Activation of Proton Pumping in Human Neutrophils Occurs by Exocytosis of Vesicles Bearing Vacuolar-type H\(^+\)-ATPases*

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Proton pump activity is not measurable in the plasma membrane of unstimulated neutrophils but becomes readily detectable upon activation by soluble agonists. The mechanism of pump activation was investigated in this report. V-type H\(^+\) pump activity, estimated as a baflimycin A\(_2\)-sensitive elevation of the cytosolic pH, was stimulated in suspended neutrophils by chemotactic peptides and by phorbol esters. Stimulation of pump activity induced by the agonists was greatly enhanced by cytochalasin B, an agent known to potentiate granular secretion in neutrophils. We therefore compared the rate and extent of pump activation with the pattern of exocytosis of the four types of secretory organelles present in neutrophils, using flow cytometry and enzyme-linked immunosorbent assay. The kinetics of exocytosis of secretory vesicles and secondary and tertiary granules but not primary granules paralleled the appearance of pump activity. The subcellular localization of the pump was defined by cellular fractionation and immunoblotting using an antibody to the C subunit of the V-type ATPase. The pump was abundant in tertiary granules, with significant amounts present also in primary granules and secretory vesicles. The pump was scarce in secondary granules and not detectable in the cytosol. Finally, the agonists failed to stimulate pump activity in neutrophil cytoplasts, which are intact cell fragments devoid of acidic granules. Together, our results suggest that the V-type H\(^+\)-ATPase is not constitutively present in the plasma membrane of neutrophils but is delivered to the surface membrane by exocytosis during cellular activation. Tertiary granules and secretory vesicles are the most likely source of V-ATPases. Following insertion in the plasma membrane, the pump is poised to effectively extrude the excess metabolic acid that is generated during chemotaxis and bacterial killing.

In response to chemoattractants, polymorphonuclear leuко"
V-type $H^+$ pump activity is not measurable across the plasma membrane of resting neutrophils but becomes evident after stimulation with agonists such as chemotactants or phorbol esters (17, 20). The mechanism leading to this apparent activation of the pump in leukocytes is not clear. However, progress has been made in other systems toward understanding the regulation of pump activity. Polypeptide activators and inhibitors have been purified from the kidney and brain that directly alter pump activity (18, 21). In addition, recruitment of intracellular H+$^+$-ATPases to the plasma membrane is a well documented means of initiating pumping across the plasma membrane. In tissues along the urinary tract, exocytosis of ATPase-bearing vesicles produces insertion of active pumps into the apical membrane, triggering H+$^+$ exclusion from the cells (26, 27). Termination of H+$^+$ pumping then occurs by retrieval of ATPases into endocytic vesicles. Because neutrophils are endowed with a variety of diverse intracellular organelles that are secreted upon stimulation, it is possible that exocytosis is the mechanism responsible for the acquisition of V-type pump activity by the plasma membrane.

The purpose of the experiments described in this report was to investigate the mechanism of activation of H+$^+$ pumping in neutrophils. Specifically, we wished to determine whether the V-type H+$^+$ pump is constitutively present in the plasma membrane of neutrophils or whether it is delivered to the surface membrane during stimulation via exocytosis.

