Acidification Activity of Human Neutrophils

TERTIARY GRANULES AS A SITE OF ATP-DEPENDENT ACIDIFICATION*

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The acidification activity of human neutrophils, known to occur extracellularly and intraphagolytically, was studied in intact and in fractionated cells. The subcellular location of the acidification activity was investigated by rate zonal sedimentation of post-nuclear supernatants from resting cells on continuous sucrose gradients. The acidification measurements indicated a dominance of activity in gelatinase-rich tertiary granules. On the other hand, ATPase activities were located in plasma membrane and in the membranes of the cytoplasmic granules (specific, azurophilic, and tertiary). All of these activities were diminished by the inhibitors dicyclohexylcarbodiimide and diisothiocyanostilbene disulfonic acid; however, studies with other inhibitors, especially N-ethylmaleimide and duramycin, suggested ATPase enzymatic differences depending on location. The results taken together provide direct and strong indication of involvement of a proton pump ATPase in acidification inside neutrophils. Furthermore, the dominant location of acidification activity in tertiary granules that very readily degranulate presumably has significant implications for the importance of low pH in cidal events and the inflammatory process.

Neutrophils, the first line of defense in peripheral blood against infectious disease, first ingest and then destroy intruder organisms by releasing cidal factors and degradative enzymes against the entrapped target in the phagocytic vacuole (1, 2). In this process, neutrophils generate reactive oxygen metabolites, mainly superoxide and hydrogen peroxide, that facilitate the killing of microbes (3). Although the digestive enzymes such as the cathepsins are known to require a low pH of 4–5, a dependency of bactericidal activity on acidification has not been established (2, 4, 5). An acid pH per se is bactericidal for certain ingested microorganisms and acid pH does promote conversion of superoxide to hydrogen peroxide (6). Moreover, acidification may be required in neutrophils for the fusion of granules and the transfer of material from one compartment to another. Supporting this is the observation that protein and receptor recycling in other cells are sensitive to inhibitors of acidification (7, 8). In view of the role of acidification in these biological processes, it is important to understand the mechanism and subcellular location of acidification activity in neutrophils.

Various mechanisms of acidification in neutrophils have been proposed: (a) an increase of lactic acid formation during phagocytosis (4, 9), (b) the production by an NADPH oxidase of perhydroxyl radicals (-OOH) which subsequently split into superoxide (O2) and protons (10), (c) a proton-translocating system in association with the oxidase system (11, 12), (d) a carbonic anhydrase system (13, 14), and (e) a proton pumping ATPase (15). Here we present evidence that is strongly indicative of a proton pump ATPase involvement in neutrophil function.

There are many precedents for the establishment and maintenance of an acid pH by a proton pump ATPase in a wide variety of subcellular fractions. Such an ATPase has been directly implicated in lysosomes (16, 17), clathrin-coated vesicles (18, 19), synaptic vesicles (20), Golgi (21, 22), acrosomes (23), chromaffin granules (24, 25), and platelet granules (26–28). Although ATPase activity in human granulocytes has been reported previously (29, 30), its involvement in phagocytic vacuole acidification during phagocytosis has not been ascertained. We recently reported (31) the presence of a membrane-bound, Mg2+-dependent ATPase activity in human neutrophils that was inhibited by N,N'‐dicyclohexylcarbodiimide and suggested its function as a proton pump.

In the present extension of these studies, we show that tertiary granules, rather than the more familiar specific and azurophilic granules, are surprisingly dominant with respect to organelle acidification. The implications of this subcellular location and a role of acidification in microbicidal events are considered.

MATERIALS AND METHODS

Neutrophil Preparation—Neutrophils were prepared from fresh blood after removal of erythrocytes by sedimentation at unit gravity through dextran as described (31, 32). Subsequent centrifugation into Ficoll-Hypaque gradients was performed as indicated following published protocols (33).

