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Role of isoprenylation in intracellular pH regulation of granulocytes

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Lovastatin was used to study the role of isoprenylated GTP-binding proteins in the regulation of intracellular pH in granulocytic HL-60 cells. The cytosolic acidification that accompanies stimulation by chemoattractants was completely eliminated by lovastatin. The subsequent activation of Na^+/H^+ exchange was partially inhibited by the isoprenylation antagonist. In contrast, the osmotic activation of the Na^+/H^+ antiport was unaffected. The data indicate that the osmotically- and receptor-induced activation of the antiport utilize divergent signalling pathways. The results also provide evidence supporting the notion that cytosolic acidification by chemoattractants results from accumulation of metabolic acid generated during the respiratory burst.

Na^+/H^+ exchange; Antiport; NADPH oxidase; Lovastatin; HL-60 cell

1. INTRODUCTION

Superoxide production is a vital mechanism whereby granulocytes protect the host from microbial infection. NADPH oxidase, the enzyme responsible for superoxide generation, can also be activated in vitro by addition of synthetic chemotactic peptides [1]. Protons, a byproduct of the oxidase reaction, tend to accumulate in the cytosol during the respiratory burst. This tendency is effectively counteracted by pH regulatory mechanisms, including Na^+/H^+ exchange [2,3]. It is thought that chemotactic peptides activate the Na^+/H^+ antiporter directly by shifting its affinity for intracellular H^+ [2,3]. However, the molecular details of this stimulation are poorly understood.

The antiporter can also be activated osmotically, when cells shrink in hypertonic solutions [4]. As in the case of receptor-mediated activation, the stimulatory process is incompletely understood. However, recent evidence obtained in muscle cells from invertebrates suggested that GTP-binding proteins may be involved [5].

A number of small molecular weight GTP-binding proteins, as well as the γ-subunit of heterotrimeric G proteins undergo post-translational processing by isoprenylation [6]. This modification alters the solubility of the GTP-binding proteins and appears to be essential for their role in signal transduction. To assess the role of isoprenylated proteins in pH_i regulation in granulocytes we have used lovastatin, an agent reported to inhibit the activity of hydroxymethylglutaryl CoA reductase, the enzyme responsible for biosynthesis of mevalonate, which is the substrate for protein isoprenylation [7]. The comparatively long incubations required for lovastatin to exert its effect precluded the use of blood neutrophils for these studies. Instead, we utilized the promyelocytic cell line HL-60. These cells, which grow in an undifferentiated state, cease to proliferate and undergo granulocytic differentiation developing a fully activatable NADPH oxidase when treated with dimethyl sulfoxide [8,9]. By inhibiting isoprenylation in these cells, we obtained evidence that different pathways mediate the osmotic and receptor-induced activation of the antiport. In addition, the data provide further evidence that the respiratory burst is the main source of cytosolic acidification during neutrophil activation.

2. MATERIALS AND METHODS

2.1. *Reagents and solutions*

Formyl-methionyl-leucyl-phenylalanine (FMLP) and mevalonic acid were from Sigma. RPMI-1640 medium was purchased from Gibco. Nigericin was from Calbiochem-Behring. 2,7-Biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) acetoxymethyl ester was obtained from Molecular Probes. *N*-Methyl-*N*-(2-methyl-2-propenyl-amino) amiloride (MMPA) was the kind gift of Merck, Sharp and Dohme. Lovastatin was the generous gift of Dr. A. Prat (Merck Frosst, Montréal). The Na^+-solution contained (in mM): 140 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 *N'*-2-hydroxyeth-ylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.3 at 37°C (300 ± 10 mosM).

2.2. *Culture of HL-60 cells*

HL-60 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in an atmosphere of 95% a and 5% CO_2. The cells (4–5 × 10^5/ml) were differentiated into neutrophil-like cells by treatment with 1.3% dimethyl sulfoxide for 6 days [8,9]. The cells

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were treated with lovastatin by addition of a 16-fold concentrated stock solution to a final concentration of 35 μM on day 4 of differentiation. In order to study the effect of mevalonic acid, the compound was added to the cell culture as the mevalonolactone at a concentration of 1 mM on day 4 of differentiation.

