Alkalinizing the Intralysosomal pH Inhibits Degranulation of Human Neutrophils

MARK S. KLEMPNER and BARBARA STYRT, *Department of Medicine, Division of Experimental Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111*

ABSTRACT Degranulation of lysosomes is one of the consequences of neutrophil activation. Regulatory mechanisms of lysosomal secretion are thought to be localized largely in the plasma membrane and cytosol, with the lysosome playing a passive role in secretion. Recent evidence indicates that the intralysosomal pH is highly acidic (pH $\simeq$ 5.5) and is maintained by active transport of H^+. We investigated whether changes in the intralysosomal pH altered the availability of lysosomes for exocytosis. Intralysosomal pH in intact neutrophils was monitored with the weakly basic fluorescent probe, 9-aminoacridine (9AA). The weak bases, methylamine, chloroquine, clindamycin, propanolol, and ammonium chloride (0.1–50 mM), caused an alkalinization of the intralysosomal pH as determined by reversal of quenching of 9AA fluorescence. Similarly, each of the weak bases, including ammonium chloride, methylamine, chloroquine, ethylamine, propylamine, propanolol, clindamycin, and dansylcadaverine, inhibited neutrophil degranulation in response to the calcium ionophore A23187, phorbol myristate acetate, or the chemotactic peptide, formylmethionine-leucine-phenylalanine plus cytochalasin B. These studies indicate that an acid intralysosomal pH is important to the neutrophil secretory response and suggest that the lysosome may play an active part in control of degranulation.

INTRODUCTION

Fusion of intracellular granules (lysosomes) with the plasma membrane is a fundamental event in cell biology related to normal endocytic and exocytic functions. This process underlies such diverse phenomena as intracellular digestion, neurotransmitter release from synaptic vesicles, and secretion of a wide variety of essential hormones, digestive enzymes, and vasoactive amines. For human neutrophils (PMN),[1] the fusion of lysosomes with the plasma membrane subserves both microbicidal and inflammatory functional responses. The non-oxygen-dependent killing of ingested bacteria depends on exposure of microorganisms to the toxic environment resulting from fusion of lysosomes with the organism containing phagosome. Recent evidence also suggests that oxygen-dependent microbicidal mechanisms may be partly dependent on lysosome-plasma membrane fusion because cytochrome *b* appears to participate in the postphagocytic "respiratory burst" and this cytochrome is largely localized in the specific granules of PMN (1, 2). The extracellular release of PMN lysosomal constituents also contributes to evolution of the acute inflammatory response. In a process that closely resembles secretion from other cells, lysosomal enzymes can be released from PMN that generate inflammatory mediators and that have proteolytic activity against extracellular substrates.

Numerous studies have addressed the regulatory mechanisms of lysosome-plasma membrane fusion in PMN. These investigations have been largely focused on events at the plasma membrane and within the cytosol. Particular attention has been directed to the movements of extracellular and membrane-bound cations and anions (3–5), the relation of these ionic fluxes to membrane potential and surface charge changes (6–8), the activation of nucleotide cyclases (9–11), and the assembly and disassembly of microtubules and microfilaments (12–14). Since, in some respects, the

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[1] *Abbreviations used in this paper:* 9AA, 9-aminoacridine; f-Met-Leu-Phe, formyl-methionine-leucine-phenylalanine; FITC-dex, fluorescein-labeled dextran; LDH, lactic dehydrogenase; pH_i, internal pH; PMA, phorbol myristate acetate; PMN, neutrophils.

lysosome is the intracellular target organelle of PMN activation, we questioned whether the lysosome plays an active role in regulating its own fate during the fusion process.

Specifically, we have recently demonstrated that in a mixed population of human PMN lysosomes, the intralysosomal pH is highly acidic and this intralysosomal pH appears to be maintained by an active proton-translocating pump (15). Because the fusion between other biologic membranes (e.g., egg-sperm fertilization [16], platelet granule release [17]) is influenced by pH and H^+ fluxes, we have examined in these experiments whether alteration of the intralysosomal pH effects the secretory response of human PMN. These studies suggest that maintenance of an acidic intralysosomal pH is important to lysosome-plasma membrane fusion and that alkalinization of the intralysosomal pH inhibits neutrophil degranulation.

METHODS

Isolation of PMN. Heparinized blood (20 U/ml) was obtained from healthy adult volunteers after obtaining informed consent. Leukocytes containing 95–98% PMN were isolated by Hypaque-Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation followed by dextran sedimentation and osmotic lysis of residual erythrocytes (18). PMN were washed once in phosphate-buffered saline and finally suspended in Hanks' balanced salt solution (HBSS).

