Internal pH of human neutrophil lysosomes

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Received 4 October 1982

Granule Lysosome Methylamine Neutrophil pH Phagocytic vacuole

1. INTRODUCTION

The ingestion of microorganisms by human neutrophils involves the formation of a particle-containing phagocytic vacuole (the phagosome). Several studies [1, 2] using microorganisms labelled with pH indicator dyes have demonstrated a progressive fall in pH of the phagocytic vacuole (possibly after a transient initial rise [3]) after phagocytosis. This acidification of the phagocytic vacuole is considered an important antimicrobial event, but its mechanism has not been elucidated. Several possibilities have been offered, including local production of lactate [4,5], a carbonic anhydrase-dependent proton-translocating system [6], NADPH-oxidase dependent formation of perhydroxyl radicals ($\cdot O_2H$) which then dissociate into superoxide radicals ($O_2^{\cdot -}$) and protons [7], and the discharge of lysosomal contents into the phagosome [8].

Two observations favor the possibility that the lysosome is the source of acidification:

(i) The kinetics of the decline in phagosome pH coincide with the kinetics of lysosome fusion with the phagosome to form the phagolysosome [9];

(ii) In other cells wherein the intralysosomal pH has been measured, it is decidedly acidic [10,11].

In an attempt to elucidate the role of the lysosome in phagosome acidification, we measured the intralysosomal pH of isolated neutrophil lysosomes, and the effect of varying external ion concentrations.

2. MATERIALS AND METHODS

^3H_2O (1 mCi/ml), [^{3}H]polyethylene glycol (M_r 4000, 1.2 μCi/g), [^{14}C]sucrose (400–700 mCi/mmol), [^{14}C]acetate (45–60 mCi/mmol), and [^{14}C]methylamine (40–60 mCi/mmol) were obtained from New England Nuclear (Boston MA). Silicone oil (type SF 1250) was obtained from General Electric Co. (Waterford NY).

2.1. *Isolation of lysosomes*

Heparinized blood was obtained from healthy volunteers after informed consent. Neutrophils were isolated by Hypaque-Ficoll density gradient centrifugation and dextran sedimentation as in [12]. Cells were disrupted by nitrogen cavitation at 350 lb.in^{-2} for 20 min at 4°C and a lysosome-rich fraction was isolated by differential centrifugation [13].

2.2. *Measurement of intralysosomal pH*

Lysosome pellets were resuspended in Hanks' balanced salt solution without added magnesium or calcium (HBSS). Aliquots of 0.5 ml lysosome suspension were incubated with 5 μl ^3H_2O, [^{3}H]PEG, [^{14}C]sucrose, or [^{14}C]methylamine solutions. Incubations were continued for 10 min at 37°C except where otherwise specified; in some experiments, indicated below, incubation was carried out in an ice bath (0°C).

After incubation, each sample was layered over 0.5 ml silicone fluid in a microfuge tube and spun at 12000 × g for 3 min in an Eppendorf tabletop centrifuge. This procedure rapidly separated the

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

lysosomes, which pelleted at the bottom of the tube, from the aqueous media, which remained above the oil. A 10 μl aliquot of supernatant was placed in 10 ml scintillation fluid (Aquasol, New England Nuclear) and counted in a LS7000 scintillation counter (Beckman Instruments, Fullerton CA). The remaining supernatant and silicone fluid were aspirated and discarded, and the pellet was resuspended in 100 μl 10% sodium dodecyl sulfate, placed in 10 ml scintillation fluid, and counted.

The volume of the lysosome pellet was calculated from supernatant and pellet radioactivity after incubation with ^3H_2O. This figure was corrected for extralysosomal media trapped within the pellet by subtracting the pellet volume obtained after incubation of duplicate lysosome aliquots with [^{3}H]PEG or [^{14}C]sucrose, to yield a net value for intralysosomal volume.

The ratio of intralysosomal to extralysosomal concentration of [^{14}C]methylamine (C_i/C_o) was determined from the calculated intralysosomal volume and the pellet was supernatant radioactivity after incubation of duplicate lysosome aliquots with [^{14}C]methylamine. Intralysosomal pH was then calculated from the following eq. [10]:

$$pH_{in} = pH_{out} - \log \frac{([^{14}C]\text{methylamine})_{in}}{([^{14}C]\text{methylamine})_{out}}$$

The same procedure was followed using [^{14}C]acetate as a weak acid which should accumulate in compartments of high pH.

2.3. *Effect of external ions*

In some experiments lysosomes were incubated with [^{14}C]methylamine after resuspension in HBSS pre-adjusted to varying pH with concentrated NaOH or HCl. In others, lysosomes were resuspended in 150 mM KCl or NaCl with 5 mM HEPES (pH 7.2).

2.4. *Effect of ionophores*

To assess the effect of ionophores and manipulation of membrane potential on intralysosomal pH, [^{14}C]methylamine distribution was determined in the presence of gramicidin (50 μg/ml), valinomycin (10 μM), or carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 1 μM).

