CO_2 . The cultures were diluted every 3-4 days so that the cell density did not exceed 2×10^6 cells/ml. To induce granulocytic differentiation, the cells were treated with 1.3% (v/v) DMSO for 7 days. The cells were harvested by centrifugation and resuspended at a density of 10^7 cells/ml in bicarbonate-free Hepes-buffered RPMI 1640 to be used for experiments.

Measurement of pH_i

pH_i was measured fluorimetrically by using BCECF. HL60 cell suspensions (10^7 cells/ml) in Hepes-buffered RPMI 1640 were loaded with 2 $\mu\text{g}/\text{ml}$ BCECF acetoxymethyl ester at 37 °C for 15 min. Cells were then sedimented and resuspended in fresh medium. Portions (10^6 cells) were sedimented and used for fluorescence determination in the indicated media by using a spectrofluorimeter with excitation and emission wavelengths of 495 nm (5 nm slit) and 525 nm (10 nm slit) respectively. Calibration of fluorescence versus pH_i was performed by the K^+ /nigericin method described by Thomas et al. (1979).

The fluorescence traces illustrated in the Figures are representative of a minimum of three separate experiments. Unless otherwise specified, data are presented in the text as means $\pm$ S.E.M. for the numbers of determinations indicated in parentheses.

RESULTS AND DISCUSSION

Effect of V^{4+} -OOH on pH_i in Na^+ -rich medium

The effect of V^{4+} -OOH on pH_i was studied in HL60 cells by using fluorescent indicator BCECF. In nominally bicarbonate-free Na^+ solution (extracellular $[\text{Na}^+] = 140$ mM) at 37 °C, the resting pH_i of DMSO-differentiated HL60 cells averaged 7.24 ± 0.002 ($n = 14$). As illustrated in Figure 1(a), exposure of these cells to V^{4+} -OOH (100 μM) resulted in a biphasic response, detectable after 1-2 min of treatment. The first phase consisted of a transient but significant cytosolic acidification (over the course of 2 min), followed by a sustained alkalinization that reached its full magnitude about 3 min later. The magnitude of the acidification varied from 0.03 to 0.07 pH unit. The maximum pH_i reached at the end of the alkalinization phase averaged 7.32 ± 0.03 ($n = 14$), 0.08 pH unit higher than the resting pH_i .

Mechanism of acidification

The process responsible for the V^{4+} -OOH-induced acidification was investigated first. In human neutrophils, a similar initial acidification was observed upon stimulation with the protein kinase C agonist phorbol 12-myristate 13-acetate (PMA; 'TPA'). This pH_i fall has been linked to the generation of intracellular H^+ accompanying the activation of both the oxidase and the hexose monophosphate shunt during the respiratory burst (Grinstein et al., 1986). Because peroxides of vanadate induce activation of the

Figure 1 Effect of V^{4+} -OOH on pH_i in Na^+ medium

pH_i was measured fluorimetrically by using BCECF. (a) DMSO-differentiated HL60 cells suspended in Na^+ medium; (b) DMSO-differentiated HL60 cells suspended in Na^+ medium containing 4 μM DPI from the outset; (c) undifferentiated HL60 cells suspended in Na^+ medium. V^{4+} -OOH (100 μM) was added to all traces where indicated. Temperature was 37 °C. The time scale applies to all traces. Traces are representative of at least three similar experiments.

Figure 2 Assessment of the role of the Na^+/H^+ antiporter in the V^{4+} -OOH-induced changes in pH_i

pH_i was measured fluorimetrically by using BCECF. (a) DMSO-differentiated HL60 cells were suspended in Na^+ medium. Where indicated, $5 \mu\text{M}$ EPA was added to both traces and DPI ($4 \mu\text{M}$) to the upper trace only. (b) DMSO-differentiated HL60 cells were suspended in NMG^+ medium. DPI was added to the upper trace when indicated. (c) Undifferentiated HL60 cells were suspended in Na^+ medium. Where noted, $5 \mu\text{M}$ EPA was added to both traces and DPI to the upper trace only. (d) Undifferentiated HL60 cells were suspended in NMG^+ medium. DPI was added to the upper trace when indicated. V^{4+} -OOH ($100 \mu\text{M}$) was added to all traces where indicated. Temperature was 37°C . The time scale applies to all traces. Traces are representative of at least three similar experiments.