Determination of intralysosomal pH changes. Changes in intralysosomal pH were monitored by using the distribution of the fluorescent weak base, 9-aminoacridine (9AA, Sigma Chemical Co., St. Louis, MO), across the membrane. This indicator is concentrated within compartments that are acid relative to the media with consequent quenching of fluorescence (19). A stock solution of 9AA was made up at 10 mM in ethanol and stored at 4°C. For pH monitoring, this was diluted to 0.05 μM in HBSS (final concentration of ethanol 0.0005%). All aqueous solutions were adjusted to pH 7.4 except where otherwise indicated.

Fluorescence was measured in a Perkin-Elmer 650-10S spectrophotofluorometer (Perkin-Elmer Corp., Norwalk, CT). The excitation wavelength was 400 nm and the emission wavelength 456 nm, with slit widths of 5 nm. The cuvette compartment was maintained at 37°C by using a circulating water bath (PolyScience Corp., Niles, IL). Buffer (3 ml) containing 0.05 μM 9AA was placed in the spectrophotofluorometer cuvette and initial fluorescence was recorded. PMN ($1–5 \times 10^6$/ml) were added and the suspension was thoroughly mixed; fluorescence was followed continuously until a steady state was reached. All fluorescence readings were corrected for the effect of the volumes added (1–4%) as well as for the effect of added agents on background 9AA fluorescence.

Characteristics of 9AA fluorescence. The concentration dependence of 9AA fluorescence in HBSS (pH 7.4) was examined. Solutions of HBSS containing 9AA (0.01–0.06 μM) were prepared by appropriate dilutions from the stock solution of 9AA and the fluorescence intensity was measured as described above. The effect of pH changes on the fluorescence of a given concentration of 9AA (0.05 μM) in HBSS was also examined. Solutions of HBSS containing 0.05 μM 9AA were adjusted to pH 5.0–8.0 with concentrated NaOH or HCl and the fluorescence intensity was measured. Similarly, the effects of external pH changes on the quenching of 9AA fluorescence in the presence of PMN (4×10^6/ml) was determined over the pH range 5.2–7.5.

The intracellular locale of 9AA was investigated by using a digitonin-lysis method which differentially disrupts plasma membranes and lysosomes (20–21). PMN (5×10^6/ml in HBSS) were incubated with varying concentrations of digitonin (Sigma Chemical Co.) for 30 s at 37°C; this was followed by centrifugation at 12,000 *g* for 1 min. Supernatants were assayed for the cytosolic enzyme, lactate dehydrogenase, and the lysosomal enzyme, lysozyme, as described below. In parallel experiments, PMN (4×10^6/ml) were incubated with 9AA (0.05 μM) and fluorescence was monitored continuously until a steady state was reached. Varying concentrations of digitonin were then added and the changes in 9AA fluorescence determined. The effects of digitonin on 9AA fluorescence in the absence of PMN was also determined in control solutions and fluorescence determinations were corrected for these effects.

Effects of weak bases on 9AA fluorescence. To determine the effects of permeant weak bases on the distribution and fluorescence of 9AA in the presence of PMN, fluorescence of 9AA was measured before and after the addition of PMN. After steady state was reached, various weak bases were added and the quenching of 9AA fluorescence was continuously monitored. The weak base additions included methylamine (1.0 mM), chloroquine (0.1–1.0 mM), clindamycin (1.0 mM), propanolol (1.0 mM), and ammonium chloride (0.1–50.0 mM). The pH of all added solutions was preadjusted to produce an external pH change of no greater than 0.1 pH units at their final dilutions with PMN and 9AA.

Incubation of PMN with degranulating stimuli and weak bases. PMN were suspended in HBSS at a final density of 2×10^7/ml. Aliquots of 0.5 ml of the cell suspension were mixed with 0.5 ml of HBSS or with 0.5 ml of HBSS containing the degranulating stimuli, ionophore A23187 (5×10^{-7} M); phorbol myristate acetate (PMA, 1 ng/ml); or the synthetic chemotactic peptide, formyl-methionine-leucine-phenylalanine (f-Met-Leu-Phe, 10^{-8} M) plus cytochalasin B (5 μg/ml). All solutions were carefully adjusted to pH 7.2. After a 20-min incubation at 37°C in a shaking water bath, the cell suspensions were centrifuged; the cell-free supernates were saved for enzyme determinations. An aliquot of the cell suspension was also retained for freeze-thawing (four times) to determine total cellular enzyme contents.

