Chloride Movements in Human Neutrophils during Phagocytosis: Characterization and Relationship to Granule Release

Sara Busetto, Elisa Trevisan, Eva Decleva, Pietro Dri and Renzo Menegazzi

*J Immunol* 2007; 179:4110-4124; 
doi: 10.4049/jimmunol.179.6.4110

http://www.jimmunol.org/content/179/6/4110

References

This article cites 111 articles, 61 of which you can access for free at: http://www.jimmunol.org/content/179/6/4110.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2007 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Chloride Movements in Human Neutrophils during Phagocytosis: Characterization and Relationship to Granule Release

Sara Busetto, Elisa Trevisan, Eva Decleva, Pietro Dri, and Renzo Menegazzi

Chloride ion efflux is an early event occurring after exposure of human neutrophils to several soluble agonists. Under these circumstances, a rapid and reversible fall in the high basal intracellular chloride (Cl\(^{-}\)) levels is observed. This event is thought to play a crucial role in the modulation of several critical neutrophil responses including activation and up-regulation of adhesion molecules, cell attachment and spreading, cytoplasmic alkalization, and activation of the respiratory burst. At present, however, no data are available on chloride ion movements during neutrophil phagocytosis. In this study, we provide evidence that phagocytosis of *Candida albicans* opsonized with either whole serum, complement-derived opsonins, or purified human IgG elicits an early and long-lasting Cl\(^{-}\) efflux accompanied by a marked, irreversible loss of Cl\(^{-}\). Simultaneous assessment of Cl\(^{-}\) efflux and phagocytosis in cytochalasin D-treated neutrophils indicated that Cl\(^{-}\) efflux occurs without particle ingestion. These results suggest that engagement of immune receptors is sufficient to promote chloride ion movements. Several structurally unrelated chloride channel blockers inhibited phagocytosis-induced Cl\(^{-}\) efflux as well as the release of azurophilic—but not specific—granules. It implicates that different neutrophil secretory compartments display distinct sensitivity to Cl\(^{-}\) modifications. Intriguingly, inhibitors of Cl\(^{-}\) exchange inhibited cytotoxic Ca\(^{2+}\) elevation, whereas Cl\(^{-}\) efflux was not impaired in Ca\(^{2+}\)-depleted neutrophils. We also show that FcγR(σ-) and CR3/CR1-mediated Cl\(^{-}\) efflux appears to be dependent on protein tyrosine phosphorylation but independent of PI3K and phospholipase C activation. The Journal of Immunology, 2007, 179: 4110–4124.

Neutrophils use part of their energy to pump chloride ions from the extracellular environment into the intracellular compartment, thereby accumulating a considerable amount of this anion (1). Accordingly, a distinct feature of resting neutrophils is an unusually high (80–100 mM) intracellular Cl\(^{-}\) concentration that is 4- to 5-fold higher than predicted on the basis of the Nernst equation (1, 2). The discovery of high basal chloride ions content (Cl\(^{-}\))^\(^{2}\) (2) in human neutrophils dates back approximately 40 years ago (3), but information on the role of chloride in the neutrophil pathophysiology has only recently started to emerge. In this regard, a major breakthrough was made when it was shown that a dramatic loss of Cl\(^{-}\) occurs when neutrophils are exposed to various physiological and nonphysiological soluble agonists (4). Many groups, including our own, have contributed to identify causal relationships between changes in Cl\(^{-}\) and activation of several neutrophil responses. Accordingly, direct measurements of Cl\(^{-}\) movements in stimulated neutrophils have provided evidence that Cl\(^{-}\) fluxes are involved in the modulation of several critical neutrophil responses including activation and up-regulation of adhesion molecules, cell attachment and spreading, cytoplasmic alkalization, and activation of the respiratory burst (5–9). These findings have led to the hypothesis that Cl\(^{-}\) may act as a second messenger in human neutrophils. At present, however, the mechanisms whereby Cl\(^{-}\) fluctuations regulate the complex events leading to neutrophil activation have not been elucidated.

Compared with other inflammatory cells, neutrophils have the largest repertoire of secretory compartments containing a number of proteins involved in cell adhesion, recognition of opsonized particles, generation of reactive oxygen species, and killing and degradation of microorganisms. In this regard, at least four distinct types of granules have been described within the cytoplasm, namely azurophilic or primary granules, specific or secondary granules, gelatinase-rich tertiary granules, and secretory vesicles (10, 11). These compartments are dispersed throughout the cytoplasm in unstimulated neutrophils. However, upon activation with phagocytosable or soluble agonists, granules fuse with the plasma membrane, thereby discharging their content from the cell (12–20). It has been previously demonstrated that secretory compartments are released following a precise hierarchy that largely depends on their distinct sensitivity to cytosolic Ca\(^{2+}\) concentrations. Specifically, it has been shown that secretory vesicles display the lowest Ca\(^{2+}\) threshold followed—in increasing order—by tertiary, specific, and azurophilic granules (21, 22).

Weissmann and coworkers first reported that anion channel blockers have the capacity to inhibit lysosomal enzyme secretion in human neutrophils stimulated by either immune complexes or...
the calcium ionophore A23187 (23, 24). Although these results pointed to a role of permeant anions in neutrophil degranulation, both the identity of the anions and the direction of fluxes remained unclear. Subsequently, a role for anion fluxes in exocytosis of neutrophil secretory compartments other than lysosomes has been hypothesized (25, 26).

The objectives of the present study were: 1) to examine Cl⁻ movements in human neutrophils exposed to particulate stimuli, 2) to investigate the potential relationships between Cl⁻ fluxes and degranulation, and 3) to characterize signal transduction pathways involved in the activation of Cl⁻ fluxes. To achieve these goals, we devised an experimental setting that may allow simultaneous assessment of both Cl⁻ fluxes and neutrophil functional responses. We provide evidence, for the first time, that phagocytosis of opsonized bacteria, fungi, and zymosan, elicits a Cl⁻ efflux similar to that induced by known soluble Cl⁻ releasers, such as TNF and fMLP. Such an efflux is due to a net outward movement of Cl⁻, because it is accompanied by a marked, irreversible loss ofCli.

We also show that Cl⁻ efflux regulates the release of azurophilic granules and the changes in cytosolic Ca²⁺ levels, and depends on protein tyrosine phosphorylation.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia Biotech. BSA, [2,3-dichloro-4-(2-methylene-butyryl)phenoxy]acetic acid (ethyranic acid, EA), 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS), tr-glucuronic acid sodium salt, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 2-[3-(trifluoromethyl)phenyl]-amino)-3-pyridinecarboxylic acid (niflumic acid, NA), 3-trifluoromethylphenoxy-3-phenyl-N-methyl-propylamine hydrochloride (fluoxetine, FLX), atracene-9-carboxylic acid (9-AC), (6-[3-hydroxymercuri-2-methoxy- propyl]carbamoyl)phenoxyacetic acid (mersa-ly, MA), α-cyano-4-hydroxycinnamic acid (CHC), 5(6)-carboxyfluorescein succinimidyl ester (5(6)-CFSE) were purchased from Molecular Probes Europe BV. 5(6)-carboxyfluorescein succinimidyl ester (5(6)-CFSE) was obtained from Merck KgaA. The dye was dissolved in distilled H₂O and filtered through a Millipore filter to remove non-solubilized material. Na³⁶Cl was obtained from Sigma-Aldrich. 1-(6-[17³⁵S]amino)hexyl)-1-pyrroline-2,5-dione (U73122) and 1-(6-[17³⁵S]amino)hexyl)-pyrrolidine-2,5-dione (U73343) were purchased from BIOMOL. Recombinant human TNF produced in Escherichia coli was obtained from Santa Cruz Biotechnology. Fura 2-AM, 5(6)-carboxyfluorescein succinimidyl ester (5(6)-CFSE) was purchased from Molecular Probes Europe BV. 5(6)-CFSE was dissolved in DMSO at 20 mg/ml and stored as a stock solution in the dark at ~20°C. High-purity trypan blue (TB) (color index 23850) was obtained from Merck KgaA. The dye was dissolved in distilled water at 5 mg/ml and filtered through a Millipore filter to remove non-solubilized material. Na³⁶Cl (sp. act., 14–15 μCi/g Cl⁻) was purchased from Amersham International. All other reagents and chemicals were of the highest purity grade available. All solutions were made in LPS-free water or physiologic saline (0.9% NaCl) for clinical use.

