Biochimica et Biophysica Acta, 1022 (1990) 203–210
Elsevier

BBAMEM 74717

Regulation of cytoplasmic pH in resident and activated peritoneal macrophages

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(Received 20 July 1989)
(Revised manuscript received 13 October 1989)

Key words: pH, cytoplasmic; Sodium/proton antiport; Monocyte; Macrophage; Amiloride

Cytoplasmic pH (pH_i) has been shown to be an important determinant of the activity of the NADPH oxidase in phagocytic cells. We hypothesized that a difference in pH_i and/or its regulation existed between activated and resident macrophages (RES MOs) which might explain the increased NADPH oxidase activity observed in the former. The pH_i of RES and lipopolysaccharide (LPS)-elicited MOs was examined using the fluorescent dye BCECF. Resting pH_i did not differ between resident (RES) and elicited (ELI) MOs (7.16 ± 0.05 and 7.20 ± 0.05, respectively). pH_i recovery after intracellular acid loading was partially dependent on the presence of Na^+ in the extracellular medium, and was partially inhibited by the Na^+/H^+ antiport inhibitor, amiloride. At comparable pH_i, the rate of acid extrusion during recovery was not different in RES and ELI MOs (1.48 ± 0.12 and 1.53 ± 0.06 mM/min, respectively). In both RES and ELI MOs, approx. 40% of total pH_i recovery was insensitive to amiloride and independent of extracellular Na^+. In both RES and ELI MOs, stimulation with TPA resulted in a biphasic pH_i response: an initial acidification followed by a sustained alkalinization to a new steady-state pH_i. This alkalinization was Na^+-dependent and amiloride-sensitive, consistent with a TPA-induced increase in Na^+/H^+ antiport activity. The new steady-state pH_i attained after TPA stimulation was equivalent in RES and ELI MOs (7.28 ± 0.04 and 7.31 ± 0.06, respectively), indicating comparable stimulated Na^+/H^+ antiport activity. However, the initial acidification induced by TPA was greater in ELI than in RES MOs (0.18 ± 0.02 vs. 0.06 ± 0.02 pH unit, respectively, $P < 0.05$). The specific NADPH oxidase inhibitor diphenylene iodonium (DPI) completely inhibited the respiratory burst but reduced the magnitude of this pH_i reduction by only about 50%. This suggested that the TPA-induced pH_i reduction was due in part to acid produced via the respiratory burst, and in part to other acid-generating pathways stimulated by TPA.

Introduction

Because of the marked pH-sensitivity of many intracellular enzymes, alterations in cell function can be mediated by small changes in cytosolic pH (pH_i). For example, the 0.1 to 0.2 pH unit alkalinization induced by insulin treatment of frog skeletal muscle has been shown to increase phosphofructokinase activity, significantly enhancing glycolytic flux [1,2]. This insulin-induced alkalinization is mediated by an increase in the activity of the Na^+/H^+ antiport. The Na^+/H^+ antiport, which is the best-described mechanism of pH_i regulation in mammalian cells, normally exchanges extracellular Na^+ for intracellular H^+ [3,4]. A Na^+/H^+ antiport-mediated cytoplasmic alkalinization has been documented during differentiation in several cell types [5–12]. Ladoux et al. found that retinoic acid-induced differentiation of HL60 cells from the promyelocytic to the mature granulocytic stage was accompanied by a 0.3 unit increase in pH_i, due to an increase in the activity of the Na^+/H^+ antiport [12]. Similarly, differentiation of both immature HL60 cells and promonocytic U937 cells into monocyte/macrophage-like cells was associated with an increase in pH_i resulting from an activa-

Abbreviations: MO, macrophage; RES, resident; ELI, elicited; LPS, lipopolysaccharide; pH_i, cytoplasmic pH; BCECF, 2′,7′-bis-carboxyethyl-5(6)-carboxyfluorescein; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; O_2^-, superoxide; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DPI, diphenylene iodonium; Hepes-RPMI, bicarbonate-free solution RPMI 1640 buffered with 20 mM Hepes; G-6-*P*, glucose 6-phosphate.

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tion of Na^+/H^+ exchange [13,14]. These findings suggest that an alteration in pH_i may be implicated in the acquisition of specialized functions by the differentiating phagocytic cell.