**EXPERIMENTAL PROCEDURES**

Reagents and Solutions—Bafilomycin A$_1$ was the generous gift of Dr. K. Altendorf (Universität Osnabrück, Germany). Diphenylene iodonium (DPI) was synthesized in our laboratory according to the method of Colette et al. (28). Powdered medium RPMI 1640 (HCO$_3$-$^{-}$-free, Heps-buffered, and containing L-glutamine), the tripeptide formyl-Met-Leu-Phe (fMLP), 12-O-tetradecanoylphorbol 13-acetate (TPA), and cytochalasin B (CB) were from Sigma. Nigericin and the acetychymotrypsin of BEECF were obtained from Molecular Probes. Valinomycin was obtained from Calbiochem. All other chemicals were of reagent grade and were obtained from Sigma, Fisher, BDH, or Pharmacia (M). Inosine, prepared as above but lacking CaCl$_2$ and containing 5mM EDTA. (HCO$_3$-$^{-}$-free, medium at 37°C, and after 1 min the cells were stimulated with either 1mM fMLP or 100nM TPA. At selected time points, aliquots containing 4 × 10$^6$ cells were removed and added to ice-cold K+$^+$ medium, prepared as above but lacking CaCl$_2$ and containing 5mM EDTA. Where indicated, cells were pretreated with 5μM CB for 3 min prior to stimulation. The cells were fixed in 1% paraformaldehyde for 15–30 min, followed by washing and incubation with monodonal anti-CD63 or CD67 antibodies in carbonate buffer (50mM Na$_2$CO$_3$/NaHCO$_3$, pH 9.6), followed by blocking with 1% bovine serum albumin saline buffer (0.5mM NaCl, 3mM KCl, 8mM Na$_2$HPO$_4$/K$_2$HPO$_4$, pH 7.2, and 1% Triton X-100). Samples and standards were applied for 1h, followed by washing with saline buffer and addition of biotinylated anti-mouse antibody conjugated to fluorescein isothiocyanate for 30 min at 4°C. After washing, the fluorescent label was quantified by counting 10,000 cells using a Becton-Dickinson FACScan flow cytometer. The cells were counted without gating, and the mean fluorescence intensity was determined using the Lysis II analysis software. The fluorescence intensity of cells incubated with only fluorescein isothiocyanate-labeled secondary anti-body was negligible and was not subtracted.

ELISA Assays—Secretion of tertiary granule and secretory vesicles was assessed by measuring the appearance of the markers CD63 (primary granules) and CD67 (secondary granules) on the external face of the plasma membrane (see Ref. 33 for rationale). Intact neutrophils (2 × 10$^6$) were sedimented and resuspended in 1mL of K+$^+$ medium at 37°C, and after 1 min the cells were stimulated with either 1mM fMLP or 100nM TPA. At selected time points, aliquots containing 4 × 10$^6$ cells were removed and added to ice-cold K+$^+$ medium, prepared as above but lacking CaCl$_2$ and containing 5mM EDTA. Where indicated, cells were pretreated with 5μM CB for 3 min prior to stimulation. The cells were fixed in 1% paraformaldehyde for 15–30 min, followed by washing and incubation with monodonal anti-CD63 or CD67 antibodies in carbonate buffer (50mM Na$_2$CO$_3$/NaHCO$_3$, pH 9.6), followed by blocking with 1% bovine serum albumin saline buffer (0.5mM NaCl, 3mM KCl, 8mM Na$_2$HPO$_4$/K$_2$HPO$_4$, pH 7.2, and 1% Triton X-100). Samples and standards were applied for 1h, followed by washing with saline buffer and addition of biotinylated anti-mouse antibody conjugated to fluorescein isothiocyanate for 30 min at 4°C. After washing, the fluorescent label was quantified by counting 10,000 cells using a Becton-Dickinson FACScan flow cytometer. The cells were counted without gating, and the mean fluorescence intensity was determined using the Lysis II analysis software. The fluorescence intensity of cells incubated with only fluorescein isothiocyanate-labeled secondary anti-body was negligible and was not subtracted.

**Subcellular Fractionation**—Individual granule fractions were purified from resting neutrophils as described in detail previously (35). Briefly, neutrophils were subjected to nitrogen cavitation followed by centrifugation to remove unbroken cells and nuclei. The resulting post-nuclear supernatant was applied on top of a discontinuous, three-layer Percoll gradient (1.05, 1.09, 1.12g/ml) and centrifuged at 37,000 × g for 30 min. 1-mL fractions were removed from the bottom of the gradient and assayed for the presence of marker proteins corresponding to individual compartments (indicated in parenthesis): myeloperoxidase (primary granules), lactoferrin (secondary granules), gelatinase (tertiary granules), and human leukocyte antigen class I (plasma membrane), all as described previously (36). Secretory vesicles were purified from the post-nuclear membrane fraction on the Percoll gradient were further purified by free-flow electrophoresis following neuraminidase treatment, as detailed earlier (37). Finally, Percoll was removed by centrifugation, and the samples were solubilized in boiling Laemmli sample buffer.