Neutrophil isolation

Neutrophils were isolated from peripheral blood anticoagulated with EDTA (final concentration, 4 mM) obtained from blood donors or laboratory personnel. Isolation was made according to the method described by Metcalf et al. (27), with slight modifications. In brief, 4 ml of anticoagulated fresh blood was layered onto a discontinuous Percoll gradient consisting of 4 ml of 62% and 4 ml of 75% Percoll in PBS and centrifuged at 200 × g for 10 min and then, without interruption, at 400 × g for an additional 15 min. Neutrophils were collected at the interface between the 62 and 75% Percoll and washed once at 250 × g for 7 min with Ca²⁺- and Mg²⁺-free HBSS buffer, 5 mM HEPES buffer, 5 mM glucose, pH 7.4. A brief (10-s) hypotonic shock contaminating erythrocytes was performed by suspending the cells in 3 ml of 1 mM phosphate buffer. Isotonicity was then restored by adding 7 ml of 1.3% NaCl in 1 mM phosphate buffer. After an additional washing in HBS containing 0.2% BSA (HBS-BSA), the isolated neutrophils were resuspended in the same medium at the desired concentration.

The resulting cell population contained 95–97% neutrophils, 2–3% eosinophils, and 1–2% lympho-monocytes. Before starting the functional assays, CaCl₂ and MgCl₂ (final concentration of both cations, 1 mM) were added to the neutrophil suspension. Incubation procedures were performed in poly(2-hydroxyethyl methacrylate) (poly[HEMA]) test tubes to avoid cell sticking to the tube walls. Where indicated, neutrophils suspensions were preincubated with chloride transport blockers, or control buffer, for 15 min at 37°C.

Measurement of ³⁶Cl⁻ efflux

Neutrophils, suspended at 10–15 × 10⁶/ml in Ca²⁺- and Mg²⁺-free HBS-BSA, were loaded with ³⁶Cl⁻ as previously described by Simchowitz and De Weer (1) by incubating the cells with ³⁶Cl⁻ (3.0–4.0 μCi/ml) for 2 h at 37°C in a shaking water bath. After loading, the cells were washed twice with prewarmed unlabeled buffer to remove the tracer and suspended in the same medium at 2.5 × 10⁶/ml. Measurements of ³⁶Cl⁻ efflux were performed in cells at rest or exposed to particulate or soluble stimuli. At the desired times, 800-μl aliquots of cell suspension were collected from duplicate tubes and centrifuged for 4 min at 400 × g. Then, 750-μl aliquots of the supernatants were withdrawn, and their radioactivity was counted by liquid scintillation counting in a beta counter (LS6000TA; Beckman Instruments, Fullerton, CA). The percentage of efflux was calculated as follows: [(cpm in the supernatant of time, sample) – (cpm in the supernatant of t₀ sample)/[(total cpm of cell suspension) – (cpm in the supernatant of t₀ sample)] × 100.

Measurement of Cl⁻ efflux

Changes in Cl⁻ efflux, that reflect net Cl⁻ movements, were measured using either ³⁶Cl⁻ (4) or a chemical colorimetric method (28). In the former case, the assay was performed exactly as previously described (8). In brief, neutrophils were suspended at 4–6 × 10⁶/ml in HBS-BSA and incubated with 3.0–4.0 μCi/ml ³⁶Cl⁻ for 2 h at 37°C. After loading, the cells were used without washing them free of the tracer. At the selected times of incubation, 200 μl of the cell suspensions were collected from duplicate tubes, diluted into 1400 μl of HBS-BSA prewarmed at 37°C, and centrifuged for 15 s at 12,000 × g. The pellet was suspended in 60 μl of HBS-BSA, and 50-μl aliquots were diluted into 500 μl of the same buffer layered on 600 μl of a mixture (ratio 4:1, density 1.005 ± 0.001 g/L) of silicone oil (density 1.041 g/L; silicone AR 200 fluid, 200 centistokes; Serva Electro- pheresis) and paraffin oil (density: 0.873 g/L; Merck). After 1 min of centrifugation at 12,000 × g, the supernatants were discarded, the bottom of the tubes were cut, and the cell pellet-associated radioactivity was counted as described above. Background values of ³⁶Cl⁻ trapped within the intracellular spaces in the cell pellet was measured by centrifugating an aliquot of the cell suspension immediately after the addition of Na³⁶Cl for the loading procedure. This radioactivity was found to account for <0.5% of the total radioactivity of the cell pellet after 2 h of loading with the tracer, and therefore, it was omitted from the calculations of ³⁶Cl⁻ efflux. The ³⁶Cl⁻ that remained associated to neutrophils at the selected incubation time was expressed as a percentage of ³⁶Cl⁻ associated to neutrophils at t = 0.

Cl⁻ efflux was determined also by means of a commercial kit (Chlorofix; Menarini Diagnostics) based on the colorimetric chemical assay described by Schoenfeld and Lowellen (28). This method was used instead of the radioactivity-based technique described above to avoid overestimation of cell-associated ³⁶Cl⁻ caused by entrapment within the phagosome(s) of the radiotracer present in the extracellular medium. The Chlorofix chemical assay is based on the reaction of Cl⁻ with merciric thiocyanate with generation of SCN⁻. This anion, in turn, reacts with Fe³⁺ and produces a reddish-brown end product that is proportional to Cl⁻ concentration and

![FIGURE 1. Determination of standard chloride concentrations by means of Chlorofix reagent.](http://www.jimmunol.org/Downloaded from)
can be read at 460 nm. Fig. 1 shows a calibration curve obtained with different concentrations of NaCl. The Chlorofix chemical assay proved to be sensitive enough to detect Cl⁻ concentrations in the range expected to be measured in our experimental conditions. Preliminary experiments showed that changes in Cl⁻, in TNF- or PMA-stimulated neutrophils as measured by using either 36Cl⁻ or the Chlorofix reagent, gave similar results, thus proving the reliability of the chemical assay (data not shown).

The assay was performed as follows. At the selected incubation times, aliquots of neutrophil suspensions (5·10⁹ to 10¹⁰/ml in HBS-BSA) were collected and centrifuged for 4 min at 400 g. The resulting pellets were suspended in 2 ml of isotonie sucrose and subsequently centrifuged for 30 s at 3500 g to wash out extracellular Cl⁻. The supernatants were carefully removed and discarded whereas the pellets were suspended in 100 to 200 µl of distilled water to lyse the cells and free intracellular Cl⁻. After 20 min, the solutions at room temperature, the cell lysates were subjected to a freeze-and-thaw cycle to fully disrupt the cells and disperse cell debris. The samples were centrifuged for 15 s at 12,000 × g and aliquots of the supernatants (25–75 µl) were subsequently transferred in duplicate wells of a microtiter plate. After addition of the Chlorofix reagent (200 µl), the plate was incubated for 2–3 min at room temperature, and absorbance was then read at 460 nm in a microplate reader (Power Wave X; BioTek Instruments, Inc.). Cl⁻ measured in neutrophils at the selected incubation time was expressed as a percentage of Cl⁻ at t = 0. Mean basal Cl⁻ concentration (Cl⁻i, at t = 0), as assessed by Chlorofix reagent, was 82.5 ± 19.8 mM (mean ± SD, n = 5), a value in agreement with previously published data (1, 2). In the experiments with C. albicans, control assays were performed in the absence of neutrophils to determine the contribution of candida-associated Cl⁻ to the overall Cl⁻ content of the samples. Candida-associated Cl⁻ was found to account for <1.5% of the total Cl⁻ content, and therefore, it was neglected in the calculations of Cl⁻i.

Microorganisms

Staphylococcus aureus 502A (American Type Culture Collection 27217) was a generous gift of Dr. J. Iandolo (Department of Microbiology and Immunology, University of Oklahoma Health Science Center, Oklahoma City, OK). C. albicans was a clinical isolate kindly provided by Dr. E. Crevatin (Unità Clinico Operativa di Igiene e Medicina Preventiva, University of Trieste).