Production of toxic oxygen radicals during the respiratory burst is necessary to the activated MO's effective antimicrobial and tumoricidal function [15–17]. One of the functional properties which distinguishes the activated from the resident MO is its ability to undergo an enhanced respiratory burst in response to stimulation. NADPH oxidase, the enzyme directly responsible for production of toxic oxygen radicals, displays a higher V_{max} and a lower K_m in activated compared to RES MOs [18–21]. The underlying mechanisms of this increase in NADPH oxidase activity have not been fully elucidated [22–25]. Since the NADPH oxidase activity of phagocytic cells has been shown to be sensitive to small changes in pH_i [26,27], we hypothesized that a difference in pH_i and/or its regulation by the Na^+/H^+ exchanger might exist between RES and activated MOs. The purpose of these experiments was to compare pH_i and its regulation in RES and activated peritoneal MOs.

Materials and Methods

Materials and solutions. MEM (Eagle) with Earle's salts without L-glutamine and L-leucine, heat inactivated FCS, RPMI 1640 medium (with L-glutamine, HCO_3^--free), Ca^{2+}- and Mg^{2+}-free HBSS, and PBS were obtained from Gibco. All solutions were found to be endotoxin-free (< 0.03 ng/ml) using *Limulus* amebocyte lysate assay from Associates of Cape Cod, Wood's Hole, MA. Superoxide dismutase (from human erythrocytes), cytochrome *c* (from horse heart, Type VI), formic acid 95–97%, Triton X-100, TPA, 2-deoxy-D-glucose, nigericin, monensin, Mes, Trizma base, and Hepes were from Sigma. LPS B (*E. coli* 0.111:B4) was purchased from Difco. Heparin sodium (1000 U.S.P. U/ml) came from Organon Canada, and Bio-Rad protein assay dye reagent concentrate from Bio-Rad. Amiloride and *N*-(2-methoxy-5-nitrobenzyl)amino-amiloride were the gift of Dr. E.J. Cragoe Jr., Merck, Sharp and Dohme Laboratories. The acetoxymethyl ester of BCECF was from Molecular Probes, Eugene, OR. DPI was the gift of Dr. A.R. Cross, University of Bristol. Hepes-RPMI was prepared by titrating RPMI with 20 mM Hepes-Na to pH 7.35 at 37°C. Na^+ solution contained (in mM) 140 NaCl, 5 KCl, 10 glucose, 2 $CaCl_2$, and 10 Hepes, pH 7.35 at 37°C. K^+ solution was prepared by isoosmotic replacement of NaCl by the KCl, but was otherwise identical. The osmolarity of all media was adjusted to 290 ± 5 mosM with the major salt.

Cell isolation and characterization. Peritoneal MOs were harvested from 6–8-week-old female Swiss Webster mice by peritoneal lavage with 10 ml MEM containing 10% FCS and 10 U/ml sodium heparin. RES MOs were obtained from untreated mice, while ELI MOs were obtained by lavage 4 days after i.p. injection with 30 μg *E. coli* LPS. The resultant peritoneal cells were washed twice with cold HBSS (5°C), counted using a Coulter Counter model Z_F, and resuspended in MEM with 10% FCS. The proportion of peritoneal cells identified as MOs on Wright's stain and by non-specific esterase staining was 25% for RES cells, and 40% for ELI cells. MO populations were enriched by adherence. Cells were loaded onto either plastic tissue culture wells (Flow Laboratories) for measurement of O_2^- production, or onto glass coverslips (Bellco Glass Co) for pH_i measurements, incubated for two hours at 37°C in 5% CO_2, and washed twice with warm HBSS (37°C). MO enrichment to > 93% was confirmed by Wright's stain and non-specific esterase staining following incubation and washing, for both RES and ELI cells. Peritoneal cells were loaded onto wells or coverslips to achieve a final number of approx. $5 \cdot 10^5$ MOs per well, and approx. $2 \cdot 10^6$ MOs per coverslip, following incubation and washing. Cells adherent to wells or coverslips were found to be > 95% viable by Trypan blue exclusion prior to O_2^- or pH_i measurements.

Measurement of O_2^- production. O_2^- release was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c*. A reaction mixture of 1 ml HBSS containing 80 μM cytochrome *c* was added to $5 \cdot 10^5$ plated MOs, with or without superoxide dismutase (40 μg). The reaction was initiated with 2 μg TPA, with an equivalent volume of HBSS added instead to control wells. Preliminary studies investigating the time course of O_2^- release showed that for both RES and LPS ELI MOs, O_2^- release had reached a plateau by 2 h of incubation with this dose of TPA. Following a 2-h incubation at 37°C in 5% CO_2, reduced cytochrome *c* in the supernatant was evaluated by absorbance at 550 nm. O_2^- production was calculated using an absorption coefficient of 21 $mM^{-1} \cdot cm^{-1}$ for reduced cytochrome *c*. Protein content of each well was determined according to the Bradford method [28]. Results are expressed as nmol O_2^-/mg protein.