Measurements of pH Changes in Neutrophil Suspensions—All the incubations were made in a solution containing 150 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 1.3 mM CaCl2 adjusted to pH 7.0. Neutrophils (2 × 10⁸ cells) were preincubulated with 5 μg/ml cytochalasin B and the respective agent for 5 min in a total volume of 2 ml, and the suspension was mixed with a magnetic stirrer. The pH changes were detected with a pH meter attached to a recorder. The pH was brought to 8.0 with sodium hydroxide and the buffering capacity of the suspension determined by addition of 50–100 mM of sodium hydroxide. Proton extrusion was calculated from the slope of the recorded pH curve during the first minute after addition of the stimulus (5 μg/ml phorbol myristate acetate or 10⁻⁹ M N-formyl-t-methionyl-t-leucyl-t-phenylalanine). Stock solutions of DCCD (10 mM) and mercuric chloride (10 mg/ml) were prepared daily in dimethyl sulfoxide. The effects on proton extrusion of these compounds were compared to

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‡ The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; DIDS, diisothiocyanostilbene-2,2'-disulfonic acid.
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controls containing an equivalent (1%) amount of dimethyl sulfoxide.

Subcellular Fractionation—Cell suspensions were diluted with TS (0.25 mM sucrose, 10 mM Tris-SoK, pH 8) and resuspended by centrifugation at 8000 × g for 3 min. The packed cells, about 1 g, wet weight, were resuspended in TS to a final volume of 2 ml. Disruption was accomplished with 10 strokes of a 10 ml syringe and, after 2.5 min on ice, homogenization in a Dounce tissue grinder using a type A (tight) pestle (10 strokes). Iso-osmolality was rapidly restored by addition of 1.25 ml of 2 M sucrose. The homogenate was centrifuged at 2000 × g for 4 min to obtain nuclear and postnuclear supernatant or extract fractions. For rate zonal gradient analyses, 9 ml of the extract was layered on a 25-mL, 14.3-34.2% (w/w) continuous sucrose gradient, above a 1.0-mL cushion of 60% sucrose. Gradients were centrifuged at 20,000 rpm (70,000 × g) for 15 min at 4 °C in a Beckman ultracentrifuge using a SW 27 rotor. For all gradients, 8 ml of the sample layer (fraction 1) and seven subsequent 4-ml fractions were collected from the top by pumping 60% sucrose into the bottom.

Assays for Marker Enzymes and Protein—Lactate dehydrogenase (31), lysozyme (31), 5'-nucleotidase (31), protein (31), N-acetyl-β-D-glucosaminidase (31), and lactoferrin (34) were assayed as described.

Gelatinase activity was determined by slight modification of the described method (35). Samples were treated for 15 min at 37 °C with inhibitors, 3 mM diisopropylphosphofluoridate and 0.1 mM N-ethylmaleimide, quercetin, and DIDS were effective inhibitors, and demonstrates

DISCUSSION

Acidification activity to inhibitors.

Effect of Inhibitors on the ATPase and Acidification Activities—As shown in Table II, a number of inhibitors were tested with the ATPase activities present in different fractions isolated from human neutrophils. Ouabain and vanadate, well known inhibitors of (Na⁺,K⁺)-ATPase (38), had no significant effect on the ATPase activity of any of the particulate fractions. These results are consistent with previous suggestions that a minor fraction of the ATPase activity in neutrophils is due to the presence of (Na⁺,K⁺)-ATPase (15, 30). Conversely, DCCD and DIDS, nonspecific inhibitors of proton-translocating ATPases (39), inhibited greatly all the particulate ATPases. Incubation with duramycin, an inhibitor of coated vesicle ATPase (40), resulted in a partial inhibition of the plasma membrane ATPase, whereas the other organelle ATPases were activated.

N-Ethylmaleimide, an inhibitor of the coated vesicle proton pump (19), partially affected the plasma membrane ATPase activity at 0.1 mM and had less of an effect on the other ATPases. At 1 mM, N-ethylmaleimide inhibited completely all acidification activity and inhibited about 40% of the tertiary granule ATPase activity. It is worth noting a previous report (30) in which the Mg²⁺-ATPase activity of human granulocytes was inhibited 50% by 0.2 mM N-ethylmaleimide.