Preparation of microorganisms and opsonization procedures

S. aureus was collected from single colonies grown on Luria-Bertani (LB)-agar plates, inoculated into LB broth, and grown for 18 h at 37°C. Frozen aliquots of C. albicans blastospores were diluted in Sabouraud broth and grown overnight at 30°C. Microorganisms were centrifuged by pelleting at 2000 × g for 5 min, transferred into a microtube, washed once in 0.9% NaCl solution by centrifugation at 12,000 × g for 10 s, and suspended in 1.5 ml of 0.9% NaCl. S. aureus concentration was assessed by measurement of turbidity at 500 nm. Blastospore concentrations were determined by microscopic counting in a cell-counting chamber. S. aureus and C. albicans suspensions were diluted to 1 × 10⁹ cells/ml and to 6 × 10⁹ cells/ml, respectively, in HBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ (Ca²⁺/Mg²⁺-HBS) and opsonized with 10% pooled human serum for 30 min at 37°C in a shaking water bath. Opsonized microorganisms were kept on ice until use. Where indicated, C. albicans blastospores were opsonized with human purified Ig (>95% IgG) (IG VENA; Kedrion S.p.A.) or IgG-free pooled human serum provided by Dr. L. Rigonat (Department of Immunology and Pathology, University of Trieste). A forward scatter threshold was set to gate out debris electronically by analyzing sample buffer alone. Neutrophils and free C. albicans particles were discriminated by combined measurements of forward scattering and side scattering and logarithmic amplification for FL1 and FL3. Each sample was analyzed for 30 s at the slowest flow rate to minimize the coincidental appearance of free yeast and neutrophils in the laser beam. The data were then analyzed by using CellQuest software from BD Biosciences. A forward scatter threshold was set to gate out debris electronically by analyzing sample buffer alone. Neutrophils and free C. albicans particles were discriminated by combined measurements of forward scattering and side scattering and gated in R1 and R2 regions, respectively. The percentage distribution of neutrophil subsets was calculated from dot plot analysis (FL1 vs FL3) of R1-gated events.

Assay of degranulation

Degranulation was measured after exposure of ³⁶Cl⁻-loaded neutrophils to opsonized C. albicans or FMLP to simultaneously assess granule release and chloride movements. Assay conditions were as described for ³⁶Cl⁻ efflux evaluation. In the experiments with FMLP, neutrophils were preincubated for 5 min at 37°C with cytochalasin D (2.5 µg/ml, final concentration). At the selected incubation times, aliquots of the cell suspensions were withdrawn and immediately centrifuged for 4 min at 400 g. The supernatant fluids were collected and stored on ice. Neutrophils contained in the pellets were disrupted in 0.1% (v/v) of cetyltrimethylammonium bromide. Both supernatants and disrupted cells were transferred into duplicate wells and the activity of the selected granule markers was assayed as detailed below. Release of granular proteins was expressed as a percentage of total activity.

Assays of enzyme activities

Colorimetric quantitative assays of enzyme activities were conducted in duplicate wells of microtiter plates. Myeloperoxidase (MPO) activity was measured as previously described (32), using 3,3′,5,5′-tetramethylebenzidine (TMB) as peroxidase substrate. In brief, 75 µl of 20 mM acetate buffer (pH 5.5) containing 2 mM TMB and 0.1% (w/v) cetyltrimethylammonium bromide were deposited into the wells. Then, 75 µl of either supernatant fluid or 20-fold diluted cell lysate were added. The peroxidatic reaction was started with H₂O₂ (0.3 mM final concentration) and blocked after 2–3 min with 100 µl of 2 N H₂SO₄. Absorbance was then read at 405 nm. Elastase activity was assayed according to the method described by Ciesla et al. (33), by cleavage of the specific elastase substrate N-methoxy-succinyl-ala-ala-pro-val-p-nitroanilide. The incubation mixture included: 270 µl of 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM CaCl₂, 0.5 mM NaCl, 40% (v/v) DMSO, 30 µl of either supernatant fluid or diluted cell lysate, and 18 µl of N-methoxy succinyl-ala-ala-pro-val-p-nitroanilide dissolved at 1 mg/ml in DMSO. After incubating the plate overnight at 37°C, absorbance was read 405 nm.

β-glucuronidase was assayed according to Gianetto and de Duve (34), using phenylphthalein glucuronide as substrate. Aliquots (50–100 µl) of supernatant fluid or diluted neutrophil lysates were transferred into the wells containing 90 µl of 200 mM sodium acetate buffer (pH 5.0) and 10 µl of phenolphthalein glucuronide dissolved at 10 mM in distilled water.
After incubating the plate overnight at 37°C, absorbance was read at 550 nm.

**Assay of lactoferrin release**

Release of the specific granule marker lactoferrin (LF) was measured by ELISA using anti-human LF rabbit polyclonal Abs (Sigma-Aldrich). All steps were conducted at room temperature. Immobilization of Abs onto plate microwells with hydrazide surface (HZ wells) (carbohydrate-binding 8-well Strip Plate, catalog no. 2508; Costar), which allows site-specific binding of Abs through the carbohydrate moieties of the Fc region (35), was performed exactly as described by Menegazzi et al. (9). Supernatant fluids, or cell lysates were diluted 300-fold in PBS containing 0.1% (w/v) BSA and 0.1% (v/v) Tween 20 (Merck) (PBS-T-BSA). 10 to 50 μl of the diluted samples were transferred into duplicate HZ-wells and incubated for 45 min. After 3 washings with PBS-T-BSA, biotinylated anti-human LF rabbit polyclonal Abs were added to the wells and the plate was incubated for further 45 min. After three additional washings, 50 μl of HRP-conjugated streptavidin (diluted 1000-fold in PBS-T-BSA) were added to the wells. After 45 min, HZ-wells were extensively rinsed with PBS-T-BSA and the peroxidatic reaction was subsequently conducted as described (32), using TMB as substrate. After blocking the peroxidatic reaction with 2 N H₂SO₄, absorbance was read at 405 nm with a microplate reader (Power Wave X; BioTek Instruments). LF concentrations in the samples were calculated using a calibration curve set up with known amounts of human LF. In our experimental conditions, LF cellular content was nearly 2.8 μg/10⁶ cells.

**Immunofluorescence flow cytometry**

Surface expression of CD18, a marker of secretory vesicles, secondary and tertiary granules (36), was measured in neutrophils incubated in suspension for 30 min at 37°C in either Ca²⁺/Mg²⁺-HBS or Ca²⁺/Mg²⁺-HBS-gluconate buffer. On completion of incubation, the cell suspensions were cooled on ice for 10 min and subsequently incubated for a further 60 min with 5 μg/ml mAb TS1-18 recognizing the CD18 subunit (common β-chain) of the CD11/CD18 Ag complex (β₂ integrin) (37). The cells were then washed free of the mAbs in ice-cold PBS and incubated for 45 min with a FITC-labeled rabbit anti-mouse IgG F(ab')₂ (Sigma-Aldrich). After two washings in PBS, the cells were suspended in 1% formaldehyde and analyzed by FCM using a FACSCalibur (BD Biosciences) flow cytometer.

**Lactate dehydrogenase assay**

The release of the cytoplasmatic enzyme lactate dehydrogenase was assayed as an index of cytotoxicity. The assay was performed by measuring the consumption of NADH during the conversion of pyruvate to lactate as described by Bergmeyer et al. (38).