The effects of various weak bases on degranulation in response to the ionophore A23187, PMA, and f-Met-Leu-Phe plus cytochalasin B were also examined. PMN (final density 1×10^7/ml) were preincubated for 10 min at 37°C in HBSS or HBSS supplemented with the following bases: ammonium chloride, methylamine, chloroquine, ethylamine, propylamine, propanolol, clindamycin, and dansylcadaverine. All solutions were adjusted to pH 7.2. Buffer or the degranulating stimuli were then added at the concentrations indicated above and the incubation was continued for 20 min at 37°C in a shaking water bath. The cell-free supernatants were then obtained by centrifugation and used for enzyme determinations. Each condition was run in duplicate.

To determine whether the effects of weak bases on PMN uptake of 9AA and secretion were reversible, PMN (1×10^7/ml) were incubated in HBSS (pH 7.2) or HBSS supplemented with ammonium chloride (10 mM, pH 7.2) for 5 min at 37°C in some experiments. The cell suspensions were then centrifuged (400 *g* for 5 min at 4°C) and resuspended in HBSS or HBSS containing A23187 (5×10^{-7} M). The quenching

of 9AA fluorescence and enzyme release in response to A23187 by PMN that was preincubated in HBSS or HBSS with ammonium chloride were compared as described above.

Enzyme determinations. Degranulation was monitored by the extracellular release of the lysosomal enzymes, lysozyme and β-glucuronidase. The cytosolic enzyme, lactic dehydrogenase, was also measured to determine whether enzyme release was the result of cell death.

Lysozyme activity was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, NJ) at pH 6.2 according to a turbidimetric method (22). β-glucuronidase was assayed by measuring the release of phenolphthalein from its β-glucuronate (Sigma Chemical Co.) after 4 h of incubation at pH 4.5 (23). Lactic dehydrogenase (LDH) was assayed by spectrophotometric measurement of the consumption of β-nicotinamide adenine dinucleotide during the conversion of pyruvate to lactate (24). Enzyme activities are expressed as a percent of total enzyme activity in a sample of PMN (1×10^7/ml) in the various media freeze-thawed four times. None of the weak bases affected the activities of lysozyme, β-glucuronidase, or lactic dehydrogenase.

RESULTS

Monitoring intralysosomal pH with 9AA fluorescence. To use 9AA fluorescence as a weakly basic probe to monitor pH changes within PMN lysosomes, several prerequisites must be fulfilled. First, the fluorescence of 9AA must be concentration-dependent so that its accumulation in acidic compartments will result in concentration changes that can be detected by changes in the intensity of fluorescence. As shown in Fig. 1, 9AA fluorescence was highly concentration-dependent over the range 0.01–0.06 μM. For subsequent studies with PMN, the concentration of 9AA incubated with the cells was 0.05 μM. Second, the fluorescence of the probe itself must be pH-independent. As shown in Fig. 2, the absolute fluorescence of 9AA (0.05 μM) was completely unaffected by suspension in HBSS preadjusted to pH that vary from 5 to 8. Third, if the accumulation of 9AA in lysosomes is dependent on a ΔpH (i.e., 9AA behaves as a weak base), then changing the extracellular pH should affect the lysosomal uptake of 9AA and thereby, modify the fluorescent signal. When the gradient between extracellular pH and the intralysosomal pH is minimal (e.g., at acidic extracellular pH), small quantities of 9AA should accumulate within the lysosomes and the fluorescence should not be quenched. Conversely, at neutral or alkaline extracellular pH, 9AA should concentrate within the lysosomes and increase the quenching of 9AA fluorescence. Fig. 3 shows the relationship between modifying the extracellular pH and the quenching of 9AA fluorescence in the presence of intact PMN. In accordance with the predictions that 9AA behaves as a weak base, there was a progressive increase in the quenching of 9AA fluorescence as the extracellular pH was raised from 5.2 to 7.5. Finally, to use 9AA as a probe of intralysosomal

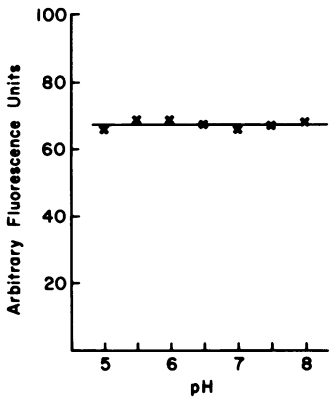

FIGURE 2 pH independence of 9AA fluorescence. Solutions of 9AA (0.05 μM) were made in HBSS and adjusted to pH 5–8. Fluorescence was measured as in Fig. 1.