2.3. *Cytoplasmic pH measurements*

Cytoplasmic pH was determined fluorimetrically using intact differentiated HL-60 cells, essentially as described earlier for human neutrophils [10]. Cells (10^7/ml) were loaded with the probe BCECF by incubation with the parent acetoxymethyl ester (3 μg/ml, final) for 10 min at 37°C. After washing, 2.5×10^6 cells were used for fluorescence measurement with excitation at 495 nm and emission at 525 nm using 5- and 10-nm slits, respectively. Calibration of the fluorescence signal was performed using nigericin by the method of Thomas et al. [11]. At the concentrations used dimethylsulfoxide, the solvent for delivery of FMLP, had no effect on pH_i.

2.4. *Other methods*

The activity of the NADPH-oxidase was measured in the presence of cytochalasin B (5 μg/ml) as the rate of oxygen consumption, as described earlier [12]. Results are presented as representative traces or as the mean ± S.E. of the number of experiments indicated. Statistical comparisons were made using Student's *t*-test for unpaired samples with a Bonferoni correction.

3. RESULTS

Recently, Bokoch and Prossnitz [13] demonstrated that treatment of HL-60 cells with lovastatin inhibited stimulation of the respiratory burst by chemoattractants, suggesting that activation of the NADPH oxidase is mediated by isoprenylated GTP-binding proteins. To confirm the effectiveness of lovastatin in our experiments, the rate of FMLP-induced oxygen consumption was compared in control cells and in cells pretreated with the drug for 3 days. Lovastatin inhibited the chemoattractant-induced respiratory burst by 92.8%, consistent with the findings of Bokoch and Prossnitz [13]. This inhibition was entirely reversed by mevalonate.

The mean pH_i of differentiated HL-60 cells suspended in Na^+-solution at 37°C was 7.17 ± 0.01 (Table I), similar to values recorded in human neutrophils under comparable conditions [10]. Pretreatment with lovastatin reduced resting pH_i slightly, to 7.05 ± 0.01 ($P < 0.01$). This effect was reversed nearly completely by the addition of exogenous mevalonate (1 mM), the product of the pathway blocked by lovastatin (Table I). The reduction in the resting pH_i is not due to complete obliteration of Na^+/H^+ exchange, since antiport activity could be readily elicited by osmotic stress or by treatment with chemotactic peptide (see below).

As shown in Fig. 1A, stimulation of the cells with FMLP induced a biphasic pH_i change: an incipient acidification followed by a larger, sustained alkalosis. The latter is attributable to activation of the Na^+/H^+ antiporter, as it is eliminated by removal of external Na^+ (not shown) or by addition of the potent amiloride analog MMPA (Fig. 1D). In the presence of MMPA, a sizeable acidification is unmasked, which has been tentatively attributed to generation of metabolic acid by the NADPH oxidase and/or the hexose monophosphate shunt [10]. As shown in Fig. 1E, this acidification is virtually eliminated by lovastatin, in parallel with inhibition of the respiratory burst. Moreover, this inhibition was bypassed by inclusion of mevalonate (Fig. 1F), validating the specificity of the effect of lovastatin. These findings provide additional circumstantial evidence supporting the notion that metabolic acid generated by the respiratory burst is responsible for the drop in pH_i triggered by FMLP.

Inhibition of the metabolic acid burst by lovastatin was also apparent in the absence of MMPA (Fig. 1B). The small and rapid acidification noted in Fig. 1A was absent when isoprenylation was impaired (Fig. 1B). Despite the absence of the acidifying component, however, the rate and extent of the alkalinization mediated by Na^+/H^+ exchange were *decreased* after lovastatin treatment (cf. Fig. 1A and B). The maximal rate of alkalinization in response to FMLP decreased from 0.26 ± 0.02 to 0.15 ± 0.01 pH units/min ($P < 0.001$; Table I). As before, this effect of lovastatin was prevented by the concomitant addition of mevalonate. These findings suggest that isoprenylation is at least partially required for activation of the antiporter by the chemoattractant-receptor complex. Failure of lovastatin to completely

Table I

Effect of lovastatin and mevalonate on pH_i in resting and stimulated HL-60 cells

Condition	Resting pH_i (*n*)	Maximum rate of change of pH_i (units/min)	
		FMLP-stimulated (*n*)	Osmotically-stimulated (*n*)
Untreated	7.17 ± 0.01 (18)	0.26 ± 0.02 (7)	0.32 ± 0.04 (6)
Lovastatin	7.05 ± 0.01 (21)	0.15 ± 0.01 (9)	0.37 ± 0.05 (6)
Mevalonate	7.12 ± 0.01 (9)	0.28 ± 0.05 (3)	0.43 ± 0.02 (3)
Lovastatin + mevalonate	7.13 ± 0.01 (11)	0.29 ± 0.03 (4)	0.33 ± 0.043 (4)

Cells were loaded with BCECF and suspended in Na^+-solution as described in section 2. The cells had been pretreated for 48 h without or with 35 μM lovastatin or a mixture of 35 μM lovastatin and 1 mM mevalonate, as indicated. Where indicated, the cells were stimulated with 0.1 μM FMLP or by increasing the osmolarity with an additional 100 mM NaCl as in Figs. 1 and 2. The absolute pH_i (first column) or the maximal rate of alkalinization (second and third columns) are shown. Values are the mean ± S.E.M. of the number of determinations specified (*n*).