Oligomycin used at 0.5 µg/ml (0.6 μM), which inhibits 90% of the mitochondrial ATPase (41), had little effect on neutrophil ATPase activity, 3% inhibition (31). This insensitivity to oligomycin is in agreement with the relative scarcity of mitochondria in mature granulocytes (42) and demonstrates that the ATP hydrolytic activity in neutrophils is not due to mitochondrial contamination. High concentrations of oligomycin (9 µg/ml or 11 μM) known to cause nonspecific inhibition of ATPases (43), affected in different degrees the particulate ATPases. Low mitochondrial contamination was found to be located between specific and azurophilic granules in rate zonal gradients (31), and this fraction was relatively sensitive to oligomycin. However, even incubation of the mitochondrial fraction with 9 µg/ml oligomycin resulted in only 40% inhibition of the ATPase activity. This lack of more than 40% inhibition indicates that the mitochondrial fraction contains other ATPases.

Incubation of the different fractions with azide, a mitochondrial inhibitor, showed an inhibitory effect on the granule ATPases, this being higher in the azurophilic granules. In contrast, plasma membrane ATPase was not affected by azide under the conditions of the assay. 5,5'-Dithiobis(2-nitrobenzoic acid), at 0.3 mM, had negligible effects on the ATPase activity in neutrophils.

Acidification activity was affected by ATPase inhibitors as shown in Table III. Dicyclohexylcarbodiimide, N-ethylmaleimide, quercetin, and DIDS were effective inhibitors, whereas vanadate was not. In general the results of acidification assays parallel those of ATPase activity, especially with regard to sensitivity to the inhibitors DCCD and DIDS and insensitivity to vanadate. However, in regard to the
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FIG. 1. Distribution of ATPase activity, acidification activity, and markers of human neutrophils compartments in rate zonal gradients. Neutrophils were isolated from fresh blood and homogenized, extracts were analyzed by rate centrifugation on 14.3 to 34.5% sucrose gradients for 15 min at 20,000 rpm, and fractions were assayed as described under “Materials and Methods.” The tops of the gradients are depicted on the left of each panel (fractions 1), the bottoms on the right (fractions 8). With the exception of ATP-dependent acidification activity (ACID), the values plotted are relative activities versus relative volume. The panels represent gelatinase (GELase), diacyclohexylcarbodiimide-sensitive ATPase (DCCD-ATPase), N-ethylmaleimide-sensitive ATPase (NEM-ATPase), lactoferrin (Lf), glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH), 5′-nucleotidase (5′), lysozyme (LYSOZ), and myeloperoxidase (PEROX).

The Possible Role of Tertiary Granules in Early Events of Phagocytosis—Resting human neutrophils showed a proton release of about 20 nmol of H+/min·mg and upon activation with phorbol myristate acetate, protons were extruded at a rate of 34–38 nmol of H+/min·mg (see Fig. 2). The presence of DCCD had an inhibitory effect on proton release by phorbol myristate acetate-activated cells (right panel), despite a stimulatory effect of DCCD on resting cells (left panel). Similar results were obtained when cells were activated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine. Furthermore, incubation of neutrophils with 50 µg/ml (0.2 mM) quercetin completely abolished the stimulation of proton extrusion by activated cells, and vanadate, at 60 or 100 µM, did not inhibit the stimulation in proton extrusion (data not shown). Inhibition of proton secretion by DCCD and quercetin imply the involvement of a proton pump ATPase and raises the possibility of tertiary granule involvement in the extracellular release of protons. It therefore is of interest to consider the degree to which tertiary granule components are present in the cell membrane. Accordingly, gelatinase release and the reactivity of intact cells with impermeant reagents were tested as probes of tertiary granules.