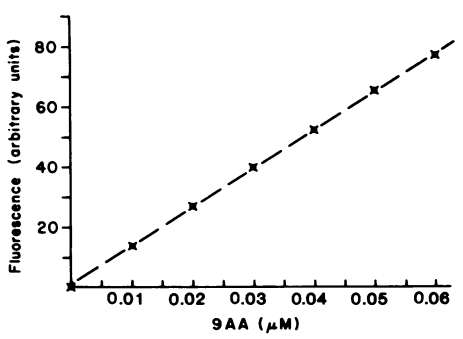

FIGURE 1 Concentration dependence of 9AA fluorescence. Solutions of 9AA were made in HBSS (pH 7.2). Fluorescence was measured with excitation at 400 nm and emission at 456 nm.

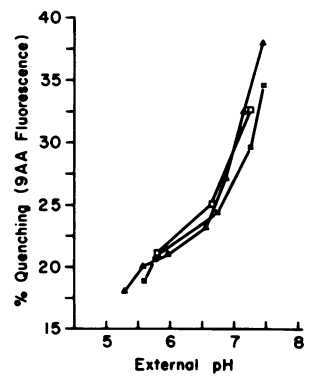

FIGURE 3 Effect of external pH on 9AA fluorescence with intact PMN. PMN (4×10^6/ml) were incubated with 9AA (0.05 μM) in HBSS adjusted to various pH and the quenching of 9AA fluorescence was measured. Results from three separate experiments are shown.

pH, it is necessary to verify that 9AA is largely localized within this intracellular compartment. Our approach has been to examine the correlation between release of a cytoplasmic marker (LDH), which is indicative of plasma membrane disruption, and release of a lysosomal marker (lysozyme), which is indicative of lysosomal membrane disruption with release of 9AA induced by the detergent digitonin. As shown in Table I, LDH release was dependent on the concentration of digitonin and was maximal at 0.025 mM. While lysozyme release by digitonin was also concentration-dependent, maximal lysosomal disruption did not occur until 0.1 mM. Digitonin-induced release of 9AA (as assessed by reversal of fluorescence quenching) showed a remarkable correlation with the lysozyme release. Whereas 0.025 mM digitonin resulted in maximal LDH release, only 33.8% of lysozyme and 15.4% of 9AA was released at this concentration. A higher digitonin concentration (0.10 mM) was required to induce maximal release of lysozyme and 9AA.

The ability of various weak bases to raise the intralysosomal pH as monitored by reversal of the quenching of 9AA fluorescence is shown in Fig. 4. At the concentrations shown, each of the weak bases reversed the PMN quenching of 9AA fluorescence. This results from redistribution of the 9AA out of the lysosome as the weak bases increase the intralysosomal pH and thereby, reduce the transmembrane pH gradient. As would be predicted from this model, reversal of fluorescence quenching of 9AA by the weak bases was dose-dependent. As shown in Fig. 5, increasing concentrations of ammonium chloride progressively reversed the quenching of 9AA fluorescence.

Effects of weak bases on degranulation. In the absence of a secretory stimulus, spontaneous release of LDH and lysozyme was similar for PMN incubated in HBSS or HBSS containing the various weak bases (<4.0%, data not shown). Figs. 6–8 demonstrate the effects of various weak bases on secretion of lysozyme