respiratory burst in differentiated HL60 cells (Trudel et al., 1991), we determined whether the V^{4+} -OOH-induced acidification similarly resulted from the activation of the NADPH oxidase. The first approach tested the effect of V^{4+} -OOH on pH_i in the presence of DPI, a competitive antagonist of the flavo-protein component of the NADPH oxidase (Ellis et al., 1988). DPI has previously been demonstrated to inhibit NADPH oxidase activity by more than 95% in human neutrophils, resulting in almost complete suppression of metabolic acid production. As shown in Figure 1(b), the transient acidification preceding the rise in pH_i was completely abolished by pretreatment with DPI, indicating that this acidification is likely to result from accumulation of metabolic acid produced when the oxidase is activated by V^{4+} -OOH. Consistent with this hypothesis, the extent of the V^{4+} -OOH-induced alkalization was slightly increased by pretreatment with DPI: $\Delta\text{pH}_i = 0.11 \pm 0.01$ ($n = 14$), $\Delta\text{pH}_i = 0.08 \pm 0.01$ ($n = 15$) in the absence of DPI.

A second line of evidence supporting the involvement of the NADPH oxidase in the cytoplasmic acidification was obtained by using undifferentiated HL60 cells. Unlike their granulocytic counterparts, undifferentiated HL60 cells are reported to possess only marginal NADPH oxidase activity (Newberger et al., 1979). The effect of V^{4+} -OOH ($100 \mu\text{M}$) on pH_i in undifferentiated cells is illustrated in Figure 1(c). Only a monophasic alkalization ($\Delta\text{pH}_i = 0.16 \pm 0.02$; $n = 10$) was recorded, resembling the response observed in differentiated cells treated with DPI (cf. Figure 1b). The failure of undifferentiated cells to undergo acidification upon V^{4+} -OOH treatment confirms the results

obtained with DPI in differentiated cells, and reinforces the notion that in HL60 granulocytes V^{4+} -OOH induces activation of the NADPH oxidase, resulting in the production of metabolic acid.

Mechanism of V^{4+} -OOH-induced alkalization

Role of the Na^+/H^+ antiporter

The acidification induced by V^{4+} -OOH was only transient and followed by a significant cytosolic alkalization phase, indicating that V^{4+} -OOH activates one or more compensatory H^+ -extrusion system(s). The following experiments aimed at elucidating the mechanisms responsible for this alkalization. The presence of the Na^+/H^+ antiporter in the plasma membrane of phagocytic cells (HL60 granulocytes, neutrophils) and its role in pH_i homeostasis have been well documented (Grinstein and Furuya, 1986; Restrepo et al., 1987; Costa-Casnellie et al., 1988). To test the involvement of the exchanger in the V^{4+} -OOH-induced alkalization, HL60 granulocytes were treated with V^{4+} -OOH under conditions precluding Na^+/H^+ antiporter activity, e.g. in the presence of the amiloride analogue EPA, a potent and specific inhibitor of the antiporter, or in Na^+ -free media. As shown in Figure 2(a) (lower trace), when differentiated cells suspended in Na^+ -rich solution were pretreated with $5 \mu\text{M}$ EPA, V^{4+} -OOH no longer induced a cytoplasmic alkalization, but instead promoted a pronounced cytosolic acidification: $\Delta\text{pH}_i = -0.40 \pm 0.05$ ($n = 4$). It is noteworthy that addition of the inhibitor alone induced a small acidification (leftmost portions of Figure

2a), indicating that the antiport is constitutively active in unstimulated differentiated cells to compensate for basal metabolic acid generation.

Similar results were obtained when differentiated cells were suspended in media devoid of Na^+ : a sizeable acidification was induced by V^{4+} -OOH in both NMG^+ solution (0.19 ± 0.01 , $n = 3$; lower trace in Figure 2b) and in K^+ solution (0.29 ± 0.04 , $n = 4$; lower trace in Figure 3a). This dependence on external Na^+ and the sensitivity to the amiloride analogue indicates that the V^{4+} -OOH-induced alkalinization is mediated, at least in part, by Na^+/H^+ exchange. The cytosolic acidification unmasked by inhibiting the antiport likely reflects excess metabolic acid generated during activation of the oxidase. Indeed, the magnitude of the acidification was markedly decreased by pretreating the cells with DPI before challenge with V^{4+} -OOH, both in Na^+ medium containing EPA (Figure 2a, upper trace) or in Na^+ -free media (upper traces of Figures 2b and 3a). Taken together, these results demonstrate that V^{4+} -OOH not only stimulated the NADPH oxidase, but also induced activation of the Na^+/H^+ antiport.