**Spectrofluorometric measurement of Ca²⁺ fluxes**

In these experiments, fura-2 AM (Molecular Probes) was used as the fluorescent, Ca²⁺-sensitive indicator. Neutrophils (5–10 × 10⁶/ml in HBS buffer) were incubated in suspension with 2 μM fura-2 AM for 20 min at 37°C, diluted 5-fold and further maintained at 37°C for an additional 20 min. On completion of the loading procedure, the cells were diluted 1:1 with HBS buffer and washed twice in the same buffer. Neutrophils were suspended at 2.5 × 10⁶/ml in HBS buffer containing 1.0 mM Ca²⁺ and kept in the dark at room temperature. In the experiments in which Ca²⁺ and ⁴⁶Cl⁻ fluxes were measured in the same cell suspension, loading with fura-2 AM was conducted during the last 40 min of incubation of neutrophils with Na⁴⁶Cl⁻ (see above). For each assay, 1 ml of the cell suspension was put in a thermostated cuvette in a 650-10S fluorescence spectrophotometer (PerkinElmer) equipped with a device for continuous stirring of the incubation mixture. Excitation and emission wavelengths were set at 340 and 510 nm, respectively. Before starting the assay, MgCl₂ (final concentration, 1 mM) was added to the cell suspension. After a stable baseline was obtained, the cells were exposed to either opsonized C. albicans blastospores or FMLP. Increase in fura-2 fluorescence was monitored over a 5- to 10-min period. To assay the effect of CI⁻ transport inhibition on changes in cytosolic Ca²⁺, neutrophils were preincubated with the selected inhibitors, or control buffer, for 15 min at 37°C in the cuvette under continuous stirring. Such a preincubation did not modify the neutrophil basal Ca²⁺ levels (data not shown).

**Transmission electron microscopy**

Neutrophils were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 15 min at 4°C. For peroxidase staining, the cells were rinsed twice with 0.05 M Tris-HCl buffer (pH 7.5) at room temperature and were then incubated in the same buffer containing 0.05% 3,3’-diaminobenzidine tetrahydrochloride and 0.02% H₂O₂ (freshly prepared) for 60 min. On completion of the incubation, the cells were rinsed with 0.1 M cacodylate buffer (pH 7.4) for 10 min and postfixed in 2% OsO₄ for 60–90 min at room temperature. Neutrophils were then dehydrated through a series of graded ethanol and embedded in Dow epoxy resin (DER 332, Unione Chimica Europea). Ultrathin sections were cut by an Ultrathome III (Pharmacia LKB), stained with lead citrate, and examined with a transmission electron microscope (TEM) (EM208; Philips). For quantitative evaluation, at least 150 cell sections showing nuclear lobes were scored.

**Statistical analysis**

Statistical significance was tested by Student’s t tests calculated using GraphPad Prism 3.0 (GraphPad Software). Values of p < 0.05 were considered statistically significant.

**Results**

**Cl⁻ movements during phagocytosis: comparison with soluble agonists**

It is well known that human neutrophils respond to both soluble physiologic agonists (such as cytokines and chemotactic substances) and nonphysiologic activators (i.e., phorbol esters, A23187) with a rapid Cl⁻ efflux (4–8). Because Cl⁻ movements in phagocytosing neutrophils have not yet been rigorously evaluated, we have performed a series of experiments to address this issue. Specifically, ⁴⁶Cl⁻-loaded neutrophils were exposed to three distinct serum-opsonized phagocytosable particles, namely STZ, S. aureus, and C. albicans. Fig. 2A shows that these particles triggered a similar efflux of ⁴⁶Cl⁻. Notably, this efflux was comparable to that observed in cells exposed to the soluble agonists TNF and FMLP. To establish whether phagocytosis-induced Cl⁻ efflux is accompanied by a decrease in Cl⁻, or otherwise results from activation of Cl⁻/CI⁻ exchange (1), we subsequently measured variations in Cl⁻. As shown in Fig. 2B, Cl⁻, of resting neutrophils did not change significantly throughout the incubation. In contrast, phagocytosis caused a marked decrease of Cl⁻, thereby indicating a net outward flux of Cl⁻. Additionally, Fig. 2B demonstrates that the Cl⁻ ions lost after phagocytosis were not regained. In contrast, the markedly reduced levels of Cl⁻, caused by treatment with FMLP and TNF returned to normal at 60 min after exposure.

**Cl⁻ efflux during phagocytosis: role of cytoskeleton and immune receptors**

Opsonophagocytosis encompasses two main steps, namely 1) recognition, i.e., particle binding to phagocyte surface receptors including FcγR(s) and/or complement receptors (CR1/CR3), and 2) particle internalization requiring dynamic modifications of the actin cytoskeleton (39–43). To assess the relative role of binding and internalization in opsonophagocytosis-induced Cl⁻ efflux, we have conducted experiments in which binding, ingestion and Cl⁻ movements were simultaneously measured. We used C. albicans in such experiments inasmuch as we have recently developed a cytofluorometric assay to simultaneously assess its recognition and ingestion (31). Neutrophil-candida mixtures were incubated in suspension, and after a predetermined time period, aliquots were withdrawn and assayed for ⁴⁶Cl⁻ efflux, recognition and ingestion. Fig. 3A shows that the microfilament-disrupting agent cytochalasin D did not inhibit phagocytosis-induced Cl⁻ efflux. In contrast, this agent abrogated internalization of yeasts without affecting binding to neutrophils (Fig. 3B). Altogether these findings indicate that opsonin receptor-mediated recognition is sufficient to elicit Cl⁻ efflux even in the absence of internalization. As demonstrated in Fig. 4, serum-
tionally, opsonization with purified IgGs or IgG-depleted serum, reader. Cl
at room temperature and absorbance was read at 460 nm in a microplate
move the tracer and suspended at 2.5
neutrophils were washed twice with prewarmed HBS-BSA buffer to re-
taining 1 mM CaCl2 and 1 mM MgCl2.

ditions were collected from duplicate tubes and centrifuged for 4 min at
incubation at 37°C in a shaking water bath, 800-
efflux in human neutrophils. After loading with 36Cl
FIGURE 2. Serum-opsonized phagocytosable particles stimulate a net
outward chloride efflux in human neutrophils. After loading with 36Cl
radioactivity counting. Data represent the mean

FIGURE 3. Recognition, but not ingestion, is required to trigger 36Cl-
efflux in neutrophils during phagocytosis of serum-opsonized C. albicans.

Relationship between Cl− efflux and phagocytosis-induced
degranulation

The regulatory role of Cl− movements in several neutrophil func-
tions is well established. Accordingly, fluctuations of Cl−, have been shown to promote conformational changes and to increase expression of β2 integrins, TNF- and β2 integrin-mediated spreading and metabolic activation, as well as cytoplasmic alkalization (5–9). However, little is known concerning the relationships be-
tween Cl− efflux and degranulation (7). We have addressed this issue in an experimental model of neutrophils phagocytosing serum-opsonized C. albicans. Under these circumstances, both de-
granulation (44–46) and Cl− ions efflux occur. First, several struc-
turally unrelated chloride transport blockers (1, 47–52) were

used as a source of complement-derived opsonins, was almost as effective as opsonization with whole serum in inducing Cl− efflux.

derived opsonins are required to induce Cl− efflux by C. albicans.
Accordingly, nonopsonized yeasts did not elicit Cl− efflux. Addi-
tionally, opsonization with purified IgGs or IgG-depleted serum,
lowered Cl\(^-\) concentrations. In fact, suspension of resting cells for 90 min at 37°C. Assay of 36Cl\(^-\) efflux was as in Fig. 2. Data represent the mean ± SEM of three experiments performed with neutrophils isolated from different donors.

Fig. 5 shows that five of six compounds (namely EA, NA, 9-AC, NPPB, and FLX) coordinately inhibited 36Cl\(^-\) efflux (A) as well as the release of the azurophilic granule marker MPO (B). These compounds, however, did not significantly affect the release of the specific granule marker LF (C). DIDS did not alter 36Cl\(^-\) efflux or granule release at the tested concentration. None of the compounds affected lactate dehydrogenase release or inhibited the enzymatic activity of purified human MPO (data not shown). These results indicated that inhibition of MPO release could not be due to drug-induced cell toxicity or alterations in enzymatic activity of MPO.

The effect of Cl\(^-\) transport inhibitors on neutrophil degranulation during phagocytosis was subsequently investigated at the ultrastructural level. Neutrophils pretreated with either solvent (0.3% DMSO) or 150 μM EA, a broad spectrum and potent inhibitor of Cl\(^-\) movements (1, 4, 6–9), were incubated for 15 min with C. albicans. Preparations were then fixed, stained for peroxidase, and analyzed by TEM. The results of these analyses showed that the percentage of phagocytosing cells in EA-treated neutrophils (calculated as the number of cell sections showing at least one ingested candida particle) was similar to that observed in control neutrophils (nearly 60%). These data are in keeping with the results of cytofluorometric studies (Table I). One representative experiment is shown in Fig. 6. In >90% of control neutrophils, the peroxidase-positive granules gathered around and/or fused with the phagosome membrane (Fig. 6, a–c). Conversely, azurophilic granules were scattered within the cytoplasm of EA-treated cells and only occasionally they were fused with the phagosomal membrane (Fig. 6, d–f). In contrast, specific granules fused with phagosomes were evident in EA-treated neutrophils (Fig. 6f and inset therein).