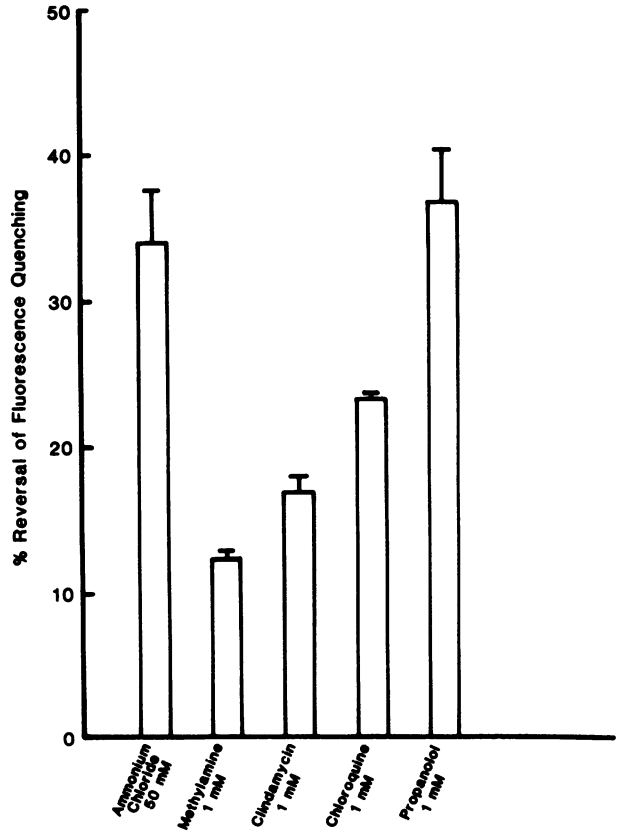

FIGURE 4 Effect of weak bases on intralysosomal pH. PMN were equilibrated with 9AA (0.05 μM) and quenching of 9AA fluorescence was measured (excitation 400 nm, emission 456 nm). Each of the bases was then added and reversal of fluorescence quenching was determined. None of the bases increased 9AA fluorescence in the absence of PMN.

TABLE I
Release of Indicators from PMN By Digitonin Lysis

	Digitonin (mM)			
	0	0.001	0.025	0.10
Lactate dehydrogenase	11.1±1.8*	7.6±0.8	79.9±6.2	83.7±4.6
Lysozyme	8.3±1.1	8.8±1.0	33.8±2.7	84.7±0.7
9AA	5.8±3.8	0	15.4±0.4	81.3±4.4

* Release of indicators is expressed as a mean±SEM of the percentage of maximal release obtained by treating PMN with 0.02% Triton X-100 in two to seven experiments that were each run in duplicate.

by PMN in response to the Ca^{++} ionophore A23187 (Fig. 6), PMA (Fig. 7), and f-Met-Leu-Phe plus cytochalasin B (Fig. 8). Under control conditions, which were without any weak base present, these secretory stimuli induced lysozyme release between 24.2 and 38.0% of total cellular lysozyme. All supernates contained <4% of the cytosolic enzyme, lactate dehydrogenase; this indicates that lysozyme release was not due to cell death. In the absence of a weak base, β-glucuronidase release was 33.9±1.1, 8.0±0.8, and 15.7±0.5% of total cellular enzyme for cell suspensions treated with A23187, PMA, and f-Met-Leu-Phe plus cytochalasin B, respectively (not shown). At the concentrations shown, each of the weak bases inhibited lysozyme release in response to the secretory stimuli. The most profound inhibitory effect was consistently seen with the weakly basic antibiotic, clindamycin. At a clindamycin concentration of 10^{-3} M, there was a 92.2 and 91.5% inhibition of lysozyme release in re-

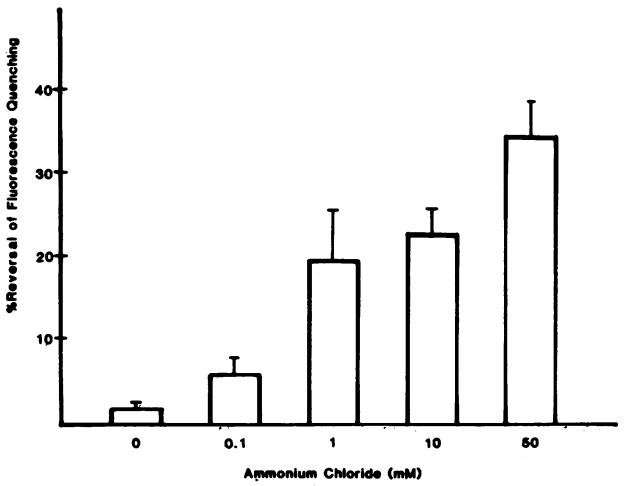

FIGURE 5 Alkalinization of intralysosomal pH by ammonium chloride. PMN (5×10^6/ml) were incubated with 9AA (0.05 μM) and the reversal of 9AA fluorescence quenching by increasing concentrations of ammonium chloride was measured as in Fig. 4. Volume additions were constant for each concentration of ammonium chloride (1% of total volume) and reversal of fluorescence quenching was corrected for this dilution effect.

sponse to A23187 and PMA, respectively, and at 10^{-4} M, lysozyme release in response to the chemotactic peptide was inhibited by 76.4%. β-glucuronidase release in response to A23187 and the chemotactic peptide was also consistently inhibited by the weak bases at the same concentrations (not shown). Although PMA-induced β-