It is noteworthy that activation of the antiport upon challenge with V^{4+} -OOH brought pH_i above the basal pre-stimulation level. This implies that activation of Na^+/H^+ exchange does not occur simply in response to the decrease in pH_i subsequent to the stimulation of the oxidase, but instead represents a direct stimulatory effect of V^{4+} -OOH on the antiporter. Consistent with this notion, the V^{4+} -OOH-induced cytosolic alkalinization persisted even under conditions where the metabolic acid production was suppressed by the presence of DPI (Figure 1b), as well as in the oxidase-deficient undifferentiated cells (Figure 1c).

In undifferentiated cells the V^{4+} -OOH-induced alkalinization was completely abolished under conditions precluding Na^+/H^+ activity (i.e. removal of extracellular Na^+ or addition of amiloride analogue; Figures 2c and 2d). Of note, the large acidification observed under these conditions in differentiated cells was not detected in the undifferentiated cells. Moreover, as expected, since these cells lack oxidase activity, pretreatment with DPI did not affect the response to V^{4+} -OOH. The results obtained with undifferentiated cells therefore confirm that peroxides of vanadate directly activate the Na^+/H^+ antiporter and that the occurrence of acidification requires the presence of a functional NADPH oxidase. It is also noteworthy that, in contrast with differentiated cells, treatment of undifferentiated cells with EPA failed to induce a significant spontaneous acidification (c.f. leftmost portion of traces in Figures 2a and 2c). This may reflect a slower rate of metabolic acid generation by the undifferentiated cells compared with their granulocyte counterparts, or the presence in the former of other effective pH -regulatory processes.

The above results demonstrate that maintenance of pH_i in the physiological range in V^{4+} -OOH-activated HL60 granulocytes is due, in part, to concurrent activation of the Na^+/H^+ antiporter. However, the possible activation of additional pH_i -regulatory processes must be considered. In addition to the Na^+/H^+ antiporter, two other independent pathways have been shown to contribute to H^+ extrusion in activated neutrophils: a H^+ conductance (Henderson et al., 1987, 1988; Nanda and Grinstein, 1991; Kapus et al., 1992) and a vacuolar (V)-type H^+ -ATPase (Nanda et al., 1992). It was therefore of particular interest to test whether peroxides of vanadate induce activation of either or both of these H^+ -extrusion processes in HL60 granulocytes.

Because the V-type H^+ -ATPase and the H^+ conductance are both electrogenic processes, their activity is best appreciated in depolarized cells. We therefore investigated the possible involvement of these extrusion pathways by analysing the effects of V^{4+} -OOH on pH_i in depolarizing (i.e. K^+ -rich) media. As

Figure 3 Assessment of the role of V-type H^+ -ATPases and H^+ conductance in the V^{4+} -OOH-induced pH_i changes in DMSO-differentiated HL60 cells

(a) Differentiated HL60 cells were suspended in K^+ medium in the presence (upper trace) or absence (lower trace) of $4 \mu\text{M}$ DPI from the outset. (b) Differentiated HL60 cells were suspended in K^+ medium containing $4 \mu\text{M}$ DPI from the outset. Where indicated, 100 nM bafilomycin A_1 (Baf) was added to the lower trace. (c) Differentiated HL60 cells were suspended in K^+ medium containing $4 \mu\text{M}$ DPI from the outset. Where noted, 100 nM bafilomycin A_1 (Baf) was added to the upper trace. Bafilomycin A_1 (100 nM) and ZnCl_2 ($50 \mu\text{M}$) were added to the lower trace where indicated. V^{4+} -OOH ($100 \mu\text{M}$) was added to all traces where indicated. Temperature was 37°C . The time scale applies to all traces. Traces are representative of at least three similar experiments.

mentioned above, when differentiated cells were suspended in K^+ medium, V^{4+} -OOH treatment induced intracellular acidification (Figure 3a, lower trace). In order to unmask concurrent H^+ -extruding processes, we prevented metabolic acid generation by blocking the oxidase with DPI. As illustrated by the upper trace of Figure 3(a), in cells suspended in K^+ -rich medium with DPI, treatment with V^{4+} -OOH elicited a sizeable cytosolic alkalinization, averaging $0.12 \pm 0.02 \text{ pH unit}$ ($n = 17$). Because the medium was devoid of Na^+ , this alkalinization is not mediated by the Na^+/H^+ antiport. Therefore alternative H^+ extrusion mechanism(s) must be responsible for the pH_i increase. The following experiments were designed to investigate the possible involvement of two H^+ -efflux processes: the V-type H^+ pump and the H^+ conductance.