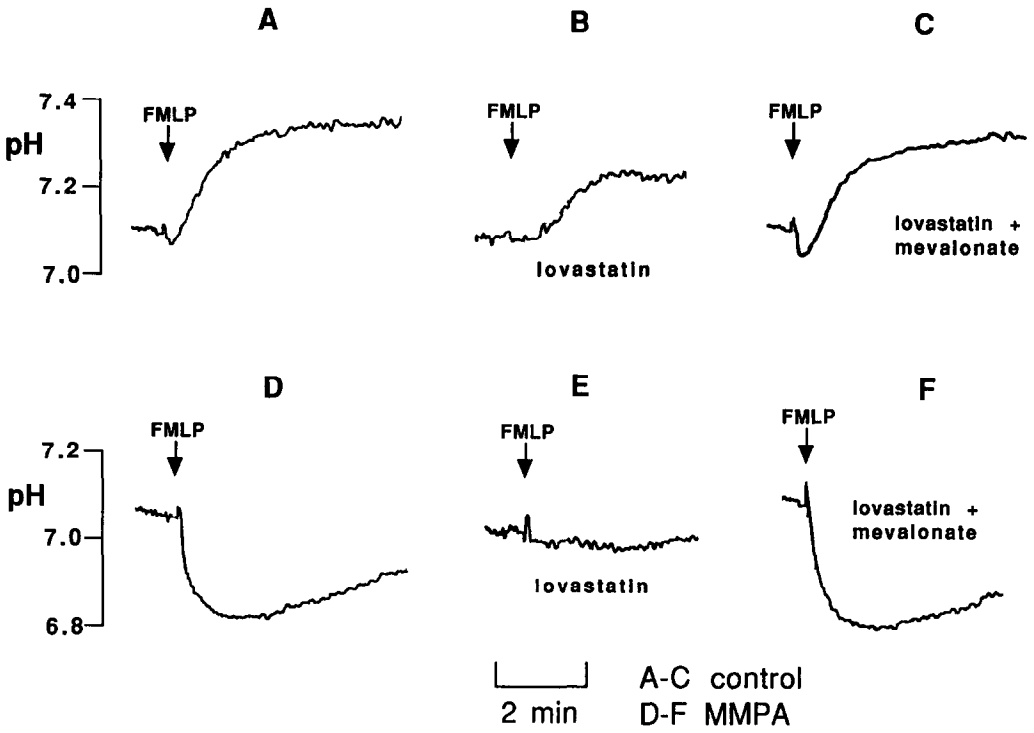

Fig. 1. Effects of lovastatin and mevalonate on the FMLP-induced pH_i changes of HL-60 cells. Fluorimetric pH_i determinations performed in cells suspended in Na^+-solution in the absence (A–C) or presence of 1 μM MMPA (D–F). The cells had been pretreated for 48 h without (A and D) or with 35 μM lovastatin (B and E) or a mixture of 35 μM lovastatin and 1 mM mevalonate (C and F). Where indicated, the cells were stimulated with 0.1 μM FMLP. Traces are representative of at least three experiments of each type.

inhibit stimulation of the antiport may indicate incomplete suppression of isoprenylation or the existence of a separate, isoprenylation-independent activation pathway.

In contrast to the findings in chemoattractant-treated cells, the cytoplasmic alkalinization induced by exposing cells to hypertonic medium (Fig. 2A) was not influenced by pretreatment with lovastatin or mevalonate (Fig. 2B). The rates of osmotically-induced alkalinization averaged 0.32 ± 0.04 pH units/min and 0.37 ± 0.05 pH units/min in the absence and presence of lovastatin, respectively (Table I). These findings indicate that treatment with the drug did not directly interfere with the activity of the Na^+/H^+ antiporter. In addition, the data suggest that different pathways mediate the stimulation of the antiport by receptors and by hypertonic shrinking.