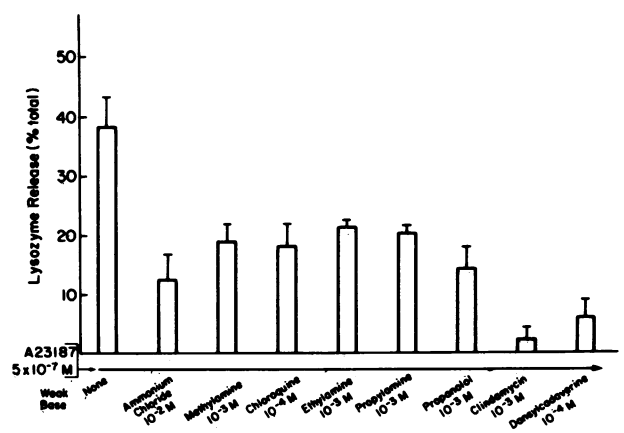

FIGURE 6 Effects of weak bases on lysozyme release by PMN stimulated with A23187. PMN (1×10^7/ml) were preincubated with HBSS or HBSS supplemented with the weak bases at the concentrations indicated (pH 7.2) for 10 min at 37°C. Lysozyme release in cell-free supernates was measured after stimulation with A23187 (5×10^{-7} M) for 20 min at 37°C. LDH release was <4% in all supernates. Results are the mean±SEM of four separate experiments.

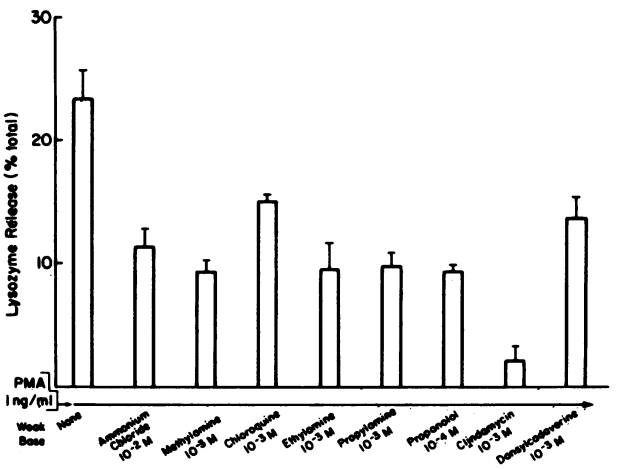

FIGURE 7 Effects of weak bases on lysozyme release by PMN stimulated with phorbol myristate acetate. Conditions were as in Fig. 6 with the secretory stimulus, phorbol myristate acetate (1 ng/ml). Results are the mean±SEM of three separate experiments.

glucuronidase release was <10% in the absence of a weak base, this small degree of enzyme release was further inhibited by clindamycin (1 mM), methylamine (1 mM), propanolol (1 mM), and chloroquine (0.1 mM) (data not shown).

The dose-response relationship between increasing concentrations of ammonium chloride and A23187-stimulated lysozyme release is shown in Fig. 9. In the absence of any weak base, A23187 stimulated 47.3±2.9% of total cellular lysozyme release. Ammonium chloride at 0.1 and 0.5 mM did not inhibit

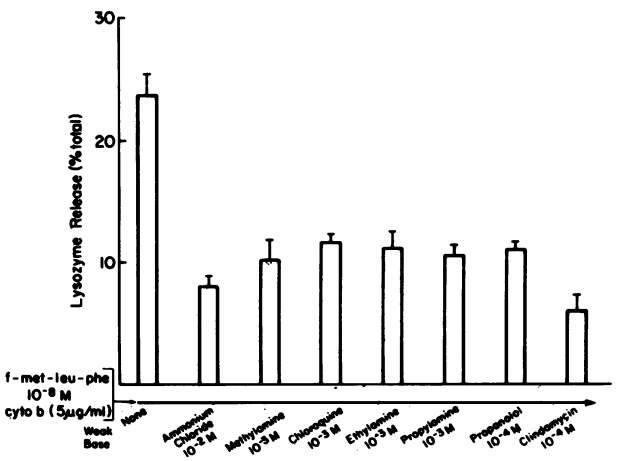

FIGURE 8 Effects of weak bases on lysozyme release by PMN stimulated with f-Met-Leu-Phe (10^{-8} M) plus cytochalasin B (5 μg/ml). Conditions were as in Fig. 6. Results are the mean±SEM of three separate experiments.