The data in Table IV indicate that gelatinase activity, known to mark tertiary granules (37), was readily released from neutrophils. This presumably involves fusion of tertiary granules with the cell membrane, release of soluble gelatinase, and insertion of granule membrane into the cell surface. Activation by addition of phorbol ester lead to 65% release, in considerable excess of lysozyme from specific granules. It is noteworthy that specific granules are known to join the

TABLE I
Specific activities of ATPase in different particulate fractions
Fractions were prepared and analyzed as described under “Materials and Methods.” Plasma membrane (PM) corresponds to rate zonal centrifugation fraction 2, tertiary granule to fraction 4, specific granule to fraction 6, and azurophilic granule to fraction 8. The values given are the mean ± standard error of at least three replicates.

| Fraction    | ATPase activity |
|-------------|-----------------|
|             | DCCD-sensitive  | Total           |
|             | nmol/min·mg protein |         |
| Cell        | 14 ± 2          | 25 ± 6         |
| Extract     | 20 ± 4          | 28 ± 4         |
| PM          | 41 ± 1          | 57 ± 5         |
| Tertiary    | 50 ± 2          | 60 ± 3         |
| Specific    | 48 ± 3          | 60 ± 3         |
| Azurophilic | 16 ± 2          | 20 ± 3         |

distribution of activities on gradients, parallelism was lacking as plasma membrane fractions were relatively active in ATPase and inactive in acidification activity. The possible significance of such differences will be discussed below.

The Possible Role of Tertiary Granules in Early Events of Phagocytosis—Resting human neutrophils showed a proton release of about 20 nmol of H+/min·mg and upon activation with phorbol myristate acetate, protons were extruded at a rate of 34–38 nmol of H+/min·mg (see Fig. 2). The presence of DCCD had an inhibitory effect on proton release by phorbol myristate acetate-activated cells (right panel), despite a stimulatory effect of DCCD on resting cells (left panel). Similar results were obtained when cells were activated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine. Furthermore, incubation of neutrophils with 50 µg/ml (0.2 mM) quercetin completely abolished the stimulation of proton extrusion by activated cells, and vanadate, at 60 or 100 µM, did not inhibit the stimulation in proton extrusion (data not shown). Inhibition of proton secretion by DCCD and quercetin imply the involvement of a proton pump ATPase and raises the possibility of tertiary granule involvement in the extracellular release of protons. It therefore is of interest to consider the degree to which tertiary granule components are present in the cell membrane. Accordingly, gelatinase release and the reactivity of intact cells with impermeant reagents were tested as probes of tertiary granules.

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TABLE II
Effects of inhibitors on the ATPase activities of different fractions
Fractions from rate zonal gradient centrifugation of neutrophil extracts were collected and assayed (after a 5-min preincubation with the indicated inhibitor) as described under “Materials and Methods.” The results shown are either the mean ± standard error of at least three determinations or a representative value of duplicate determinations. In the absence of inhibitors the fractions exhibited the specific activities shown in Table I.

| Inhibitor        | Plasma membrane ATPase activity % | Tertiary ATPase activity % | Specific ATPase activity % | Azurophilic ATPase activity % |
|------------------|-----------------------------------|---------------------------|---------------------------|-------------------------------|
| None             | 100                               | 100                       | 100                       | 100                           |
| DCCD, 0.1 mM     | 35 ± 2                            | 24 ± 1                    | 26 ± 2                    | 27 ± 2                        |
| DIDS, 0.1 mM     | 37 ± 2                            | 40 ± 3                    | 41 ± 2                    | 43 ± 4                        |
| 5′-Dithio-bis(\(\text{nitrobenzoic} \) acid), 0.3 mM | 84 ± 5                            | 84 ± 3                    | 87 ± 4                    | 86 ± 11                        |
| N-Ethylmaleimide, 0.1 mM | 83                                | 98                        | 94                       | 95                            |
| Ouabain, 1 mM    | 99                                | 102                       | 102                      | 115                           |
| Vanadate, 0.3 mM | 101                               | 97                        | 97                       | 123                           |
| Duramycin, 40 µg/ml | 78                                 | 129                       | 151                      | 116                           |
| Oligomycin, 9 µg/ml | 82                                 | 75                        | 73                       | 70                            |
| Azide, 0.1 mM    | 93                                | 71                        | 75                       | 48                            |
phagosome during the early phases of phagocytosis (44). The data suggest that tertiary granules join the phagosome at least as fast as the specific granules.