Role of V-type H⁺ ATPases

The existence of vacuolar or V-type H⁺-ATPases has been well documented in phagocytic cells such as macrophages and human neutrophils. These H⁺ pumps are primarily responsible for acidification of several intracellular organelles, an event which appears to be crucial in a variety of cellular processes, including receptor recycling, protein sorting and intracellular microbial degradation (Mellman et al., 1986). The hallmark of V-type H⁺ pumps is their sensitivity to bafilomycins, macrolide antibiotics that exert little or no inhibition of other classes of ATPases (Bowman et al., 1988). Studies using bafilomycin A₁ have provided evidence for the presence of V-type H⁺-ATPases in the plasma membrane of macrophages and neutrophils (Swallow et al., 1988, 1990; Nanda et al., 1992; reviewed in Grinstein et al., 1992). We therefore considered the possibility that similar pumps were responsible for the Na⁺-independent alkalization observed in DPI-treated HL60 cells. Figure 3(b) illustrates the effect of bafilomycin A₁ on the V⁴⁺-OOH-induced alkalization of depolarized cells. At 100 nM, a concentration reported to inhibit fully the V-type H⁺-ATPases of macrophages and human neutrophils, bafilomycin A₁ exerted a partial and somewhat variable inhibitory effect on this alkalization. Out of 14 individual experiments, an inhibition was seen in 9. On average, the extent of the V⁴⁺-OOH-induced alkalization was significantly inhibited by the macrolide: ΔpH_i was 0.05 ± 0.01 ($n = 14$) in bafilomycin A₁-treated cells, versus 0.12 ± 0.02 ($n = 17$) in control cells. In contrast, the maximal rate of alkalization was only marginally affected: 0.024 ± 0.005 ($n = 14$) in bafilomycin A₁-treated cells, versus 0.030 ± 0.004 ($n = 17$) in control cells ($P > 0.05$). These findings suggest that V-type pumps contribute to the alkalization observed in Na⁺-free media, but that additional, more rapidly activating, pathways are also engaged.

Role of the H⁺ conductance

The persistence of a residual alkalization under conditions precluding the activity of both the Na⁺/H⁺ antiporter and the V-type H⁺-ATPase suggests the involvement of a third H⁺-extrusion pathway. H⁺-conductive pathways have been detected in the plasma membrane of a variety of cell types, including neurons (Byerly et al., 1984; Thomas and Meech, 1982), oocytes (Barish and Baud, 1984), alveolar cells (DeCoursey, 1991) and phagocytes (Demaurex et al., 1993; Kapus et al., 1993), where they are poised to play a significant role in pH_i homeostasis (for review, see Lukacs et al., 1993). By analogy with human neutrophils, where recent studies point to the activation of a H⁺-conductive pathway after stimulation with phorbol esters (Nanda and Grinstein, 1991; Kapus et al., 1992), it is conceivable that V⁴⁺-OOH similarly activates a H⁺ conductance in HL60 granulocytes. In neutrophils, the H⁺-conductive pathway is insensitive to amiloride and bafilomycin, but is blocked by Zn²⁺, which has been reported to block H⁺ currents in all the biological systems studied to date (Barish and Baud, 1984; Mahaut-Smith, 1989; DeCoursey, 1991; Thomas and Meech, 1982; for review, see Lukacs et al., 1993). We therefore tested the effect of Zn²⁺ on the residual alkalization observed in cells that were stimulated with V⁴⁺-OOH in the presence of bafilomycin. As illustrated in Figure 3(c), the V⁴⁺-OOH-induced alkalization was completely abolished by pretreating the cells with 50 μM Zn²⁺, providing evidence that V⁴⁺-OOH also activates a H⁺-conducting pathway in HL60 granulocytes.

In summary, our results demonstrate that, when stimulated by peroxides of vanadate, three H⁺-extrusion pathways are concomitantly activated and contribute importantly to the regulation

of pH_i in HL60 granulocytes: the Na⁺/H⁺ antiporter, a bafilomycin-sensitive H⁺-extrusion process and a Zn²⁺-sensitive process, likely the H⁺ conductance. It is noteworthy that in undifferentiated cells only the Na⁺/H⁺ antiporter was activated by V⁴⁺-OOH, since little effect on pH_i was noted under conditions allowing detection of the pump and conductance (results not shown). The contribution of V-type H⁺-ATPases and the H⁺-conductive pathway to H⁺ extrusion upon stimulation with V⁴⁺-OOH therefore appears to be restricted to mature phagocytes. A H⁺-conductive pathway is particularly relevant in active mature phagocytes, where it has been suggested to play a dual role: as a mechanism for acid extrusion during cellular activation, and as a pathway for counter-ions to neutralize the activity of the electrogenic NADPH oxidase (reviewed in Lukacs et al., 1993). It is noteworthy in this respect that the H⁺ conductance appears to develop in parallel with expression of the NADPH oxidase during granulocytic differentiation of HL60 cells (A. Y. Qu, A. Nanda, S. Grinstein and J. T. Curnette, unpublished work).