3. RESULTS

3.1. *Calculation of intralysosomal pH*

When lysosomes were incubated with [^{14}C]methylamine for 10 min at 37°C, the mean pH difference (ΔpH) across the membrane was 2.02 ± 0.14 pH units (mean ± standard error of the mean, 11 determinations). The mean pH of the incubation media was 7.57 ± 0.03, giving a mean intralysosomal pH of 5.55 ± 0.13. It was not possible to calculate a ΔpH using [^{14}C]acetate distribution because [^{14}C]acetate was excluded from the lysosomes (not shown).

3.2. *Effects of temperature on measured intralysosomal pH*

When the determination of [^{14}C]methylamine was carried out in an ice bath, uptake was much slower than at 37°C, and the measured ΔpH was correspondingly lower. Fig. 1 shows comparative kinetics of uptake at 37°C and 0°C. In contrast to uptake at 37°C, which was maximal at the 10 min time point, uptake at 0°C continued for up to 1 h.

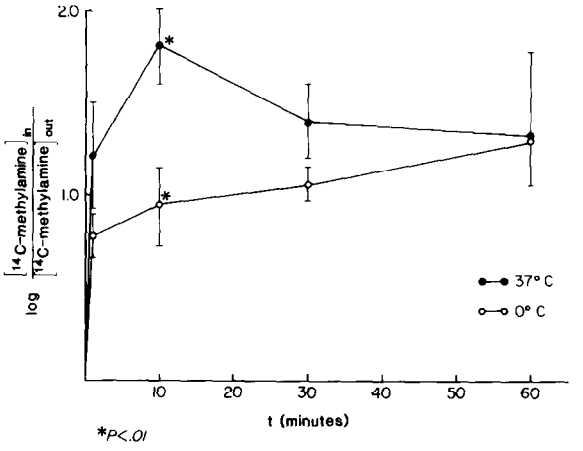

Fig. 1. Effect of temperature on lysosomal [^{14}C]-methylamine uptake: log C_i/C_o after incubation at 37°C or in ice bath (0°C) (mean ± SEM of 2–3 expt).

3.3. *Effect of extralysosomal pH*

When lysosomes were suspended in HBSS adjusted to varying initial pH and the ΔpH across the membrane was measured, a close correlation was found between the pH gradient and the external

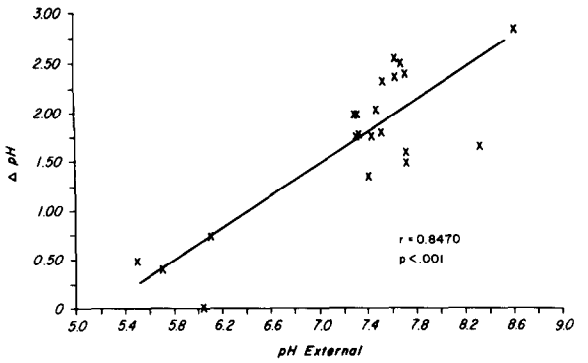

Fig. 2. Variation of pH gradient with ambient pH: expressed as $\Delta pH = \log C_i/C_o$ of [^{14}C]methylamine.

pH (fig. 2). Increasing the external proton concentration led to a decrease in the measured pH gradient between intralysosomal and extralysosomal compartments.

3.4. *Effects of external ion availability*

Lysosomes incubated with [^{14}C]methylamine in the presence of 150 mM KCl or NaCl showed no significant difference in calculated internal pH, which was 5.94 ± 0.06 in KCl and 6.03 ± 0.09 in NaCl solution.

3.5. *Effect of ionophores*

The protonophore CCCP reduced the transmembrane pH gradient from 1.28 ± 0.09 to 0.76 ± 0.03 ($P < 0.05$ by Student's t-test). Gramicidin or valinomycin alone had no significant effect (not shown), but the combination of valinomycin and CCCP further reduced ΔpH to 0.25 ± 0.05 ($P < 0.001$ compared with CCCP alone).

4. DISCUSSION

An accepted method of measuring pH differences across a membrane is determination of the distribution of a weak acid or base across the membrane [14]. In the case of a compartment with acidic interior relative to the environment, a weak base which penetrates the membrane in its uncharged form will reach an equilibrium distribution determined by the Henderson–Hasselbalch equation. The pH difference across the membrane can then be determined by measuring interior and exterior concentrations of the base. This method has been used to measure an interior pH of 6.3–6.4 in rat liver lysosomes [10,11]. In these experiments we applied similar methodology to human neutrophil lysosomes using the weak base [^{14}C]methylamine. At near-physiologic external pH, our results indicate an intralysosomal pH of 5.6 with a gradient of 2 pH units across the membrane of the isolated lysosome. As noted in [14], measurements using a weak acid, which is excluded from acidic compartments, are not accurate in this setting. The finding that [^{14}C]acetate was almost completely excluded from lysosomes and did not provide a useable ΔpH value is compatible with the assumption of a highly acidic lysosomal interior.