**Immunoblotting**—Aliquots from purified granule fractions were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with a polyclonal rabbit anti-serum against the C subunit of the mammalian V-type H+$^+$-ATPase (molecular mass, 39–42 KDa). After washing and incubating with peroxidase-labeled goat anti-rabbit antibody, the blots were developed using enhanced chemiluminescence (ECL, Amersham Corp.).

**Confocal Microscopy**—Intact neutrophils or cytoplasts were incubated with 2.5 μM acridine orange for 10 min at room temperature.
After washing, an aliquot of cells was applied to a coverslip, which was quickly mounted on a microscope slide and placed in the stage of a Leica TCS 4D laser confocal microscope with a 63× objective. The sample was excited at 488 nm, and emitted light was collected after passing through a long pass 540 nm filter. Digitized images (Nomarski and fluorescence) were cropped in Adobe Photoshop and imported into Adobe Illustrator for assembly and labeling.

RESULTS

Activation of V-type H⁺ pump activity by fMLP and Potentiation by CB—Activation of the V-type H⁺ pump in human neutrophils was assessed by monitoring the rate of H⁺ extrusion from the cytosol. To this end, changes in pH were detected fluorimetrically using BCECF (see “Experimental Procedures”). To analyze the activity of the pump in isolation, other pH regulatory mechanisms were inhibited by performing ionic substitutions and by pharmacological means. BCECF-loaded neutrophils were suspended in a Na⁺-free, high K⁺ medium to preclude Na⁺/H⁺ exchange and were treated with 50 μM ZnCl₂ to inhibit H⁺ efflux through the H⁺ conductance (see Refs. 17 and 20 for details). DPI, an antagonist of the flavoprotein component of the NADPH oxidase (38, 39), was included to inhibit the oxidase and thereby limit acid production that would otherwise obscure detection of H⁺ extrusion. Finally, the cells were treated with 1 μM valinomycin, a K⁺ ionophore to ensure the availability of a mobile counterion to neutralize the electrogenic fluxes of H⁺. When cells treated in this manner were stimulated with 1 μM fMLP, a small but reproducible acidification was observed, followed by a significant alkalinization with a maximal rate averaging 0.026 ± 0.03 pH/min (n = 5) (Fig. 1A). The initial acidification may represent residual acid production due to incomplete inhibition of the NADPH oxidase or may be due to other DPI-insensitive metabolic pathways. More importantly, the alkalinization phase drove pH above the basal (prestimulation) value, implying that H⁺ extrusion was not simply in response to the initial acidification but was in fact directly stimulated by the chemoattractant. As shown in the bottom trace of Fig. 1A, the alkalinization phase stimulated by fMLP was completely inhibited by 100 nm bafilomycin A₁, implying mediation by a V-type H⁺ pump (18–20). Consistent with this finding, the alkalinization was also inhibited by similar concentrations of concanamycin, another selective inhibitor of V-ATPases (not shown).

To investigate the possible role of granule secretion in activation of the V-type pump, we measured H⁺ extrusion activity in cells treated with CB, an agent known to potentiate exocytosis in neutrophils (40). Pretreatment of cells with 5 μM CB did not alter the basal pH, but enhanced the rate of alkalinization in response to fMLP by 3-fold; the maximal rate of alkalinization increased to 0.090 ± 0.010 pH/min (Fig. 1B). As before, the alkalinization was virtually eliminated by bafilomycin A₁ (Fig. 1B, lower trace). Potentiation of pump activity by CB suggests that secretion of endomembrane compartments may contribute to activation of the pump.

Phorbol Ester Stimulation of V-type H⁺-ATPase—CB is thought to enhance secretion by disruption of microfilaments, thereby favoring granule translocation to the membrane. However, potentiation of the effects of fMLP by CB has also been attributed, in part, to prevention of receptor down-regulation (41). To ascertain that the effects of CB reflect increased secretion, as opposed to a receptor-associated process, we tested the effect of the microfilament-disrupting agent on a receptor-independent response. As shown previously, V-type pump activity can also be induced when neutrophils are stimulated with the phorbol ester, TPA. As depicted in the middle trace of Fig. 2A, stimulation with TPA elicited a transient acidification followed by a substantial alkalinization with a maximal rate averaging 0.094 ± 0.010 pH/min (n = 6). As with fMLP stimulation, pretreatment with CB enhanced the maximal rate of TPA-induced alkalinization. However, the potentiation of pump activity was comparatively smaller, amounting to only a 56% increase in the rate of pH increase (Fig. 2A, top trace). Consistent with the involvement of V-type H⁺ pumps, the alkalinization stimulated by TPA in both CB-treated and untreated cells was significantly inhibited by 100 nm bafilomycin (Fig. 2A, bottom trace). Together with the results shown in Fig. 1, the potentiation of pumping activity by CB pretreatment is highly suggestive that exocytosis of intracellular granules contributes to the activation of the pump.