Measurement and manipulation of pH_i. pH_i of cells adherent to coverslips was measured fluorimetrically using the pH-sensitive, cytoplasmic, fluorescent dye BCECF. Coverslip-adherent cells were loaded by incubation in Hepes-RPMI with 2 μg/ml of the precursor acetoxymethyl ester of BCECF for 15 min at 37°C. Fluorescence microscopy studies confirmed that BCECF was uniformly distributed in the cytoplasmic compartment, demonstrating its usefulness as an indicator of pH_i. After washing with fresh Hepes-RPMI, the coverslip was placed in 2 ml of the indicated medium in a plastic cuvette, so that the surface of the coverslip was oriented at 45° to the incident excitation beam. Stabilization of the coverslip was accomplished by designing a

cover for the plastic cuvette which had a shallow groove oriented diagonally on its undersurface within which the coverslip fit snugly. The lower edge of the coverslip rested on a raised platform at the bottom of the cuvette. This permitted the use of a stir bar to ensure uniform mixing of the medium. Chemicals were added to the medium through an aperture in the corner of the cuvette cover. Fluorescence was measured at 37 °C with magnetic stirring using a Perkin Elmer LS-5 fluorescence spectrometer, with excitation at 495 nm and emission at 525 nm, using 5 and 10 nm slits, respectively. Calibration was done using a combination of monensin and nigericin for Na^+ medium, and nigericin alone for K^+ medium, followed by titration with aliquots of 1 M Tris or 1 M Mes, as described [29].

Acid-loading was accomplished by transfer to NH_4^+-free K^+ medium following preincubation of adherent cells in Hepes-RPMI containing 10 mM NH_4Cl for 10 or 15 min at 37 °C. The principles behind this NH_4^+ 'prepulse' method of acid loading are as follows: during an exposure to NH_4^+, pH_i at first rises rapidly, due to the influx of NH_3 into the cytoplasmic compartment where it combines with H^+ to form NH_4^+. This is followed by a slow decline in pH_i, due to passive influx of NH_4^+, which dissociates to form NH_3 and H^+. When cells are then transferred to a medium free of NH_4^+, NH_3 rapidly leaves the cell, leading to a further rapid dissociation of internal NH_4^+ into NH_3 and H^+, and a fall in pH_i to a level lower than the initial one. The degree of acidification produced by this technique can be controlled by the choice of $[NH_4^+]_o$ and length of exposure.

Other methods. G-6-*P* was depleted by preincubating in PBS containing 10 mM deoxyglucose for 30 min at 37 °C. All measurements were carried out at 37 °C.

Statistics. Results are presented as representative traces of at least three experiments, or as mean ± 1 S.E. of the number of experiments indicated in parentheses. Statistical significance was established using the *t*-test for unpaired samples.

Results

Resting pH_i

To determine whether a difference in resting pH_i existed between RES and ELI MOs, pH_i was measured in physiological Na^+ medium at 37 °C. These studies demonstrated no difference in resting pH_i between RES and ELI MOs (7.16 ± 0.05 and 7.20 ± 0.05, respectively, $n = 10$).

pH_i Recovery from cytoplasmic acidification

In other cell types, Na^+/H^+ exchange has been found to be important in protecting against cytoplasmic acidification. Reduction in cytoplasmic pH to a level below resting pH_i activates the antiport, which mediates

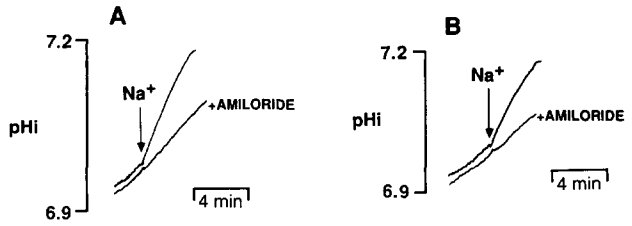

Fig. 1. Na^+-dependent and Na^+-independent cytoplasmic alkalinization of acid-loaded macrophages. BCECF-loaded macrophages were preincubated with 10 mM NH_4Cl for 10 min at 37 °C. pH_i of resident (A) or LPS-elicited (B) macrophages was measured fluorimetrically as described under Materials and Methods. Traces start upon transfer of cells to NH_4^+-free K^+ medium with (bottom traces) or without (top traces) amiloride (200 μM). 40 mM NaCl was added where indicated by arrows. Traces are representative of at least three experiments. Temperature: 37 °C.