If membrane fusion occurs during activation of neutrophils, an increase in surface protein available for reaction with impermeant reagents might be expected. Accordingly, the impermeant reagent diazobenzenesulfonate was used to label resting and phorbol myristate acetate-activated cells. Resting cells were labeled to a significant extent when incubated with diazobenzenesulfonate, indicating the presence of exposed components, presumably membrane proteins (Table V). Pre-treatment of neutrophils with phorbol myristate acetate caused an increased reactivity (Table V). In addition, sonication of the cells during reaction with diazobenzenesulfonate resulted in more than an additional 10-fold greater reactivity (data not shown), indicating that a relatively small proportion of cellular components reactive to diazobenzenesulfonate are exposed in intact cells.

Thus, enzyme release and impermeant labeling, used, as probes for the granules, indicate that membrane fusion occurs during activation. Therefore, it seems reasonable to suggest that the increased proton secretion that occurs in activated neutrophils is due to a significant extent to proton pump activity derived from the tertiary granules as a consequence of fusion between the granule and cell membranes.

**DISCUSSION**

The ATPase and Proton Pumping Activities of Neutrophils—The present results further demonstrate both a plasma membrane and granule localization of the ATPase activity in human neutrophils. This is in agreement with previous reports (13, 30, 45). About 30% of the total ATPase is localized in plasma membrane, and, interestingly, only 10% is located in the azurophilic granules, even though these contain most of the acid hydrolases that would presumably require an ATP-driven proton pump to maintain an acid pH favorable to hydrolytic action. The remainder of the ATPase activity is recovered on sucrose gradients in the region of tertiary and specific granules. In all cases, inhibition of the Mg2+-dependent ATPase by the inhibitors DCCD and DIDS, suggests the presence of a proton pump functioning in phagocytic vacuole acidification. Inhibition of ATPase and acidification activities by millimolar concentrations of N-ethylmaleimide is further evidence for the presence of a "vacuolar" type of proton pump, similar to that of lysosomes and coated vesicles (18, 19).

Some differences between the different membrane populations emerge from the results obtained (a) using inhibitors and (b) measuring acidification. The tertiary and specific granules differ from plasma membrane in that their ATPase activity is more sensitive to azide inhibition and less sensitive to N-ethylmaleimide. Interestingly, duramycin inhibits partially the ATPase located in gradient fractions containing plasma membrane. As duramycin is an inhibitor of coated vesicle ATPase (40), the effect may be due to the presence of this ATPase either in plasma membrane per se or in coated vesicles that co-fractionated with plasma membrane.

The acidification activity of the different membranes of resting neutrophils is clearly greatest in gradient fractions containing the gelatinase-rich tertiary granules (Fig. 1). The acidification activity of all active fractions exhibited a similar sensitivity to inhibitors, however, and thus provide no direct evidence for the existence of different proton pump systems in different membranes. The presence of distinct ATPases in plasma membrane must be taken into account and may contribute to the different behavior of the ATPase activity located there. Thus, the presence of a (Na+,K+)-ATPase (15, 46) and of a Ca2+ pump (47, 48) in the plasma membrane of...
neutrophils has been reported. As noted above, plasma mem-
brane fractions from the gradients exhibited relatively more
ATPase than acidification activity (Fig. 1). Some of this
difference can be attributed to the presence of ATPases that
do not pump protons. In addition, unsealed plasma membrane
vesicles are perhaps present and these would be expected to
show maximum ATPase activity without acidification activ-
ity, as proton gradients cannot be established in leaky vesicles.
In regard to disrupted membranes, it is also necessary to point
out that the membrane fragments derived from disrupted
granules might also be located in the upper region of the rate
zonal gradients with the plasma membrane.