Taken together, these results suggest a causal relationship between Cl\(^-\) efflux and degranulation. Moreover, they indicate that degranulation of diverse secretory compartments are differentially sensitive to changes in Cl\(^-\). This hypothesis was further substantiated by data shown in Table II, obtained in cells with artificially lowered Cl\(^-\), concentrations. In fact, suspension of resting cells for 30 min in Cl\(^-\)-free buffer resulted in a nearly 40% decrease of basal Cl\(^-\), as well as in different mobilization of secretory organelles. Specifically, compared with cells suspended in Cl\(^-\)-containing buffer, the release of secretory vesicles, tertiary and secondary granules—measured as surface expression of CD18 (36)—was markedly higher (+430%) compared with the release of LF (+187%) as well as of MPO (+98%).

<table>
<thead>
<tr>
<th>Table I. Effect of Cl(^-) transport inhibitors on phagocytosis of serum-opsonized C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Treatment</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Control buffer(^b)</td>
</tr>
<tr>
<td>EA (μM)</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>150</td>
</tr>
<tr>
<td>250</td>
</tr>
<tr>
<td>DIDS (μM)</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>120</td>
</tr>
<tr>
<td>MA (μM)</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>2500</td>
</tr>
<tr>
<td>CHC (μM)</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>2500</td>
</tr>
<tr>
<td>9-AC (μM)</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>2500</td>
</tr>
<tr>
<td>NA (μM)</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>NPPB (μM)</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>150</td>
</tr>
<tr>
<td>FLX (μM)</td>
</tr>
<tr>
<td>12.5</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>50</td>
</tr>
</tbody>
</table>

\(^a\)Neutrophils (2.5 \times 10^6/ml) were incubated for 15 min at 37°C in a shaking water bath with the indicated substances and subsequently mixed with serum-opsonized, 5(6)-FAM-SE-labeled C. albicans (1:5 cell-to-candida ratio). After 30 min of incubation at 37°C, aliquots of the incubation mixtures were diluted with an equal volume of 250 μg/ml TB dissolved in ice-cold 0.1 M citrate buffer (pH 4) and analyzed by flow cytometry. Quantitation of recognition and ingestion were performed as described in detail in Materials and Methods. Data are the means of two independent experiments performed with neutrophils isolated from different donors.

\(^b\)Control assays were performed with cells suspended in HBS buffer containing the appropriate working dilution of the solvent. Stock solutions of all inhibitors were made in DMSO, except MA, which was dissolved in physiologic saline.

Relationship between Cl\(^-\) movements and cytosolic Ca\(^{2+}\) elevation

Ca\(^{2+}\) plays a crucial role in the regulation of neutrophil degranulation. Accordingly, neutrophil secretory compartments have been shown to degranulate in hierarchical order depending on their sensitivity to cytosolic Ca\(^{2+}\) concentrations. In this regard, azurophilic granules display the highest Ca\(^{2+}\) threshold. In fact, proteins contained within these granules are released only when cytosolic Ca\(^{2+}\) concentrations rise to 700 nM or higher (21, 53).

Because inhibition of Cl\(^-\) efflux influenced the release of azurophilic but not specific granules, we asked whether such difference could be ascribed to a regulatory effect of Cl\(^-\) fluxes on cytosolic Ca\(^{2+}\) levels. To address this issue, the effects of chloride channels
blocks on changes in cytosolic Ca\(^{2+}\) and 36Cl\(^{-}\) efflux were simultaneously assessed. In these experiments, EA and FLX—belonging to the phenoxyacetate and propylamine family, respectively—were used. Neutrophil suspensions were loaded with both 36Cl\(^{-}\) and fura-2 AM, washed, transferred into a cuvette, and then incubated with or without EA or FLX under continuous stirring. After the addition of opsonized C. albicans, changes in cytosolic Ca\(^{2+}\) were recorded in a spectrofluorometer. An aliquot of the cell suspension was centrifuged and the resulting supernatant was assayed for 36Cl\(^{-}\).

Fig. 7, A–C shows that both EA and FLX inhibited 36Cl\(^{-}\) efflux and cytosolic Ca\(^{2+}\) elevation in a dose-dependent manner. Intriguingly, the calculated IC\(_{50}\) values for inhibition of cytosolic Ca\(^{2+}\) and 36Cl\(^{-}\) efflux were similar (EA, 117.1 ± 3.5 μM and 125.0 ± 1.2 μM; FLX, 31.1 ± 3.5 μM and 34.3 ± 3.1 μM for Ca\(^{2+}\) and 36Cl\(^{-}\), respectively, mean ± SD, n = 3–6). As expected, both compounds inhibited azurophilic granules release by nearly 50% as determined by MPO release (data not shown).

Ca\(^{2+}\) mobilization from inositol 1,4,5-triphosphate (Ins(1,4,5)P\(_3\)) sensitive intracellular stores may occur upon engagement of FcyR(s) and CR3 (54, 55) but not of CR1 (56, 57). Store-derived Ca\(^{2+}\) mobilization is an early event contributing to the overall Ca\(^{2+}\) elevation and the subsequent activation of several neutrophil responses, including phagocytosis-induced degranulation (58, 59). To investigate whether Cl\(^{-}\) efflux may play a role in the mobilization of Ca\(^{2+}\) from intracellular stores, neutrophils were preincubated with either EA or FLX in Ca\(^{2+}\)-free, EGTA-containing buffer and exposed to opsonized C. albicans particles. Fig. 7 shows that inhibition of 36Cl\(^{-}\) efflux (E) was accompanied by inhibition of cytosolic Ca\(^{2+}\) elevation (F). It is worth noting that these experimental conditions did not influence particle recognition (D).

Studies with cells exposed to a soluble secretagogue (fMLP) further support a regulatory role of Cl\(^{-}\) fluxes in Ca\(^{2+}\) homeostasis. Fig. 8A shows that four chemically unrelated Cl\(^{-}\) transport blockers inhibited fMLP-induced 36Cl\(^{-}\) efflux. Specifically, EA, NA, and NPPB showed the most potent inhibitory effect (nearly 70% inhibition). Inhibition by FLX was less marked although remaining statistically significant (nearly 30% inhibition, p < 0.025, n = 3). The effects of two Cl\(^{-}\) transport blockers, namely EA (strong inhibitor) and FLX (less potent inhibitor) on intracellular Ca\(^{2+}\) changes were then examined. Fig. 8 shows that, similar to the effects observed during phagocytosis of C. albicans (Fig. 7), inhibition of 36Cl\(^{-}\) efflux was paralleled by an inhibition of cytosolic Ca\(^{2+}\) elevation in the presence (Fig. 8, B and C) or in the absence (Fig. SD) of extracellular Ca\(^{2+}\). The data reported in Table III indicate that both EA and FLX inhibited fMLP-induced neutrophil degranulation. As expected, MPO release was more markedly affected than LF release, because azurophilic granules are less sensitive to Ca\(^{2+}\) elevation than the specific ones (21). With fMLP as a stimulatory agent, higher concentrations of EA (300 μM) could be used compared with phagocytosis experiments. Under these circumstances, EA strongly inhibited both 36Cl\(^{-}\) efflux and cytosolic Ca\(^{2+}\) elevation (92% and 89%, respectively; means of two independent experiments). Accordingly, the release of both MPO and LF was also markedly reduced (95.7 ± 5.7% and 92.2 ± 7.4% inhibition, respectively; means ± SD, n = 4).