net exchange of extracellular Na^+ for intracellular H^+ until the steady-state pH_i is regained [3,4]. To determine whether differences in Na^+/H^+ activity exist between RES and ELI MOs, intracellular acid loading was used to stimulate Na^+/H^+ exchange. Preincubation in 10 mM NH_4Cl for 10 min at 37 °C followed by transfer to NH_4^+-free K^+ medium consistently yielded acidification to a pH_i of approximately 6.95 in both RES and ELI MOs. pH_i recording commenced upon transfer to K^+ medium (Fig. 1). Even in the absence of extracellular Na^+, a significant rate of pH_i recovery was observed in both RES and ELI cells. Investigation of the mechanism of this Na^+-independent pH_i recovery is discussed below. In order to compare rates of Na^+/H^+ exchange-mediated pH_i recovery, a bolus of 40 mM NaCl was added to acid-loaded RES or ELI cells in K^+ medium, to initiate Na^+/H^+ exchange (Fig. 1). In both RES and ELI MOs, addition of 40 mM NaCl resulted in a rapid increase in the rate of pH_i recovery. In order to quantitate the contribution of the Na^+/H^+ antiport to pH_i recovery, and to quantitatively compare pH_i recovery in RES and ELI MOs, the rates of acid extrusion by RES and ELI MOs during recovery from cytoplasmic acidification were determined in the absence or presence of the Na^+/H^+ antiport inhibitor, amiloride (200 μM). Acid extrusion rates (in mM/min) were calculated as the product of the rate of change of pH_i (in pH unit/min) and the buffering capacity (in mM/pH unit), and represented the rate at which acid equivalents were eliminated from the cytoplasm. Buffering capacity, which was estimated independently by pulsing RES or ELI MOs with 5 mM of the weak base NH_4Cl, did not differ between the two cell types (approximately 20 mM/pH unit, in the pH_i 6.5 to 7.0 range). As shown in Table I, the rate of acid extrusion during recovery from acid loading to pH_i 6.95 did not differ between RES and ELI MOs, either in the absence (0.99 ± 0.05 vs. 1.02 ± 0.06 mM/min, respectively, $n = 3$) or presence (0.42 ± 0.02 vs. 0.40 ± 0.01 mM/min, respectively, $n = 3$) of amiloride. In both MO

TABLE I

Effect of amiloride on acid extrusion by acid-loaded macrophages

Initial pH_i	Cell type	Acid extrusion rate [a] (mM/min)	
		without amiloride	with amiloride
6.95	RES	0.99 ± 0.05	0.42 ± 0.02 [c]
	ELI	1.02 ± 0.06 [b]	0.40 ± 0.01 [b,c]
6.65	RES	1.48 ± 0.12 [d]	0.53 ± 0.04 [c,d]
	ELI	1.53 ± 0.06 [b,d]	0.54 ± 0.03 [b,c,d]

[a] BCECF-loaded macrophages were preincubated with 10 mM NH_4Cl at 37 °C for either 10 or 15 min, to achieve acid loading to a pH_i of 6.95 or 6.65, respectively. Cells were transferred to NH_4^+-free K^+ medium, with or without 200 μM amiloride. Rate of pH_i recovery in resident or LPS-elicited macrophages was measured immediately after addition of 40 mM NaCl, as indicated in Fig. 1. Acid extrusion rate was calculated as the product of pH_i recovery rate (in pH unit per min) and cytosolic buffering capacity (approx. 20 mM/pH, estimated independently using weak electrolyte pulses). The data are means ± S.E. of three experiments. Temperature: 37 °C.

[b] n.s. (not significant) vs. RES at same initial pH_i, with or without amiloride.

[c] $P < 0.01$ vs. without amiloride, at same initial pH_i.

[d] $P < 0.05$ vs. pH_i 6.95, with or without amiloride.

types, amiloride (200 μM) reduced the rate of pH_i recovery by approximately 60% (Table I, $P < 0.01$ without vs. with amiloride, $n = 3$). To compare Na^+/H^+ exchange activity in RES and ELI MOs under a greater acid stress, the cells were acid loaded to a pH_i of approx. 6.65 by preincubating in 10 mM NH_4Cl for 15 min. The rate of acid extrusion was faster during recovery from acid loading to pH_i 6.65 than to pH_i 6.95 (Table I, $P < 0.05$, $n = 3$). This was consistent with the known pH_i-dependence of the rate of Na^+/H^+ exchange in other cell types [3,4,30]. However, as found at pH_i 6.95, there was no difference in the rate of acid extrusion by RES and ELI cells either in the absence (1.48 ± 0.12 vs. 1.53 ± 0.06 mM/min, respectively, $n = 3$) or presence (0.53 ± 0.04 vs. 0.54 ± 0.03 mM/min, respectively, $n = 3$) of amiloride. In both RES and ELI MOs, amiloride (200 μM) reduced the rate of pH_i recovery from acid loading to pH_i 6.65 by approximately two-thirds (Table I, $P < 0.01$ with vs. without amiloride, $n = 3$). Taken together, these data suggested that the rate of pH_i recovery mediated by Na^+/H^+ exchange was equivalent in the two cell types.