Several features of the activation of H⁺ pumping are noteworthy. Upon stimulation by either fMLP or TPA, a small but significant initial acidification occurs, followed by a larger alkalinization that is noticeable after approximately 2 min and drives pH above the prestimulation value. In control, untreated cells bafilomycin does not appreciably affect the rate or extent of the initial acidification but completely inhibits the secondary alkalinization. This suggests that the onset of the alkalinization is delayed by approximately 2 min from the time of addition of the stimulus (see Fig. 1A and cf. middle and lower traces of Fig. 2A).

Fig. 1. V-type H⁺ pump activity stimulated by fMLP. Intracellular pH was monitored fluorimetrically using the pH-sensitive dye BCECF, as described under “Experimental Procedures.” Neutrophils (2 \( \times \)10⁶) were suspended in 2 ml of K⁺ medium containing 2 μM DPI, 1 μM valinomycin, and 50 μM ZnCl₂. The bottom trace of each panel contained 100 nm bafilomycin A₁ (+ bal) and 5 μM CB from the outset. Cells in B were pretreated with 5 μM CB (+ CB) for 3-5 min prior to stimulation. Where indicated, 1 μM fMLP was added to all cell suspensions. Temperature was 37 °C. The data are representative of at least three similar experiments of each type.

Fig. 2. V-type H⁺ pump activity stimulated by TPA. Cells were suspended in K⁺ medium containing 2 μM DPI, 1 μM valinomycin, and 50 μM ZnCl₂. The cells in the top traces of each panel were treated with 5 μM CB (+ CB) for 3-5 min prior to stimulation. The suspensions depicted in the middle traces were not pretreated. The lower traces contained 100 nm bafilomycin A₁ (+ bal) and 5 μM CB from the outset. Where indicated by the arrows, 100 nm TPA was added to each suspension. Cells in B were acidified by an NH₄⁺ prepulse, and the traces begin after resuspension in NH₄⁺-free K⁺ medium. The data are representative of at least three similar experiments of each kind.
To better appreciate the course of activation of H⁺ pumping, cells were acid-loaded by the NH₄⁺ prepulse method and then stimulated with TPA in the presence and the absence of bafilomycin. We anticipated that under these conditions, the initial phase of cytosolic acidification would be minimized, whereas the rate of pumping would be magnified. Typical results are shown in Fig. 2B. As shown in the leftmost portion of the traces, no pH recovery was observed in untreated, acid-loaded cells. The phorbol ester elicited a rapid, biphasic alkalization: a rapid bafilomycin-insensitive component was followed by a slower phase that was inhibited by the macrolide antibiotic and was therefore presumably mediated by the V-type pumps. The lag time required for development of the bafilomycin-sensitive phase (approximately 2 min) is comparable with that detected in Fig. 2A, indicating that activation of the pumps is not instantaneous. The pathway responsible for the bafilomycin-resistant H⁺ (equivalent) extrusion noted in Fig. 2B is not clear.

Pretreatment with CB did not suffice, by itself, to induce pH recovery from acid-loaded cells but accelerated the alkalization initiated by TPA (Fig. 2B, top trace). Thus, not only does CB potentiate the H⁺ pump-mediated alkalization stimulated by TPA, but the CB effect is only manifest after cellular stimulation.