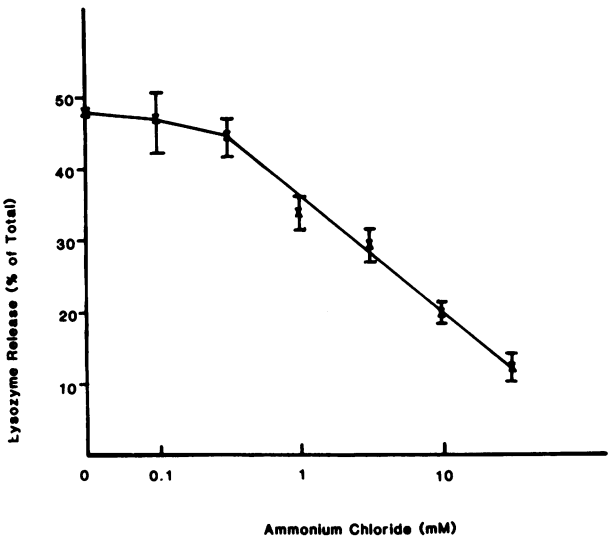

FIGURE 9 Dose response of ammonium chloride inhibition of lysozyme release by PMN stimulated with A23187 (5×10^{-7} M). Conditions were as in Fig. 6 with PMN preincubated with HBSS or HBSS supplemented with increasing concentrations of ammonium chloride. Results are the mean±SEM of three separate experiments.

A23187-stimulated lysozyme release. At higher ammonium chloride concentrations, however, there was progressive inhibition of degranulation by this weak base.

The effects of ammonium chloride on PMN quenching of 9AA fluorescence and secretion in response to A23187 were reversible. Quenching of 9AA fluorescence by control, HBSS-treated cells was 36.5±3.9%, and was 37.9±4.5% after PMN had been preincubated in ammonium chloride (10 mM) for 5 min and then resuspended in ammonium chloride-free HBSS (pH 7.2, $n = 3$, $P > 0.1$). After ammonium chloride pretreatment and removal of the weak base, PMN released 32.9±1.9% of total cellular lysozyme in response to A23187 (20 min at 37°C) and control PMN released 25.9±2.4%. Spontaneous lysozyme release (in the absence of A23187) was 3.9±1.7% for ammonium chloride-treated cells and 4.1±1.6% for control cells. These results are the mean±SEM of four separate experiments that were each run in triplicate.

DISCUSSION

In a previous study, we demonstrated that the pH within a mixed population of isolated human PMN lysosomes was highly acidic and proposed that this preformed acid was a principal source of protons for the acidification of the phagocytic vacuole after phagosome-lysosome fusion (15). That the intralysosomal pH was only partially alkalinized by the protonophore, carbonyl cyanide *m*-chlorophenyl hydrazone, and that the ΔpH was almost completely abolished by the combination of carbonyl cyanide *m*-chlorophenyl hydrazone with the K^+ ionophore, valinomycin, suggested the existence of an electrogenic H^+ pump for maintenance of the intralysosomal acid pH. In these studies, we utilized a method for monitoring the intralysosomal pH in intact PMN based on the accumulation of a weakly basic fluorescent probe, 9AA, within these acidic lysosomes. Using this method, we have investigated whether permeant weak bases, which accumulate within intracellular acidic lysosomes and alkalinize the intralysosomal pH, have an effect on the availability of lysosomal contents for extracellular release in response to several stimuli. Our studies demonstrate that alkalinization of the intralysosomal pH by weak base accumulation markedly inhibited PMN degranulation in response to A23187, PMA, or f-Met-Leu-Phe plus cytochalasin B.

The use of 9AA as a weak base fluorescent probe was first introduced by Deamer et al. (19) for the measurement of intraliposomal pH and has subsequently been used to measure the pH in several systems, including mitochondria (25), secretory granules (26), sperm cells (27), and platelets (17). Like other weak bases, 9AA accumulates in acidic compartments down a pH gradient and demonstrates a concentration-dependent quenching of fluorescence. Quenching is thought to result from self-interactions between 9AA molecules as a function of 9AA concentration (19). Thus, the fluorescence of 9AA is proportional to its concentration and it concentrates in acidic compartments, including PMN lysosomes. As emphasized by Simons et al. (17), 9AA fluorescence changes are proportional to internal pH (pH_i) changes, but do not provide a priori an accurate measure of absolute pH_i; we utilized this probe to monitor changes in intralysosomal pH.

The major requirements for using 9AA fluorescence as a probe of intralysosomal pH are that (*a*) the intensity of fluorescence be a measure of 9AA concentration in our buffer system, (*b*) that the fluorescence of a given concentration of 9AA be pH-independent over the pH range of interest, and (*c*) that the probe localizes predominantly in the lysosome. Figs. 1 and 2 verify the applicability of 9AA fluorescence as a concentration-dependent, pH-independent parameter in our system. Several observations serve to justify the interpretation of changes in 9AA fluorescence as a reflection of changes in lysosomal pH_i. The intracellular compartments of PMN include cytosol, nucleus, lysosomes, mitochondria, and endoplasmic reticulum. Of these, only lysosomes have been demonstrated to have an acidic pH_i that would lead to 9AA accumulation (15). Since the pH_i of mitochondria is alkaline in other cells (27), it is highly unlikely that 9AA would accumulate in this