The finding of an amiloride-insensitive component of pH_i recovery in RES and ELI MOs was unexpected (Table I). To rule out the possibility that this residual recovery was due to incomplete inhibition of Na^+/H^+ exchange by amiloride, recovery was studied in the presence of up to 800 μM amiloride ($K_i \approx 6$ μM in rat lymphocytes, Smith, J. and Grinstein, S., unpublished data) or 12.5 μM of the potent amiloride analogue *N*-(2-methoxy-5-nitrobenzyl)aminoamiloride ($K_i \approx 40$ nM in rat lymphocytes). Neither increased concentrations of amiloride nor this potent analogue inhibited the residual recovery, suggesting that it was not Na^+/H^+ antiport-mediated. Furthermore, in both RES and ELI MOs, a significant rate of pH_i recovery was observed in the absence of external Na^+, which was not affected by the addition of 40 mM Na^+ in the presence of amiloride (Fig. 1A, B). This Na^+-independence confirmed that the residual pH_i recovery was not mediated by Na^+/H^+ exchange. One potential alternate mechanism of recovery was Na^+-independent HCO_3^-/Cl^- exchange, which by exchanging intracellular chloride for extracellular bicarbonate, could conceivably result in Na^+-independent cytoplasmic alkalinization [31]. This possibility was unlikely because the extracellular media used throughout were nominally HCO_3^--free. However, since nominally HCO_3^--free media could potentially contain sufficient HCO_3^- in equilibrium with atmospheric CO_2 to support HCO_3^-/Cl^- exchange, this possible mechanism of pH_i recovery was tested using known inhibitors of HCO_3^-/Cl^- exchange. Neither SITS (1 mM) nor DIDS (100 μM) inhibited Na^+-independent pH_i recovery in RES or ELI cells, indicating that HCO_3^-/Cl^- exchange was not responsible for the Na^+-independent, amiloride-insensitive pH_i recovery in these cells.

Effect of TPA on pH_i

Stimulation with the phorbol ester TPA has been shown to activate Na^+/H^+ exchange in thioglycolate-elicited rat peritoneal MOs, resulting in cytoplasmic alkalinization [32]. Since phagocytic cell NADPH oxidase activity has been shown to vary directly with pH_i [26,27], we hypothesized that a difference in the degree of TPA-induced alkalinization might exist between RES and ELI MOs, that could account for the augmented respiratory burst seen in the latter cell type in response to TPA (Table II). The effect of TPA (10^{-7} M) on pH_i in the two cell types was therefore compared (Fig. 2). Stimulation with TPA in Na^+ medium induced a biphasic response in both RES and ELI MOs: an initial apparent acidification was followed by a sustained alkalinization phase (Fig. 2A, B). Before investigating the mechanisms underlying this response, it was necessary to ensure that the transient apparent acidification was genuine. The possibility existed that the decrease in fluorescence observed following TPA stimulation might reflect a nonspecific change in MO shape or adherence, rather than a genuine decrease in pH_i. To rule out this possibility, cells were stimulated with TPA in K^+ medium in the presence of the K^+/H^+ exchanging ionophore nigericin (1 μM). The effect of this ionophore in K^+ medium is to maintain pH_i at the same level as extracellular pH, so that pH_i cannot change in response to TPA stimulation. There was no change in the fluorescence signal when TPA was added to either RES or ELI MOs in K^+ medium containing 1 μM nigericin, demonstrating that the acidification phase of the TPA response was genuine.

TABLE II

Macrophage cytoplasmic pH reduction [a] and superoxide production [b] in response to TPA

Cell type	Control [c]		G-6-*P* depleted [d]	
	pH_i reduction	O_2^- production	pH_i reduction	O_2^- production
RES	0.06 ± 0.02	55.2 ± 7.8	0	5.5 ± 0.2
ELI	0.18 ± 0.02 [e]	507.5 ± 60.0 [f]	0	3.4 ± 0.7

[a] pH_i of BCECF-loaded macrophages was measured fluorimetrically as described under Materials and Methods. Resident (RES) or LPS-elicited macrophages in Na^+ medium were treated with TPA (10^{-7} M) as shown in FIg. 2A, B. The resultant transient acidification was measured at its maximum point.

[b] Superoxide production in response to TPA stimulation was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c*, as described under Materials and Methods, and expressed as nmoles produced over a 2-h incubation period/mg cell protein.