Comparison of the Kinetics of Pump Activation and Granule Exocytosis: Effect of CB—As detailed in the Introduction, at least four types of secretory granules have been described in neutrophils. One or more of these could deliver H⁺ to the membrane during stimulation with soluble agonists. Therefore, we undertook studies of secretion to define those granule type(s) that could contribute to plasmalemmal recruitment of H⁺ pumps. Candidate granules must fulfill the following criteria: (i) exocytosis must be induced by both fMLP and TPA in the absence of CB; (ii) secretion induced by fMLP or TPA should be potentiated by CB to an extent commensurate with the stimulation of H⁺ pumping; and (iii) exocytosis must precede or at least coincide with the onset of pumping.

Secretion of granules can be assessed by measuring either the appearance of granule matrix proteins in the suspension medium or the appearance of granule membrane proteins on the plasma membrane. The secretion of primary and secondary granules was assessed using the latter method by detecting the appearance on the surface membrane of the granular markers CD63 and CD67, respectively. Monoclonal antibodies against luminal epitopes of these proteins (which become exposed to the extracellular milieu upon secretion) were added to stimulated neutrophils, and the results were quantified by flow cytometry following staining with fluoresceinated secondary antibodies (see "Experimental Procedures"). As shown in Fig. 3A, stimulation with fMLP alone induced only a minute amount of secretion of CD63 over 6 min. Treatment with CB alone similarly failed to induce secretion (solid triangles in Fig. 3; partially obscured by the open squares in Fig. 3A). When cells were pretreated with CB and then stimulated with fMLP, however, the amount of staining in the plasma membrane was more than 7-fold higher than in unstimulated cells (mean of three independent experiments), in good agreement with earlier findings (33). As is apparent from Fig. 3A, the kinetics of this secretion is very rapid, with most of the exocytosis occurring within the first minute of stimulation. A comparable pattern of CD63 secretion was observed with TPA. As shown in Fig. 3B, only a modest increase in surface expression of CD63 occurred within the first 4 min after stimulation with TPA alone. Again, preincubation with CB potentiated the amount of primary granule secretion, inducing a 5–6-fold increase in CD63 expression after stimulation. The failure of fMLP to trigger CD63 secretion in the absence of CB and the disproportionately large potentiation induced by CB suggest that primary granules are not likely to provide the source of H⁺ pumps in stimulated neutrophils.

The surface expression of CD67, reflecting exocytosis of secondary granules, is shown in Fig. 3C and D. Stimulation with fMLP alone caused detectable secretion of secondary granules, inducing a 2-fold increase in CD67 expression over 6 min. CB alone had no effect on secretion, but preincubation with this agent prior to stimulation with chemoattractant resulted in a 4-fold increase in CD67 expression over the same time period (Fig. 3C). The kinetics of secretion were rapid, with most of the exocytosis occurring within the first 2 min. A somewhat different pattern of secondary granule exocytosis was observed upon TPA stimulation. When treated with the phorbol ester alone, the kinetics of secretion were slower, with no significant secretion occurring until after 1 min. A 1.8-fold increase in CD67 expression was noted after 2 min and over 3-fold increase at 6 min. (Fig. 3D). When preincubated with CB, the secretion induced by subsequent stimulation with TPA was only moderately enhanced. Thus, the responsiveness of CD67 to fMLP and TPA and the potentiation by CB are comparable with the effects of these stimuli on H⁺ pumping, suggesting that secondary granules may be the source of the ATPases.

Exocytosis of secretory vesicles and tertiary granules was assessed by measuring the appearance of the soluble proteins albumin and gelatinase, respectively, in the suspension medium of stimulated neutrophils, using ELISA (see "Experimental Procedures"). In agreement with earlier results (10), stimulation with fMLP induced a sizable release of albumin,

![Exocytosis of H⁺ Pumps in Neutrophils](image-url)
The secretion of albumin (A and B) and gelatinase (C and D) from stimulated neutrophils. The time course of albumin and gelatinase release from stimulated neutrophils was quantified using ELISA as described under “Experimental Procedures.” In A and C, the open squares show cells stimulated with 1 μM fMLP alone, whereas the closed squares represent cells pretreated with 5 μM CB for 3 min prior to stimulation. The open triangle shows the amount of protein released from unstimulated cells kept at 37°C for 6 min, whereas the closed triangle shows the secretion induced by 6 min in the presence of 5 μM CB alone. In B and D, the open circles represent cells stimulated with 100 nm TPA, whereas the cells shown by the closed circles were pretreated with 5 μM CB for 3 min prior to TPA stimulation. The time scale shown in the lower panels applies also to the top panels. Note the different scale in the ordinate of D versus C. All panels show a typical experiment and are representative of three similar experiments.