These results suggest that Cl\(^{-}\) fluxes may act as an upstream regulatory signal for cytosolic Ca\(^{2+}\) elevation triggered by activation of different receptor types. If this is the case, it is expected that Cl\(^{-}\) efflux is independent of Ca\(^{2+}\) elevation. To test whether actually Cl\(^{-}\) efflux is independent, 36Cl\(^{-}\) efflux was assayed in neutrophils loaded with the Ca\(^{2+}\) buffer agent BAPTA and subsequently suspended in Ca\(^{2+}\)-free medium containing EGTA. As expected (Fig. 9A) Ca\(^{2+}\)-depleted neutrophils did not show a rise in cytoplasmic Ca\(^{2+}\) after exposure to either opsonized candida particles or fMLP. Furthermore, Ca\(^{2+}\) depletion did not alter chloride fluxes stimulated by fMLP or by phagocytosis of IgG- or
C3b/bi-coated candida blastospores (Fig. 9B). The small decrease in chloride efflux after phagocytosis of candida is likely to be due to the slight reduction in binding (~15%) of opsonized particles to BAPTA/EGTA-treated cells (data not shown). Altogether these findings provide strong evidence that Cl\(^{-}\) efflux in stimulated neutrophils is largely independent of cytosolic Ca\(^{2+}\) changes.

**Intracellular signals involved in the activation of Cl\(^{-}\) efflux**

As previously shown, Cl\(^{-}\) ions movements triggered by particulate or soluble stimuli appear to precede and to be independent of cytosolic Ca\(^{2+}\) increase, one of the earliest events in the signaling pathways elicited by Fc\(y\)R(s), CR3, and fMLP receptor (fMLP-R) (55, 60–64). We thus sought to investigate the signals involved in the control Cl\(^{-}\) fluxes. Specifically, we focused our attention on known critical intracellular messengers that act upstream of calcium, including tyrosine kinases (TKs), PI3K, PLC, and the pertussis toxin (Ptx)-sensitive G\(_{\text{i}}\) protein (55, 60–71). To determine whether these regulatory proteins could play a role in the activation of Cl\(^{-}\) movements, \(^{36}\)Cl\(^{-}\)–loaded neutrophils were pretreated with specific inhibitors and subsequently exposed to opsonized candida particles or fMLP. Experimental results are summarized in Fig. 10.

Fig. 10 shows that the broad-spectrum protein TK inhibitor genistein almost completely abolished \(^{36}\)Cl\(^{-}\) efflux triggered by either IgG- or C3b/bi-coated C. albicans (top panel), thereby suggesting that activation of cytosolic TK is necessary for such an efflux to occur. It is worth noting that genistein, at the concentration used in these experiments, did not influence recognition and ingestion of candida particles as judged by cytofluorometric analysis (76.5 and 73.9% of cells with bound/ingested candida particles in untreated and genistein-treated cells, respectively; mean of two duplicate experiments). In contrast, fMLP-mediated Cl\(^{-}\) efflux was insensitive to genistein (bottom panel), thereby indicating that Cl\(^{-}\) channel/transporter activation is independent of the tyrosine phosphorylation wave elicited by this compound (72 and references therein).

PI3K has been shown to act downstream of cytosolic TK(s) and fMLP-R-coupled G\(_{\text{i}}\) protein but upstream of Ca\(^{2+}\) release from intracellular stores (61–64, 68). It is thus possible that this kinase may act as an activator of Cl\(^{-}\) efflux. As shown in Fig. 10, wortmannin, a specific inhibitor of PI3K when used in the nanomolar range (73), did not modify \(^{36}\)Cl\(^{-}\) efflux from neutrophils exposed to opsonized candida (top panel) or fMLP (bottom panel). As expected, wortmannin markedly inhibited Ca\(^{2+}\) elevation induced by engagement of either Fc\(y\)R(s) or CR3 (94.3 and 80% inhibition, respectively; mean of two experiments performed with cells isolated from different donors), thereby confirming its effectiveness as a PI3K inhibitor. It is therefore concluded that this kinase is not involved in the activation of Cl\(^{-}\) movements.

Engagement of opsonin receptors and fMLP-R results in activation of PLC\(_{\gamma}\) and PLC\(_{\beta}\), respectively (55, 69, 70). Both PLC isoforms catalyze the breakdown of PtdIns(4,5)P\(_{2}\) to generate Ins(1,4,5)P\(_{3}\), and diacylglycerol (DAG) (74). Besides their role in classical signal transduction, it has been recently suggested that phosphoinositides and DAG may play a role in the regulation of ion channels (75–77). Interestingly, recent data have indicated that inflammatory mediators may trigger a volume-regulated PLC-activated chloride current through a DAG-dependent Ins(1,4,5)P\(_{3}\)–independent pathway, without the involvement of PKC-dependent phosphorylation (78). To assess the possible role of PLC in phagocytosis- and fMLP-stimulated Cl\(^{-}\) efflux, we tested the effect of the PLC inhibitor U73122, a membrane-permeable aminosteroid
that interferes with other PLC-mediated neutrophil responses (55, 67, 79). Fig. 10 shows that 36Cl− fluxes stimulated by opsonized candida blastospores (top panel) or fMLP (bottom panel) were insensitive to U73122, thereby suggesting that PLC activation is not required for the occurrence of Cl− efflux. It is worth noting that, under the same experimental conditions, U73122 efficiently inhibited PLC-dependent cellular responses. In fact, this compound markedly reduced phagocytosis- and fMLP-induced increase of cytosolic Ca2+ (85.3 ± 5.2% and 88.0 ± 6.1% of inhibition for candida- and fMLP-mediated response, respectively; mean ± SD, n = 3). As expected, the inactive analog U73343 had no effect on both 36Cl− efflux and cytosolic Ca2+ elevation (data not shown).

fMLP-R is a member of the G protein-coupled receptor superfamily of seven transmembrane spanning receptors. Upon ligand binding to fMLP-R, the heterotrimeric Gα protein dissociates into a GTP-bound α-subunit (Gα-GTP) and a βγ-subunit (reviewed in Ref. 68). Several reports have clearly demonstrated that Ptx ribosylates Gα, thereby preventing Gα activation. Accordingly, Ptx blocks several fMLP-stimulated neutrophil functions (64, 80, 81). In keeping with these data, we have shown that Ptx markedly inhibited 36Cl− efflux (bottom panel). This result suggests that Gα protein activation plays a pivotal role in fMLP-induced Cl− movements.

Discussion

The present study is the first to examine Cl− movements in human neutrophils during phagocytosis. Specifically, we have shown that phagocytosis is accompanied by a massive and irreversible decrease in Cl− and that such a decrease regulates degranulation.

Cl− movements in both resting and stimulated neutrophils have been previously investigated in a host of different experimental models, including cells in suspension (1, 4–8), cells adherent to surfaces coated with extracellular matrix proteins (8), and cells laid onto surface-bound mAbs recognizing either the common β-chain (CD18) or the individual α-chains (CD11a, CD11b, CD11c) of β2 integrins (9). Altogether, these studies have provided novel insights into the role of Cl− movements in neutrophil pathophysiology. In addition, they have clearly demonstrated a positive relationship between modifications of Cl− and activation of several neutrophil responses (5–9). Under these circumstances, we undertook this study to shed light on the role Cl− movements during neutrophil phagocytosis.

In this regard, we have first shown that phagocytosis of serum-opsonized particles is accompanied by a considerable Cl− efflux which is paralleled by a marked loss of Cl− (Fig. 2). Differently from the effects observed with the use of soluble agonists, the decrease in Cl− induced by phagocytosis appeared irreversible, thereby suggesting that the mechanisms responsible for Cl− regain in phagocytosing neutrophils are unable to counteract the loss of chloride ions. In this regard, it is worth noting that Cl− movements with similar features have been reported in neutrophils adherent to fibronectin-coated surfaces (8). The mechanism of Cl− reuptake remains to be elucidated, and the question as to whether this mechanism does not occur in phagocytosing cells deserves further investigations.