[c] The data are means ± S.E. of ten experiments.

[d] Glucose 6-phosphate was depleted by preincubation in phosphate-buffered saline containing 10 mM deoxyglucose for 30 min at 37 °C. The data are means ± S.E. of three experiments.

[e] $P < 0.005$ vs. RES.

[f] $P < 0.001$ vs. RES.

Previous studies had shown that neutrophils undergo an initial cytoplasmic acidification in response to TPA [33]. Because the plated MOs were contaminated with a small number of neutrophils (approx. 2–5%), it was possible that the transient TPA-induced acidification in fact represented a change in neutrophil pH_i. To rule out this possibility, adherent RES or ELI peritoneal cells were incubated overnight and then tested for pH_i response to TPA. Following this incubation, there were

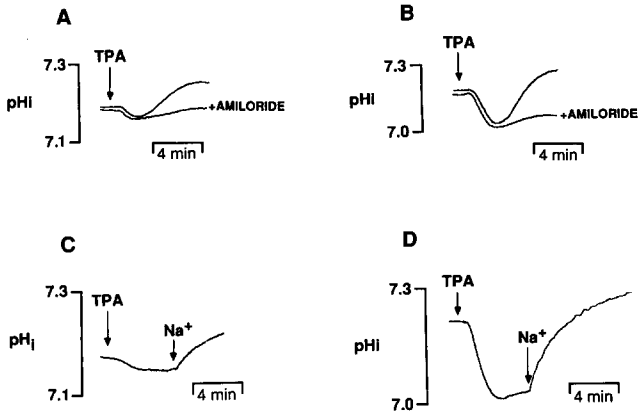

Fig. 2. Effect of TPA on macrophage pH_i. pH_i of BCECF-loaded macrophages was measured fluorimetrically as described under Materials and Methods. Resident (A, C) or LPS-elicited (B, D) macrophages were treated with TPA (10^{-7} M) where indicated by arrows. (A, B) Cells were placed in Na^+ medium with (bottom traces) or without (top traces) amiloride (200 μM). (C, D) Cells were placed in K^+ medium to which 40 mM NaCl was added where indicated by arrows. Traces are representative of at least three experiments. Temperature: 37 °C.

no remaining viable neutrophils, and the adherent cell population consisted of 100% MOs. There was no difference in the acidification phase of the response to TPA following overnight incubation for either RES or ELI cells. Thus, the observed TPA-induced acidification could not be attributed to neutrophil contamination.

Cytoplasmic alkalinization phase of the TPA response

To determine whether the cytoplasmic alkalinization which followed TPA treatment of cells in Na^+ medium was mediated by enhanced Na^+/H^+ exchange, the pH_i response to TPA was studied in the presence of amiloride. As illustrated in Fig. 2A and B, amiloride (200 μM) inhibited the TPA-induced alkalinization in both RES and ELI MOs in Na^+ medium. To further confirm that the alkalinization phase was Na^+/H^+ exchange-mediated, cells were treated with TPA in Na^+-free K^+ medium (Fig. 2C, D). In both RES and ELI MOs, the alkalinization phase was inhibited in K^+ medium, and it was only upon addition of 40 mM NaCl that significant alkalinization occurred, further demonstrating the Na^+-dependence of the alkalinization phase. Taken together, these data demonstrated that in both cell types the TPA-induced alkalinization was mediated by enhanced Na^+/H^+ exchange.

Since, in a physiological Na^+ medium, a difference in pH_i could potentially account for functional differences between RES and ELI MOs upon stimulation, the final pH_i following TPA-induced alkalinization was compared in RES and ELI cells. The final pH_i was significantly higher than the initial baseline pH_i in both RES (7.28 ± 0.04 vs. 7.16 ± 0.05, $P < 0.05$, $n = 10$) and ELI (7.31 ± 0.06 vs. 7.20 ± 0.05, $P < 0.05$, $n = 10$) MOs. Clearly, the final pH_i following this Na^+/H^+ exchange-mediated alkalinization was not different between RES and ELI MOs. Thus, there was no evidence that the extent of stimulation of the Na^+/H^+ antiport in response to TPA differed in the two cell types.