reflecting exocytosis of secretory vesicles (Fig. 4A, lower trace). Significant quantities of albumin were detected in the suspension medium even after 30 s, and release of the protein continued for at least 6 min. Treatment with CB alone failed to initiate secretion (solid triangles in Fig. 4). However, pretreatment with CB greatly accelerated the kinetics of secretion, so that maximal secretion occurred by 30 s (Fig. 4A, upper trace), with no further release noted even after 6 min. The course of albumin release upon TPA stimulation is shown in Fig. 4B. The general pattern of secretion paralleled that found with fMLP stimulation, but the kinetics of albumin release were faster, reaching a maximum by 3 min. As before, pretreatment with CB accelerated the kinetics of albumin secretion: maximal release occurred by 30 s. Thus, both fMLP and TPA trigger significant secretion of secretory vesicles, with a time course and agonist sensitivity that resemble the activation of H⁺ pumping.

The exocytosis of tertiary granules was assessed by using ELISA to measure the release of gelatinase. As shown in Fig. 4C, stimulation with fMLP alone induced significant secretion of gelatinase that reached a maximum within 3 min. Pretreatment with CB induced a 3-fold increase over this amount, with a maximal level attained within 30 s (Fig. 4C, upper trace). A strikingly different pattern of exocytosis was observed following stimulation with phorbol ester. The secretion of gelatinase induced by TPA occurred more slowly than with fMLP stimu-
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Fig. 5. Subcellular fractionation of neutrophils and immunoblotting of purified fractions. Neutrophils were fractionated by nitrogen cavitation followed by centrifugation through a three-layer Percoll gradient as described under "Experimental Procedures." A shows profiles of marker proteins in the individual fractions collected from the bottom of the gradient (i.e., fraction #4 was the most dense). Open squares, myeloperoxidase (a marker of primary granules, 1°); closed triangles, geranylgeranyl pyrophosphate (a marker of secondary granules, 2°); open circles, gelatinase (a marker of tertiary granules, 3°); closed squares, albumin (a marker of secretory vesicles, which coaggregate with plasma membranes), sv/pm. The inset of A shows the activities of human leukocyte antigen class I (a plasmalemmal marker, open diamonds). B, separation of plasma membranes from secretory vesicles by free-flow electrophoresis. Closed triangles show human leukocyte antigen activity, whereas open diamonds represent latent alkaline phosphatase activity. pm, plasma membranes; sv, secretory vesicles. C and D show immunoblots of purified fractions blotted with an antibody against the 39-kDa subunit of the V-type ATPase. All lanes in C contained 25 ng of protein except the 1° granule lane, which contained 50 ng. D contained 10 ng of protein/lane. The arrowhead indicates the immunoreactive band of -39 kDa. In D densitometric scans of blots like that shown in C were normalized to quantity of phospholipid using the protein/lipid ratios determined in Ref. 46 and standardized relative to the tertiary granule fraction, arbitrarily taken as 100%. The data are means ± S.E. of three blots.

The activation of H⁺ pumps in cytoplasts was assessed next, measuring BCECF fluorescence under conditions that favor detection of this transporter, as in Figs. 1 and 2. Fig. 6E illustrates results obtained at basal pHᵢ. Neither fMLP nor TPA stimulation could induce cytosolic alkalinization in cytoplasts, in striking contrast to that observed in whole neutrophils (e.g., Figs. 1 and 2). Moreover, pretreatment with CB failed to induce any detectable V-ATPase activity when the cytoplasts were subsequently stimulated with chemoattractant or phorbol ester (Fig. 6E). Failure to alkalinize was not due to the coexistence of an offsetting acidifying process. This was concluded because the addition of bafilomycin had little effect on pHᵢ in stimulated cytoplasts (Fig. 6E).