We then aimed to gain a better understanding of Cl− fluxes in phagocytosing neutrophils. We first attempted to elucidate the relationships between Cl− release and phagocytosis. To address this issue, 36Cl−-loaded cells incubated with serum-opsonized, fluorosecin-labeled C. albicans blastospores were used. This allowed us to evaluate simultaneously both responses. Our results showed that Cl− fluxes were largely independent of particle ingestion because cytochalasin D, that almost abolished particle internalization, only slightly affected Cl− efflux (Fig. 3). It is thus posited that recognition of opsonized particles plays a crucial role in triggering Cl− fluxes. Moreover, nonopsonized candida particles failed to stimulate chloride efflux, thereby suggesting that engagement of opsonin receptors is essential to elicit this effect. Additionally, these results ruled out a major role of other receptors involved in neutrophil-candida interactions, such as β-glucan receptors (Dec-1) or mannose receptors (82, 83) in the stimulation of Cl− efflux. Cl− fluxes induced by candida particles coated with either IgG or C3b/bi alone were lower compared with those triggered by candida opsonized with whole serum (Fig. 4). This small but reproducible difference could be ascribed to the lack of FcγR(s)/CR3 cooperation in the induction of neutrophil activation (84), because particles opsonized with whole serum, IgG or C3b/bi bound similarly to neutrophils (data not shown). These results are in agreement with our previously published data showing that CR3 (CD11b/CD18) is capable of triggering Cl− efflux in human neutrophils (9). In addition, the finding that IgG-opsonized candida particles stimulate Cl− fluxes suggested a novel signaling property for FcγR(s).

Release of granular proteins during phagocytosis plays a role in both microbicidal activity and tissue damage. A role for permeant anions in the regulation of neutrophil degranulation has been previously hypothesized based on inhibition of lysosomal enzyme secretion by anion channel blockers (23, 24). To gain new insights into the role of Cl− movements in neutrophil degranulation, we devised an experimental set-up that allowed simultaneous evaluation of both Cl− fluxes and exocytosis. Experiments with the use of several chemically unrelated Cl− transport inhibitors unequivocally showed the existence of a positive correlation between Cl− efflux and the release of azurophilic granules (Fig. 5, A and B). Interestingly, the release of specific granules appeared largely independent of Cl− fluxes, inasmuch as none of the inhibitors significantly affected LF release (Fig. 5C). Further evidence for a
distinct sensitivity of the two major types of granules to cytoplasmic Cl−/H11002− levels came from a different experimental approach, namely ultrastructural analysis of phagocytosis-induced degranulation. The TEM micrographs (Fig. 6) showed that fusion of azurophilic granules with phagosomes is markedly diminished in the presence of the Cl−/H11002− transport inhibitor EA. In contrast, fusion of specific granules was not affected, thereby confirming enzyme assays data (Fig. 5, B and C). These findings, along with the observation that chloride transport inhibitors did not affect C. albicans ingestion (Table I), seem to exclude that the reduction of azurophilic granule release might be ascribed to drug toxicity.

Altogether these results suggest that the extent of release of distinct secretory compartments may depend on Cl−/H11002− levels. This possibility is supported by the observation that degranulation of all secretory organelles occurred when Cl−/H11002− was lowered to nearly 60% of basal levels by means of incubation in Cl−/H11002−-free buffer (see Table II). It should be noted, however, that the release of small secretory vesicles, tertiary and secondary granules largely exceeded that of azurophilic granules. These data are in keeping with previously published observations on the spontaneous release of specific granules from resting neutrophils suspended in Cl−/H11002−-free gluconate buffer (85).

Although the release of primary and secondary granules may follow distinct signaling pathways (86–91), elevation of cytosolic Ca2+/H11001− is largely recognized as a crucial step in neutrophil degranulation. Additionally, the order of release of neutrophil secretory organelles is tightly controlled by the gradual increase of cytosolic calcium (21, 22). Because Cl−/H11002− fluxes appeared to be involved in the regulation of phagocytosis-induced granule secretion, we addressed the question as to whether a relationship exists between Cl−/H11002− and Ca2+/H11001− movements. To explore this possibility, we performed a series of cross-inhibition experiments using a model allowing simultaneous assessment of 36Cl−/H11002− fluxes and cytosolic Ca2+/H11001− changes. We found that Cl−/H11002− transport blockers had parallel, dose-dependent inhibitory effects on Cl−/H11002− efflux and Ca2+/H11001− elevation (Fig. 7, A–C). In contrast, depletion of intracellular calcium by

**FIGURE 7.** Effect of Cl− transport inhibitors on phagocytosis-induced cytosolic Ca2+/H11001− elevation and 36Cl−/H11002− efflux. Neutrophils were loaded with 36Cl−/H11002− and fura-2 AM, washed in HBS buffer, and suspended at 2.5 × 106/ml in HBS-BSA containing 1 mM CaCl2 and 1 mM MgCl2. A total of 1 ml of the cell suspension was then transferred into a cuvette kept at 37°C, placed in a fluorescence spectrophotometer equipped with a device for continuous stirring. A, To assay the effect of EA and FLX on cytosolic Ca2+/H11001− elevation and 36Cl−/H11002− efflux, neutrophils were preincubated without and with the compounds, for 15 min under continuous stirring. After a stable baseline was obtained, the cells were exposed to serum-opsonized C. albicans and changes in fura-2 fluorescence were monitored over a 10 min period. An aliquot of the cell suspension was withdrawn, centrifuged and the supernatant assayed for 36Cl−/H11002− efflux as described in Fig. 2. Data of intracellular Ca2+/H11001− and 36Cl−/H11002− efflux are expressed as percentage of control fura-2 fluorescence and 36Cl−/H11002− release, respectively. B and C, Representative fura-2 recordings of candida-induced cytosolic Ca2+/H11001− changes in neutrophils exposed to different concentrations of FLX and EA. The arrows indicate the addition of serum-opsonized candida blastospores. D, E, and F, 36Cl−/H11002− and fura-2-loaded neutrophils were suspended in Ca2+/H11001−-free HBS-BSA containing 1 mM MgCl2. EGTA (1 mM, final concentration) was added to the incubation mixture 2 min before the addition of candida blastospores. Changes in cytosolic Ca2+/H11001− and 36Cl−/H11002− efflux were monitored as above. Assay of recognition was as described in Fig. 3. Data are means ± SEM of three to ten experiments. Asterisks in E and F denote values significantly different from the control (*, p < 0.05; **, p < 0.01).
Materials and Methods
detailed in

significantly influence phagocytosis-induced $^{36}$Cl$^{-}$ influx. Our present findings are consistent with previous data showing that Cl$^{-}$ ions fluxes in the regulation of neutrophil calcium homeostasis. Our present findings are consistent with previous data showing that the Src family protein TK p56lck plays a crucial role in the activation of Cl$^{-}$ channels in human lymphocytes (94, 95). The results obtained with the use of Ptx indicate, for the first time, that G$i$ protein activation is required to elicit Cl$^{-}$ fluxes evoked by fMLP. Because other signal transducers downstream of G$i$ protein (TK, PI3K, PLC) do not appear to be involved in fMLP-induced Cl$^{-}$ influx (Fig. 10, bottom panel), it remains to be established whether G$i$-derived subunits may serve by engagement of immune receptors and fMLP-R depend on TK and G$i$ protein activation, respectively. With regard to the role of TK as regulators of Cl$^{-}$ movements, our data are in line with previous findings showing that the Src family protein TK p56lck plays a crucial role in the activation of Cl$^{-}$ channels in human lymphocytes (94, 95). The results obtained with the use of Ptx indicate, for the first time, that G$i$ protein activation is required to elicit Cl$^{-}$ fluxes evoked by fMLP. Because other signal transducers downstream of G$i$ protein (TK, PI3K, PLC) do not appear to be involved in fMLP-induced Cl$^{-}$ influx (Fig. 10, bottom panel), it remains to be established whether G$i$-derived subunits may serve

BAPTA/EGTA abolished Ca$^{2+}$ response (Fig. 9A) but did not significantly influence phagocytosis-induced $^{36}$Cl$^{-}$ efflux (Fig. 9B). Similar results were obtained when neutrophils were exposed to the soluble agonist fMLP (Figs. 8 and 9), thereby supporting a role of Cl$^{-}$ ions fluxes in the regulation of neutrophil calcium homeostasis. Our present findings are consistent with previous data showing that Cl$^{-}$ movements may influence Ca$^{2+}$ mobilization (92–93).