Cytoplasmic acidification phase of the TPA response

As noted, previous studies of neutrophil pH_i have demonstrated a transient initial acidification following stimulation with TPA [33]. This was attributed to the production of increased amounts of metabolic acid by neutrophils during the TPA-induced respiratory burst. We hypothesized that respiratory burst-associated acid production might also be responsible for the initial acidification observed in TPA-stimulated MOs. As shown in Table II, much higher levels of O_2^- were produced in response to TPA by ELI than by RES MOs (507.5 ± 60.0 vs. 55.2 ± 7.8 nmol/mg, respectively, over a 2-h incubation, $n = 10$ each, $P < 0.001$). In keeping with the postulated relationship between the initial pH_i reduction and respiratory burst activity, the TPA-induced reduction in pH_i was consistently greater in ELI than in RES cells (0.18 ± 0.02 vs. 0.06 ± 0.02 pH units,

respectively, $P < 0.005$, $n = 10$, Table II). When pH_i reduction was plotted against O_2^- production by cells of the same type (RES or ELI) assayed in parallel with the pH_i measurement, a significant correlation between pH_i reduction and O_2^- production in response to TPA stimulation was observed ($r = 0.8$, $P < 0.001$, $n = 20$). This result supported the hypothesis that metabolic acid generated during the respiratory burst was responsible for the cytoplasmic acidification observed upon TPA stimulation.

To further test this hypothesis, cells were depleted of the substrate for the respiratory burst and then the pH_i response to TPA studied. Normally, NADPH is generated through the hexose monophosphate shunt, which requires G-6-*P*. Presumably, impairment of the respiratory burst by depletion of G-6-*P* would inhibit the initial acidification seen after TPA stimulation. As expected, G-6-*P* depletion markedly reduced O_2^- production in response to TPA in both RES and ELI MOs (90% and 99% reductions, respectively, $n = 3$ each, Table II). Depletion of G-6-*P* also completely eliminated the TPA-induced pH_i reduction in both cell types, without altering cell viability (consistently > 95%).

Since G-6-*P* may serve as a substrate for several metabolic processes, the possibility existed that G-6-*P*-depletion could inhibit acid-generating pathways other than the respiratory burst. To investigate whether the TPA-induced pHi reduction was solely due to acid produced via the respiratory burst, the specific NADPH oxidase inhibitor, DPI [34], was used. LPS-elicited MOs were treated for 4 h with 10 μM DPI (viability > 85%). This concentration of DPI reduced O_2^- production in response to TPA from 680.5 ± 99.9 to 12.8 ± 1.4 nmol/mg protein, over a 2-h incubation ($n = 3$, $P < 0.001$). Parallel measurements of pHi response in K^+ medium demonstrated that treatment with DPI diminished the magnitude of pH_i reduction (control 0.24 ± 0.02, $n = 5$ vs. DPI-treated 0.12 ± 0.02, $n = 6$, $P < 0.001$), but not to the extent observed with G-6-*P* depletion. Taken together, these findings suggest that, while part of the TPA-induced pH_i reduction resulted from acid generated via the respiratory burst and/or the associated hexose monophosphate shunt, other acid-generating pathways may also contribute to this reduction.

Discussion

The present studies were performed to determine whether differences in pH_i or its regulation could account for functional differences noted between resident and activated MOs. The two cell types had equivalent resting pH_i levels, and displayed equivalent Na^+/H^+ antiport activity in response to acid loading, and to stimulation with TPA. The lack of evidence of a difference in Na^+/H^+ exchange activity between RES and ELI MOs found here is in contrast to the studies of Costa-Casnellie et al. [11] and Ladoux et al. [12–14] who examined Na^+/H^+ exchange in immature vs. mature HL-60 and U937 cells. This discrepancy may be due to the use of freshly harvested untransformed cells as opposed to a cell line. Perhaps more importantly, the in vivo activation of MOs with LPS may differ significantly from the in vitro induction of differentiation from immature to mature HL60 cells, given that modulation by extracellular factors could differ considerably under these two circumstances, and that RES MOs are mature cells while promyelocytic HL-60 cells are immature precursor cells.