The present results shed some light on the mechanism of activation of the H⁺-extrusion systems. V⁴⁺-OOH has been shown to promote a large increase in phosphotyrosine accumulation in HL60 granulocytes (Trudel et al., 1991), suggesting that at least some of its biological effects are likely to be secondary to inhibition of phosphotyrosine phosphatase activity. Accordingly, a good correlation was found to exist between induction of tyrosine phosphorylation and activation of the respiratory burst, suggesting a causal relationship (Trudel et al., 1991). Vanadate, a less potent tyrosine phosphatase inhibitor, was demonstrated previously to activate the Na⁺/H⁺ exchanger in a variety of systems, including A431 cells (Macara, 1986), Swiss 3T3 fibroblasts (Daniel and Ives, 1987) and in hamster fibroblasts (Paris et al., 1987). Importantly, Paris et al. (1987) found that the effects of vanadate on Na⁺/H⁺ antiport activity were linked to phospholipase C activation (however, see Macara, 1986, for discrepant interpretation). In this context, it is relevant that a unique isoform of this enzyme, phospholipase Cy2, is expressed in HL60 cells, where it can be tyrosine-phosphorylated and presumably activated upon stimulation by V⁴⁺-OOH (Bianchini et al., 1993). Accordingly, parallel elevations of inositol 1,4,5-trisphosphate and of cytosolic Ca²⁺ were recorded within minutes of addition of V⁴⁺-OOH. A concurrent release of diacylglycerol is also expected under these conditions, which is expected to stimulate protein kinase C. Results obtained previously with PMA-stimulated neutrophils suggest that activation of protein kinase C may suffice to activate the Na⁺/H⁺ antiport, as well as the H⁺ pump and conductance (Nanda and Grinstein, 1991; Kapus et al., 1992; Nanda et al., 1992). On the other hand, concentrations of staurosporine that fully inhibit protein kinase C do not eliminate stimulation of the H⁺-extrusion systems by chemoattractant peptides. Instead, activation of the transporters by agonists such as *N*-formylmethionyl-leucyl-phenylalanine is completely prevented by tyrosine kinase inhibitors at concentrations that block the receptor-induced phosphotyrosine accumulation (A. Nanda, unpublished work). These observations further support the involvement of tyrosine phosphorylation during physiological activation. It is therefore conceivable that V⁴⁺-OOH stimulates H⁺ transport by multiple, possibly redundant, signalling pathways. This possibility is currently under investigation.

Though measured independently, the following evidence suggests that the activation of all three H⁺ transporters likely occurs concomitantly, so that the mechanisms are simultaneously operative. First, pretreatment of granulocytes with Zn²⁺ partially diminishes the phorbol-ester-induced alkalization observed in Na⁺ medium (A. Nanda, unpublished work), suggesting a contribution of the H⁺ conductance even in the presence of

antiport activity. Moreover, by direct electrical recordings under patch clamp, the activity of the H^+ conductance can be detected in the presence of external NaCl, conditions conducive to Na^+/H^+ exchange (Demaurex et al., 1993). Furthermore, as described previously (Nanda et al., 1992) and in the present paper, activation of the conductance and the V-type H^+ ATPase occurs simultaneously. However, quantitative estimation of the activity of individual transporters necessitates pharmacological elimination of the others. Together, these findings indicate that the pump, conductance and antiporter are activated simultaneously. Concomitant stimulation of all three transporters ensures co-ordinated and intense H^+ extrusion to prevent deleterious acid accumulation within the cytosol, thereby preserving cell function and favouring continued microbicidal action.

This work was supported by the Medical Research Council of Canada. A.N. is supported by a Studentship from the Medical Research Council of Canada. S.G. is cross-appointed to the Department of Biochemistry of the University of Toronto and is the recipient of an International Research Scholars Award from the Howard Hughes Medical Institute.

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