It is conceivable, however, that the procedure for cytoplast preparation itself induces insertion of V-type H⁺ pumps into the plasma membrane, so that further exocytosis and therefore stimulation of V-ATPase activity become impossible. If this were the case, then active pumps inserted in the plasma membrane during cytoplast isolation would be expected to respond to acidic stress by extruding H⁺ in a bafilomycin-sensitive manner. This possibility was tested by acid-loading cytoplasts with an ammonium prepulse and monitoring pHᵢ recovery, as described for whole cells in Fig. 2. As shown in Fig. 6F, cytoplasts showed a very slow pHᵢ recovery upon acidification to pHᵢ ~6.4. However, this alkalinization could not be enhanced by TPA stimulation, nor was it inhibited by bafilomycin. This argues against the presence of active V-type H⁺-ATPases in the plasma membrane of cytoplasts. When taken together, the data presented in Fig. 6 support the contention that the acidic in-

surface) using the lipid/protein ratios of the individual granule types determined previously (46). When normalized in this fashion, most of the V-ATPase is found in the tertiary granules (Fig. 5E), with significant amounts also in the primary granules and secretory vesicles. The small amount of staining observed in the secondary granules may represent contamination with tertiary granules during the fractionation procedure.

Cytoplasts Do Not Contain Acidic Granules and Do Not Express an Activatable V-ATPase—The data in Fig. 5 indicate that in resting neutrophils, virtually all the V-ATPases reside in internal membranes with little or no pump detectable in the plasma membrane. Together with the data of Figs. 1–5, these findings support the model of recruitment of plasmalemmal V-ATPases—most of the V-ATPase is found in the tertiary granules (Fig. 5E) and secretory vesicles. The small amount of staining observed in the secondary granules may represent contamination with tertiary granules during the fractionation procedure.

Neutrophil cytoplasts are intact cell fragments devoid of nuclei and granules. They can be prepared by centrifugation of CB-treated neutrophils through a discontinuous Ficoll gradient, as described before (30). We used such cytoplasts to test whether removal of secretory granules impaired pump activation. As shown in A and C of Fig. 6 and consistent with earlier results (47), cytoplasts are smaller than the parental cells and lack the granularity readily observable by bright field microscopy. More importantly, the acidic compartments that normally accumulate acridine orange in whole neutrophils are noticeably absent in cytoplasts (cf. B and D, Fig. 6).
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mediated H\textsuperscript{+} extrusion during neutrophil stimulation.

It is now recognized that V-type H\textsuperscript{+} pumps are active in the plasmalemma of phagocytic cells. Elicited as well as resident macrophages possess V-type pumps that are constitutively active and respond to cytoplasmic acid loading by extruding excess H\textsuperscript{+} across the plasma membrane (48–50). Unlike macrophages, untreated neutrophils do not display pumping in response to cytosolic acidification. Instead, they require an additional stimulus to begin the active extrusion of H\textsuperscript{+} (Refs. 17 and 20; see also Fig. 2B). The results of the present study suggest that the mechanism underlying this stimulation is the fusion of pump-bearing vesicles with the plasma membrane.

As shown by the immunoblot experiment in Fig. 5, the plasma membrane of resting neutrophils is largely void of V-ATPases. Four lines of evidence suggest that the appearance of pump activity on cellular stimulation occurs by exocytosis of granules containing the V-ATPases: (i) CB, which is known to synergize secretion, potentiates the activation of the pumps by either fMLP or TPA; (ii) the kinetics of exocytosis of secretory granules and vesicles parallels or precedes the appearance of pump activity; (iii) primary and tertiary granules, as well as secretory vesicles contain V-type pumps, as assessed by immunobllocting of the C subunit; and (iv) the removal of acidic intracellular compartments from neutrophils (i.e. by preparing cytoplasts) eliminated the activation of the pump by fMLP or TPA.

Analysis of the immunoblotting and secretion data of Figs. 3–5 provides an indication of the class(es) of granules involved in the delivery of H\textsuperscript{+} pumps to the plasma membrane. Secondary granules are unlikely to participate in this response, because they are largely devoid of H\textsuperscript{+}-ATPases. The minute amount detected in these granules by immunoblotting can readily be accounted for by cross-contamination with tertiary granules, which are incompletely removed from this fraction. Our failure to detect ATPases in secondary granules is in agreement with earlier observations that these organelles fail to accumulate DAMP, a weak base used for electron microscopy (43). For these reasons, we regard secondary granules as unlikely sources of the V-ATPases that are translocated to the plasma membrane.