The ability to recover from an intracellular acid load is important to maintenance of optimal enzymatic activity, and thus cell function. In phagocytic cells, this capacity may be of particular importance, since they may be required to carry out their antimicrobial and tumoricidal activities under the stress of an acidic extracellular environment, such as that which prevails in an abscess or tumor. In the neutrophil, a sufficiently acidic extracellular milieu can result in intracellular acidification, which has been shown to impair the respiratory burst [35]. The present studies demonstrate that MOs possess at least two efficient mechanisms of recovery from an intracellular acid load. pH_i regulation by the Na^+/H^+ exchanger is a property common to numerous other mammalian cell types [3,4]. By contrast, the Na^+-independent, amiloride-insensitive and SITS-insensitive mechanism of pH_i recovery demonstrated here has not been documented in other non-epithelial cells. In recent studies using thioglycolate-elicited mouse peritoneal MOs in suspension, we have shown that this mechanism of pH_i recovery is sensitive to *N*-ethylmaleimide and *N,N'*-dicyclohexylcarbodiimide, which are inhibitors of the H^+-ATPase of endosomal membranes, and is also inhibited by ATP depletion [36]. Furthermore, we have also recently found that bafilomycin A_1, a specific inhibitor of H^+-ATPases of the vacuolar type [37], inhibits the Na^+-, HCO_3^--independent pH_i recovery of thioglycolate-elicited MOs [44]. These properties suggest that this alternate mode of pH_i regulation may be mediated through the action of a H^+-ATPase extruding H^+ ions from the cytoplasm. It is possible that the MO has evolved such a specialized proton pumping mechanism of pH_i regulation in order to protect itself against intracellular acidification in the acidic milieu of tumors and abscesses. Na^+/H^+ exchange via the antiporter has been shown to be inhibited at low extracellular pH [3,38]. Under these conditions extracellular HCO_3^- is also depleted, impairing exchange of extracellular HCO_3^- for intracellular Cl^-. Thus, other potential mechanisms of pH_i recovery are inhibited in acidic environments, perhaps necessitating the development of another efficient means of pH_i regulation. This Na^+- and HCO_3^--independent mecha-

nism of pH_i recovery was observed in both RES and ELI acid-loaded MOs, suggesting that both MO types may need to function in such acidic environments.

The major difference in pH_i regulation between resident and activated MOs was the degree of initial acidification that occurred following TPA stimulation. Simchowitz previously suggested that the cytoplasmic acidification observed in neutrophils stimulated with formylmethionylleucylphenylalanine in low external Na^+ medium was due to the reversal of Na^+/H^+ exchange [39]. This possibility was ruled out in the present studies both because the acidification occurred in 140 mM Na^+ medium, where the gradient would be strongly against reversed Na^+/H^+ exchange, and because the acidification was not inhibited by amiloride (Fig. 2). Based on previous studies in neutrophils demonstrating that the magnitude of the initial acidification paralleled the extent of the respiratory burst in response to TPA [30,33], it was postulated that the pH_i reduction in MOs was due to generation of metabolic acid during the respiratory burst. Indeed, there was a significant correlation between pH_i reduction and O_2^- production. However, subsequent studies using a specific inhibitor of the respiratory burst demonstrated that only part of the pH_i reduction could be attributed to acid generated via the respiratory burst. Acid produced by other metabolic processes stimulated by TPA may account for the remainder. Recent studies by Naccache and colleagues [40] have also demonstrated a lack of correlation between the magnitude of the respiratory burst and the initial pH_i reduction in response to formylmethionylleucylphenylalanine and leukotriene B_4 in human neutrophils, suggesting an alternate source for acid equivalents.

Both Simchowitz [26] and Nasmith and Grinstein [27] demonstrated that in neutrophils, the magnitude of the respiratory burst increased with cytoplasmic alkalinization, indicating the somewhat alkaline pH optimum of the NADPH oxidase. However, the similarity of the resting pH_i levels, and the new post-TPA steady-state pH_i levels in RES and LPS ELI MOs suggests that the enhanced NADPH oxidase activity characteristic of the latter cell type is not based on more optimal pH_i conditions. Furthermore, the greater cytoplasmic acidification induced by TPA in activated compared to resident MOs would not be expected to enhance NADPH oxidase activity in the former, again suggesting that differences in pH_i regulation do not underlie the difference in respiratory burst activity. This difference may instead be based on differing concentrations of one or more of the multiple components which make up the NADPH oxidase system [41], or on differences in modulation of their activity by other factors, such as kinases and phosphatases [42]. In addition, the present studies do not rule out the possibility that a transient acidification might indirectly prime MOs for the respiratory burst by elevating cytosolic calcium [36], a mechanism analogous to LPS priming of human neutrophils [43].

In summary, differences in pH_i or its regulation could not account for functional differences between resident and activated MOs. Two efficient mechanisms of pH_i recovery from intracellular acidification were demonstrated in both MO types. Although pH_i recovery via the Na^+/H^+ antiport is common to many mammalian cell types, pH_i recovery via a Na^+-independent, amiloride- and SITS-insensitive mechanism is a unique finding in nonepithelial cells. This may represent the action of a specialized proton extrusion mechanism developed by the MO to facilitate its efficient function under the stress of an acidic extracellular environment.

Acknowledgments

This work was supported by the Medical Research Council of Canada and a grant from Merck Frosst Canada, Ltd. C.S. is the recipient of an Ethicon-Society of University Surgeons Surgical Research Fellowship. S.G. is the recipient of a Medical Research Council Scientist Award.

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