The inhibition of proton release from activated cells by
DCCD and quercetin suggest the involvement of a proton pump.
Although nonspecific, DCCD does inhibit proton trans-
locating ATPases (49) and has been shown to inhibit the
Mg\(^{2+}\)-dependent ATPase activity of human neutrophils (31).
It is significant that the release of protons by activated
neutrophils is not affected by vanadate inasmuch as vanadate
inhibits not only the (Na\(^+\),K\(^+\))-ATPase but also certain
plasma membrane proton pumps, e.g. that of fungal plasma
membrane (50). The vanadate result indicates that a covariant
phosphorylated intermediate is not involved (51, 52) and
provides further evidence that neutrophils contain a distinct
ATP-driven proton pump.

The lack of inhibition of proton release from resting neu-
rophils by DCCD and quercetin agrees with the idea that
this release, unlike that of stimulated cells, can be attributed
to lactic acid production (53). The DCCD results also suggest
that the stimulated proton release from activated cells is due
to the fusion of granules with plasma membrane and concom-
itant insertion of proton pumps in the plasma membrane.
In this context, it may be significant that DCCD is able to inhibit
enzyme release from granules when neutrophils are activated
with phorbol myristate acetate (31).

A Relationship between Acidification and Respiratory Burst Activities—Although the importance of acidification in the
killing and digestive aspects of the microbicidal process in
neutrophils is not fully understood, it is clear that acidification
driven by an ATP-dependent proton pump is not required for
activation of the respiratory burst activity. Thus, the presence
of 20 \(\mu\)M monensin, 1.5 \(\mu\)M carbonyl cyanide p-trifluoromethoxyphenylhydrazone, 10 \(\mu\)M carbonyl cyanide m-chlorophenylhydrazone, 0.1 mM 2,4-dinitrophenol, 0.6 \(\mu\)M oligomycin, or 0.9 \(\mu\)M valinomycin had no effect on the
respiratory burst activity induced by treatment of cells with
phorbol myristate acetate. Furthermore, exposure of neutroph-
ils to DCCD, 50–500 \(\mu\)M, may even trigger respiratory burst
activation (Ref. 54 and Fig. 2).

The relationship of ATP-dependent proton release and
phagolyosomal acidification to the transient alkalization of
the phagolysosomal compartment observed at short inter-
vals after stimulation requires comment. First, the alkalini-
ization is presumably driven by the reactions of the active
oxygen intermediates, e.g. dismutation of superoxide con-
sumes protons (12). Second, if both processes are triggered
simultaneously, they would offset one another and diminish
any pH change. Accordingly, one should consider whether
regulation is complex with a hierarchy of events that allow
sequential triggering of the respiratory burst and alkalini-
zation and subsequent triggering of the proton pump ATPase
and acidification. The answers to these possibilities will be
facilitated when we can selectively affect the ATPase(s) of
the involved compartments.

A Probable Relationship between Membrane Fusion and
Activation of the Respiratory Burst—The gelatinase activity
of the tertiary granule is readily released from neutrophils.
To our knowledge, it is not possible to activate the respiratory
burst activity of neutrophils in the absence of gelatinase
release. This applies also to enucleated neutrophils inasmuch
as the release of gelatinase occurs during the process of
enucleation, a process that involves extensive treatment of
the cells at 37 °C (55), conditions favorable for membrane
fusion. However, the observations that release can occur prior
to activation, as in enucleated cells (55) and in cells pretreated
with low amounts of various stimuli (56), indicate that mem-
brane fusion events alone are not sufficient for activation of
the respiratory burst. Accordingly, the concept of a “priming”
step in activation has evolved (56). We interpret our results,
especially those on gelatinase release, as additional evidence
for the priming of neutrophils. Furthermore, we suggest that
such fusion events are obligatory for activation of the respi-
ratory burst. It is noteworthy that cells defective in degranu-
lation are unable to exhibit the burst (57).

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