As noted in [15], we found that the permeability of the lysosome membrane varied with temperature. The lower ΔpH measured at 0°C, and the slow uptake of [^{14}C]methylamine at 0°C as compared to the rapid equilibration observed at 37°C, may raise the question of whether an energy-requiring process is involved in ion permeability and pH maintenance.

The pH gradient across the lysosome membrane was decreased by the addition of external acid. Within the pH range examined (5.4–8.6), we were not able to collapse the gradient to zero.

Most of these experiments were carried out in a balanced salt solution wherein the predominant cation was sodium. This may be unphysiological since potassium is the major cation in the normal intracellular environment of the lysosome. Therefore, we compared intralysosomal pH in media with sodium or potassium as the major cation, and found no significant difference attributable to external cation availability.

Manipulation of the membrane potential, as with the ionophores gramicidin and valinomycin, had no significant effect on the transmembrane pH gradient. However, the protonophore CCCP reduced this gradient significantly. In 150 mM KCl, the combination of CCCP and valinomycin (to allow equilibration of H^+ and exchange of H^+ for K^+) even more profoundly diminished ΔpH. These observations support the contention that we have measured a true proton gradient rather than non-specific adherence of methylamine to the lysosome pellet.

We propose that this marked internal acidity of

the neutrophil lysosome is a principal source for the acidification of the phagocytic vacuole after ingestion of microorganisms. Among the other mechanisms proposed to account for this acidification, at least two have been partially refuted in [16]. These investigators measured a level of lactate production which was far from sufficient to account for proton release from stimulated neutrophils. They also discounted the production of superoxide and protons from perhydroxyl radicals as the principal proton source, by demonstrating that superoxide dismutase inhibited proton release by only 10%.

The intralysosomal acidity which we have measured may be of importance to many aspects of neutrophil function besides the acidification of the phagocytic vacuole. Lysosomes may serve as a 'sink' for the accumulation of pharmacologically active substances with weakly basic properties, on the analogy of methylamine uptake. For example, we demonstrated in [17] that the antibiotic clindamycin (a weak base with pK_a 7.7) is concentrated by neutrophils and by isolated lysosomes in a pH-dependent fashion. Such uptake may serve to increase the exposure of phagocytosed microorganisms to the antibiotic. The reported affinity of some cholinergic and adrenergic ligands for neutrophils may similarly represent lysosomal uptake of weak bases [18, 19]. Further investigation is in progress to elucidate possible energy-dependent mechanisms which may contribute to the maintenance of lysosomal pH, to differentiate the pH of separate granule fractions, and to explore the role of lysosomal acidity in other aspects of neutrophil function.

ACKNOWLEDGEMENTS

This work was presented in part at the 21st Interscience Conference on Antimicrobial Agents and Chemotherapy (Chicago IL, November 1981) and supported in part by grant AI 16732 from the National Institutes of Health.

REFERENCES

[1] Mandell, J.G. (1970) Proc. Soc. Exp. Biol. Med. 134, 447–449.
[2] Jensen, M.S. and Bainton, D.F. (1973) J. Cell Biol. 56, 379–388.
[3] Segal, A.W., Geisow, M., Garcia, R., Harper, A, and Miller, R. (1981) Nature 290, 406–409.
[4] Hirsch, J.G. and Cohn, Z.A. (1960) J. Exp. Med. 112, 1005–1014.
[5] Kakinuma, K. (1970) J. Biochem. 68, 177–185.
[6] Cline, M. (1973) Clin. Res. 21, 595 abstr.
[7] Allen, R.C. (1979) Front. Biol. 48, 197–233.
[8] Root, R.K. and Cohen, M.S. (1981) Rev. Infect. Dis. 3, 565–598.
[9] Bainton, D.F. (1973) J. Cell Biol. 58, 249–264.
[10] Reijngoud, D.-J. and Tager, J.M. (1973) Biochim. Biophys. Acta 297, 174–178.
[11] Goldman, R. and Rottenberg, H. (1973) FEBS Lett. 33, 233–238.
[12] Boyum, A. (1968) Scand. J. Lab. Clin. Invest. 21 (suppl 97), 77–89.
[13] Klempner, M.S., Mikkelsen, R.B., Corfman, D.H. and Andre-Schwartz, J. (1980) J. Cell. Biol. 86, 21–28.
[14] Rottenberg, H. (1975) Bioenergetics 7, 61–74.
[15] Reijngoud, D.-J. and Tager, J.M. (1977) Biochim. Biophys. Acta 472, 419–449.
[16] van Zwieten, R., Wever, R., Hamers, M.N., Weening, R.S. and Roos, D. (1981) J. Clin. Invest. 68, 310–313.
[17] Klempner, M.S. and Styrt, B. (1981) J. Infect. Dis. 144, 472–479.
[18] Dulis, B.H. and Wilson, I.B. (1981) Biochim. Biophys. Acta 643, 398–406.
[19] Styrt, B., Noble, J.T. and Klempner, M.S. (1982) Clin. Res. 30, 166A abstr.