compartment. There should also be little, if any, 9AA accumulation in the cytoplasm since the ΔpH between the cytoplasm and media (at neutral pH) has been measured at <0.01 pH U (28). Moreover, stimulation of PMN with, for example, f-Met-Leu-Phe leads to alkalinization of cytoplasmic pH, which would tend to exclude 9AA. To our knowledge, the intranuclear pH of PMN has not been directly measured. However, the high concentrations of basic histones in PMN nuclei (29) suggests that 9AA would not concentrate in this organelle. A recent report by Smolen et al. (30) also supports the lysosome as the site of 9AA accumulation in PMN. These studies demonstrated a high correlation between the extracellular release of 9AA from PMN loaded with this dye in a preliminary incubation and the release of lysosomal enzymes in response to several secretory stimuli. Our direct studies also indicate that the lysosome is, in large part, the intracellular site of 9AA uptake. We have patterned these experiments after those of Zuurendonk and Tager (20) and Akkermann et al. (21) who demonstrated differential digitonin lysis of plasma membranes and intracellular organellar membranes in rat hepatocytes and human platelets, respectively. At digitonin concentrations which maximally liberated a cytoplasmic enzyme, LDH, there was relative preservation of lysozyme and 9AA within a sequestered compartment. Although this is not direct proof that 9AA is in lysosomes, when it is taken together with the considerations above, it provides strong support that 9AA is a useful probe for studies of lysosomal pH_i.

Our finding that a variety of weak bases alkalinized the intralysosomal pH in PMN is similar to the observations of Poole and Ohkuma (31) on mouse peritoneal macrophages. These investigators used fluorescein-labeled dextran (FITC-dex), which accumulated by endocytosis in macrophage phagolysosomes over 24 h, as a pH-dependent probe to measure the effects of weak bases on intralysosomal pH. Their results demonstrated a dose-dependent alkalinization of lysosomal pH_i by chloroquine and several simple amines. The concentrations required for raising the macrophage phagolysosomal pH_i were comparable with those used in our studies, and alkalinization of the phagolysosomal pH_i by ammonium chloride was rapidly reversible once the weak base was removed from the media. It should, however, be emphasized that the FITC-dex method requires endocytosis of the probe; this could alter the composition of the compartment that contains the FITC-dex. Indeed, recent evidence demonstrates that clathrin-coated endocytic vesicles contain an ATP-dependent proton pump that acidifies the intravesicular space and may be the site of at least some of the FITC-dex localization (32). In contrast, our studies with the permeant weak base probe 9AA presumably do not involve endocytosis of the probe, but rather passage of the uncharged base across the plasma membrane and lysosomal membrane where it accumulates because of the already existent ΔpH.

The highly consistent findings that weak bases not only alkalinize the intralysosomal pH, but inhibit granule release in response to several secretory stimuli is not surprising when considered in the context of other systems requiring membrane fusion. This is perhaps best exemplified by the studies of Helenius et al. (33) on the pathway of viral genome entry into the cytoplasm of host cells. For example, Semliki forest virus and canine influenza virus are endocytosed into vesicles where an acidic pH is required for the viral membrane fusion reaction that results in transfer of the viral genome into the cytoplasm (34). Ammonium chloride, which alkalinizes this vesicular compartment, inhibits the fusion reaction and infectivity of the virus for the host cell. An acid pH also appears to be important for the intravesicular processing (dissociation) of receptor-ligand complexes and the recycling of the endocytosed receptor to the surface membrane (35).

That alkalinizing the intralysosomal pH inhibits PMN degranulation raises several questions as to the mechanisms of secretion and to the nature of the pH-sensitive process. Current concepts of lysosomal exocytosis relegate a passive role to the lysosome in the secretory response. Rather, the regulatory controls are considered to be exclusively localized to the plasma membrane, where stimulus reception and transduction occur, and in the cytosol, where alterations in the chemical environment (e.g., ionic composition, cyclic nucleotides, products of arachidonic acid metabolism, etc.) are expressed in terms of assembly and disassembly of contractile protein matrices and tubulin. Although it is possible that the lysosome exerts its influence on the secretory process through these same mechanisms, for example, as a site of mobilizable intracellular calcium, it is also possible that an acidic intralysosomal pH is required for the poorly defined biochemical reactions that lead ultimately to membrane fusion. Future investigation should be directed at identifying the nature of lysosomal participation in regulating its own fate during secretion.

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