Primary granules, which are lysosomal in nature and have been traditionally considered as the main source of H\textsuperscript{+} pumps in phagocytes, are also unlikely to provide the plasmalemmal ATPase activity in stimulated neutrophils. This was concluded mainly on the basis of the sensitivity of their secretion to agonists and to CB. Briefly, fMLP alone was virtually ineffective in triggering primary granule secretion (Fig. 3), whereas TPA had a minimal effect, which was barely detectable by 2 min. This profile differs from the appearance of the pump activity, which is clearly detectable with either agonist alone. Moreover, the large potentiation of primary granule secretion induced by CB is not commensurate with the comparatively smaller acceleration of H\textsuperscript{+} pumping. From these data, we conclude that the primary granules are unlikely to provide the source of the V-ATPase activity detected in the plasma membrane of stimulated cells.\footnote{Because primary granules contain significant amounts of V-ATPase, we cannot formally rule out their participation in the observed activation of pumping. It is conceivable that they are involved and that modulation of pump activity by other mechanisms may contribute to the quantitative discrepancies found (see "Discussion").}

When normalized to phospholipid content, tertiary granules were found to be the richest source of internal H\textsuperscript{+}-ATPases (Fig. 5E). This observation is in accordance with earlier reports that described uptake of radiolabeled weak bases into purified tertiary granules, ostensibly due to the H\textsuperscript{+} gradient generated across their membrane by an ATP-dependent pump (44, 45). Importantly, these granules are secreted upon stimulation with kinetics and an agonist sensitivity profile that closely parallel the appearance of pumping activity on the plasma membrane (see Figs. 1, 2, and 4). Similarly, secretory vesicles contain sizable amounts of V-ATPases and are secreted in response to fMLP and TPA with a pattern that could well account for the appearance of pumps on the cell surface. Taken together, these results are consistent with the idea that the majority of the pump activity in stimulated neutrophils origi-
nates primarily from the insertion of ATPases found in tertiary granules and/or secretory vesicles, with little contribution from the primary and secondary granules.

The insertion of V-type \( \text{H}^+ \) pumps into the plasma membrane of neutrophils appears analogous to the regulation of proton pumps in turtle bladder and other epithelia (26, 27). In these tissues \( \text{H}^+ \) pump-bearing vesicles are exocytosed into the apical membrane in response to acidification, a process seemingly mediated by an elevation of intracellular Ca\(^{2+} \), and is dependent on microtubules (26, 27). In neutrophils, preliminary experiments indicate that the pumping activity induced by TPA is abolished by pretreatment with vincristine, suggesting that it requires an intact microtubular array.

On the other hand, an artificial elevation of intracellular Ca\(^{2+} \) is insufficient, by itself, to elicit pump activation in neutrophils (20). Exocytosis of secretory vesicles and tertiary granules occurs under these conditions (10) with insertion of pumps into the surface membrane, suggesting the operation of additional mechanisms to regulate V-ATPases at the plasma membrane. In this regard, polypeptide activators and inhibitors of V-type H\(^+ \)ATPases, thereby providing a mechanism for the vectorial localization of pumps apoposed to the bonesurface (51). The ruffled border is enriched in V-ATPases, thereby providing a mechanism for the vectorial localization of pumps apposed to the bonesurface (51).

The insertion of V-type H\(^+ \)ATPases has been purified from other tissues (21-23). Additional evidence suggests that V-type pumps can also be regulated by dissociation of the V1 catalytic complex from the membrane-bound V0 domain that contains the H\(^+ \)-conducting pore (24, 25). Similar processes may conceivably function in neutrophils to alter the activity of pumps within the granule membrane or in those that have already been inserted into the plasmalemma.

In view of their elevated sensitivity to second messengers, tertiary granules and secretory vesicles are likely sequestered at an early stage of neutrophil recruitment (9). Indeed, the receptors in the membrane of secretory vesicles are essential for chemotaxis, and the gelatinase contained within tertiary granules is important in degrading the extracellular matrix as well as stimulating the migration of other cells.

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