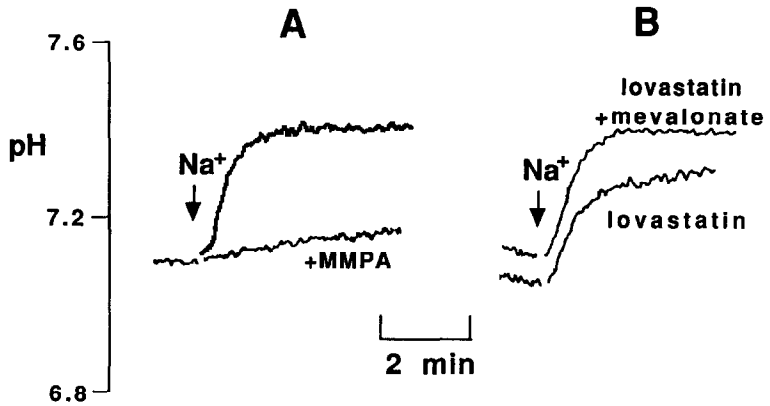

Fig. 2. Effects of lovastatin and mevalonate on the osmotically-induced alkalinization of HL-60 cells. (A) Cells suspended in isotonic Na^+-medium were challenged where indicated by addition of concentrated NaCl (an additional 100 mM). MMPA (1 μM) was added to the bottom trace from the outset. (B) Cells were pretreated with lovastatin (lower trace) or a combination of lovastatin plus mevalonate as in Fig. 1 and suspended in isotonic Na^+-medium. Where indicated, a hypertonic challenge was applied as in (A). Traces are representative of at least three experiments of each kind.

4. DISCUSSION

Prenylation of some small molecular weight GTP-binding proteins and of G proteins is necessary for signal transduction. This has been demonstrated primarily by using lovastatin. Thus, Vincent et al. [14], found that this drug impairs the progression from the G1 to the S phase of the cell cycle in cells stimulated by epidermal growth factor, insulin or insulin-like growth factor I. Similarly, lovastatin was reported to block 1,4,5-inositol triphosphate production, Ca^{2+} influx, secretion, and membrane changes in intact basophilic leukemia cells stimulated by IgE receptor cross-linking [15]. Also, as discussed above, Bokoch and Prossnitz demonstrated that activation of the phagocyte NADPH oxidase is lovastatin-sensitive [13].

The involvement of a heterotrimeric G protein in the FMLP-induced stimulation of Na^+/H^+ exchange was established earlier by means of pertussis toxin [5]. It is noteworthy that this toxin completely eliminates stimulation of the antiport. In contrast, under conditions where the respiratory burst and the associated metabolic acidosis are virtually eliminated, lovastatin only partially inhibits activation of Na^+/H^+ exchange (Table I). It is therefore unlikely that the effects of the isoprenylation antagonist are exerted at the level of the heterotrimeric G protein coupled to the chemoattractant receptor. Instead, it is more likely that inhibition is due to impairment of a small GTP-binding protein.

The data also suggest that a second, lovastatin-insensitive pathway contributes to activation of the antiport. In contrast to the receptor-mediated response, the osmotic activation of the Na^+/H^+ antiporter remained unaffected by lovastatin. This finding implies divergence of the signalling pathways and suggests that isoprenylated GTP-binding proteins are not required for the hypertonic effect. On the other hand, experiments using perfused barnacle muscles suggested earlier that GTP-binding proteins are involved in the osmotic activation of the antiporter [5]. Because some families of GTP-binding proteins are not isoprenylated, these data are not necessarily discordant. It is also possible that invertebrate cells utilize mechanisms different from those of mammals to promote Na^+/H^+ exchange. In this regard, it is relevant that multiple isoforms of the antiport exist and that the trout exchanger, the only non-mammalian form studied to date, differs markedly in structure and properties from the mammalian isoforms [16].

Finally our data provide supportive evidence to the proposal that protons generated as a byproduct of the respiratory burst are the main cause of cytosolic acidification in stimulated granulocytes [10]. Circumstantial evidence was provided by the pari passu inhibition of oxygen consumption and of the pH_i drop observed in cells preincubated with lovastatin.

In summary, the present results (a) point to the involvement of an isoprenylated protein in the activation of Na^+/H^+ exchange by chemoattractants, (b) reveal divergence of the receptor- and osmotically-induced stimulation of the antiport and (c) support the notion that the respiratory burst is responsible for cytosolic acidification during granulocyte activation.

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