In the present report we have also identified the intracellular signaling pathways regulating Cl$^{-}$ fluxes. As demonstrated by the experiments with genistein and Ptx (Fig. 10), Cl$^{-}$ fluxes triggered by engagement of immune receptors and fMLP-R depend on TK and G$i$ protein activation, respectively. With regard to the role of TK as regulators of Cl$^{-}$ movements, our data are in line with previous findings showing that the Src family protein TK p56lck plays a crucial role in the activation of Cl$^{-}$ channels in human lymphocytes (94, 95). The results obtained with the use of Ptx indicate, for the first time, that G$i$ protein activation is required to elicit Cl$^{-}$ fluxes evoked by fMLP. Because other signal transducers downstream of G$i$ protein (TK, PI3K, PLC) do not appear to be involved in fMLP-induced Cl$^{-}$ influx (Fig. 10, bottom panel), it remains to be established whether G$i$-derived subunits may serve

Table III. Effect of Cl$^{-}$ transport inhibitors on fMLP-induced degranulation $^{a}$

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>MPO Release (%)</th>
<th>Inhibition (%)</th>
<th>LF Release (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent$^{b}$</td>
<td>40.6 ± 5.3</td>
<td>-</td>
<td>56.6 ± 6.1</td>
<td>-</td>
</tr>
<tr>
<td>150 µM EA</td>
<td>8.7 ± 4.5</td>
<td>78.6</td>
<td>38.4 ± 3.2</td>
<td>32.1</td>
</tr>
<tr>
<td>50 µM FLX</td>
<td>19.7 ± 5.6</td>
<td>51.0</td>
<td>54.3 ± 5.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

$^{a}$ Neutrophils (2.5 × 10$^{6}$/ml) were preincubated for 15 min at 37°C in a shaking water bath with the Cl$^{-}$ transport inhibitors. Cytochalasin D (2.5 µg/ml, final concentration) was added during the last 5 min of preincubation. The cells were subsequently exposed to 1 nM fMLP for 15 min. On completion of the incubation, aliquots of the cell suspensions were withdrawn, centrifuged for 4 min at 400 × g and the supernatant fluids were collected and stored on ice. MPO and LF were assayed in supernatants and lysed pellets as detailed in Materials and Methods. Data are the mean ± SEM of three to five experiments.

$^{b}$ 0.3% DMSO.
as direct activators of ion channels in neutrophils, as it has been previously demonstrated for the βγ subunit in other cell types (96 and references therein).

Previous data have shown that PI3K is rapidly activated by TK following FcγR(s) or CR3 engagement (61–63). Moreover, it has been also suggested to play a role in fMLP-mediated neutrophil responses (64, 68). Working from these assumptions, we sought to determine the role of this kinase in eliciting Cl− movements. Wortmannin, an inhibitor of PI3K, did not affect 36Cl− efflux (Fig. 10). This result strongly suggests that PI3K is not involved in the modulation of Cl− fluxes.

Neutrophils express two isoforms of PtdIns(4,5)P2-specific PLC, namely PLCγ2—which is activated by tyrosine phosphorylation in response to FcγR(s) or CR3 engagement (55, 70)—and PLCβ2, whose activation depends on the Ptx-sensitive release of βγ subunit after engagement of Gs protein-coupled fMLP-R (69). Recent reports have shown that phosphoinositides and DAG may play a role in the regulation of ion channels, including a voltage-sensitive PLC-activated chloride channel evoked by inflammatory mediators through a DAG-dependent Ins(1,4,5)P3-independent pathway (75–78). We therefore aimed to investigate the potential role of PLC in the modulation of Cl− movements. In this study we have demonstrated that 36Cl− effluxes are insensitive to the PLC inhibitor U73122 (Fig. 10), thereby suggesting that the PLC/DAG system is unlikely to play a major role in the modulation of neutrophil chloride movements.

Our current report supports the concept that Cl− efflux may be considered one of the earliest events in the process of neutrophil activation (4). Of interest is also the observation that rapid activation of Cl− efflux occurs in other mammalian cells. Accordingly, a very quick (t/2 < 10 s) and dramatic (nearly 50%) loss of Cl− due to rapid efflux has been described in rat parotid acinar cells upon stimulation with a muscarinic cholinergic agonist (97). It has been also shown that progesterone-mediated tyrosine phosphorylation of the sperm γ-aminobutyric acidA (GABA_A)-like receptor/Cl− channel triggers a rapid Cl− efflux regulating Ca2+ elevation during the acrosome reaction (93).

At present, it remains to be established how changes in Cl− can modulate neutrophil Ca2+ homeostasis and degranulation. Because we have demonstrated that inhibitors of Cl− efflux interfere with Ca2+ release from intracellular stores (Fig. 7D), it is posited that cytoplasmic chloride levels may influence the interaction of Ins(1,4,5)P3 with its receptor located on store membranes. The observation that the binding of Ins(1,4,5)P3 to its receptor may be
competitively inhibited by heparin and other polyanionic polymers (98–100) is in keeping with our hypothesis. Alternatively, changes in Cl⁻→Cl⁻ could be involved in the activation of PLCγ₂ and PLCβ₂— the PLC isoforms expressed in granulocytes (55, 69, 70, 101), which in turn mediate Ins(1,4,5)P₃ formation and the increase in cytosolic calcium. In this regard, a role for chloride ion as an allosteric regulator of several different proteins has been already demonstrated (102–105).

A growing body of evidence supports the notion that Cl⁻ movements are involved in the regulation of neutrophil responses (5–9). However, several issues still remain to be elucidated. Accordingly, the mechanisms whereby Cl⁻ transport occurs across the plasma membrane remain unclear. Several chloride transporters have been described in neutrophils, including the electroneutral Cl⁻/HCO₃⁻ exchanger (1) as well as a variety of channels, such as calcium-activated channels, voltage-dependent channels, cell swelling-activated channels, and glycine-gated channels (106–109). Inhibition studies may be of aid to investigate the mechanisms involved in the regulation of Cl⁻ movements. Unfortunately, many, if not all, common chloride transport inhibitors have a broad-spectrum activity (47, 51, 106, 110–112). This phenomenon limits the potential usefulness of these compounds to shed more light on the precise molecular mechanisms involved in this process. Recently published data obtained in neutrophils isolated from mice lacking ClC-3 (Ref. 22 and our unpublished data) suggest a role for this channel in the regulation of neutrophil respiratory burst and phagocytosis (112). Identification of the mechanisms responsible for phagocytosis-induced Cl⁻ efflux was beyond the aims of this study. Nevertheless, the presence of chloride fluxes in Ca²⁺ depleted neutrophils seems to suggest that calcium-activated Cl⁻ conductance does not play a major role in this process. Although these data conflict with previous reports (5, 91), it should be noted that our findings are consistent with other studies showing that Cl⁻ efflux triggered by the cross-linking of β₂ integrins occurs in a Ca²⁺-independent manner (9).

Another issue regarding Cl⁻ movements in neutrophils concerns chloride localization within the cell. Recently, Segal and coworkers have provided preliminary evidence to suggest that this anion is contained at high concentrations within cytoplasmic granules to be transferred thereafter into phagocytic vacuoles (113). If this is the case, it is expected that inhibition of degranulation should be accompanied by reduction in Cl⁻ fluxes. However, there are at least two observations that argue against this possibility. First, we have demonstrated that ⁴⁰Cl⁻ efflux occurs normally in Ca²⁺-depleted cells (Fig. 9) despite a marked inhibition of degranulation (Ref. 22 and our unpublished data). Second, cytochalasin B, known to increase fMLP- and IL-8-induced degranulation, did not modify Cl⁻ efflux induced by these agonists (Ref. 4 and our unpublished results). Further investigations are mandatory to shed more light on the intracellular distribution of chloride ion in neutrophils.

Cation/anion trafficking across biological membranes is a complex phenomenon. Moreover, the role of ion movements in neutrophil activation remains a matter of debate (reviewed in Refs. 113 and 114). The present study may shed more light on the role of permeant anions, particularly chloride, in phagocyte activation.

Acknowledgements

We thank Dr. Francesca Vita and Dr. Maria Rosa Soranzo (Department of Physiology and Pathology, University of Trieste) and Claudio Gamboz (Centro Coordinamento e Sviluppo Progetti e Apparecchiature, University of Trieste) who helped us perform the transmission electron microscopy studies. This work was dedicated to the memory of Pierluigi Patriarca.

Disclosures

The authors have no financial conflict of interest.

References
