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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\textsuperscript{−}) 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\textsuperscript{−} efflux accompanied by a marked, irreversible loss of Cl\textsuperscript{−}. Simultaneous assessment of Cl\textsuperscript{−} efflux and phagocytosis in cytochalasin D-treated neutrophils indicated that Cl\textsuperscript{−} 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\textsuperscript{−} efflux as well as the release of azurophilic—but not specific—granules. It implicates that different neutrophil secretory compartments display distinct sensitivity to Cl\textsuperscript{−} modifications. Intriguingly, inhibitors of Cl\textsuperscript{−} exchange inhibited cytosolic Ca\textsuperscript{2+} elevation, whereas Cl\textsuperscript{−} efflux was not impaired in Ca\textsuperscript{2+}-depleted neutrophils. We also show that FcyR(−) and CR3/CR1-mediated Cl\textsuperscript{−} 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.

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2 Abbreviations used in this paper: Cl\textsuperscript{−}, intracellular chloride content; EA, [2,3-dichloro-4-(2-methylene-butyryl)]phenoxyacetic acid (ethacrynic acid); DIDS, 4,4′-disothiocyanatostilbene-2,2′-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)nbenzoic acid; NA, 2-[3-(trifluromethyl)phenyl]-amino]-3-pyridinecarboxylic acid (niflumic acid); FLX, 3-p-trifluromethylphenoxy-3-phenyl-N-methyl-proplyamine hydrochloride (fluoxetine); 9-AC, anterane-9-carboxylic acid; MA, (o-[3-hydroxymercu-2-methoxy-prop[carboxylo] phenoxyacetic acid (mersalyl); CHC, C-cyan-4-hydroxy-cinnamic acid; TB, trypto blue; HBS-BSA, HEPES-buffered saline containing 0.2% BSA; STZ, serum-treated zymosan; 5(6)-FAM-SE, 5(6)-carboxyfluorescein succinimidyl ester; FCM, fluorescence flow cytometry; MPO, myeloperoxidase; LF, lactoferrin; HZ, hydrazide; TEM, transmission electron microscopy; TK, tyrosine kinase; Ptx, pertussis toxin; U73122, 1-(6-[17β-3-methoxyoest-1,3.5(10)-tri-en-17-yl][amino][hexyl]-1H-pyrole-2,5-dione; U73343, 1-[6-([17β-3-methoxyoest-1,3.5(10)-tri-en-17-yl][amino]hexyl)-pyrrolidine-2,5-dione; PLC, phospholipase C; DAG, diacylglycerol.

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the calcium ionophore A23187 (23, 24). Although these results pointed to a role of perempt 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 IMLP. Such an efflux is due to a net outward movement of Cl−, because it is accompanied by a marked, irreversible loss of Cl−. 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 (ethacryninic acid, EA), 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS), n-glucuronic acid sodium salt, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPP), 2-[3-(trifluoromethyl)phenyl]-amino-3-pyridinedicarboxylic acid (niflumic acid, NA), 3-trifluoromethylphenoxycarbonyl-3-phenyl-propyl)carbamoyl]phenoxyacetic acid (mersalyl, MA), α-cyano-4-hydroxycinnamic acid (CHC), IMLP, cytocalasin D, pertussis toxin (Ptx), dextrorotatory glucose, and physiologic saline (0.9% NaCl) for clinical use.

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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) × 100.

Measurement of Cl− movements

Changes in Cl− movements, 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 washed without warming 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 0.9% NaCl, and 100-μl aliquots were diluted into 500 μl of the same buffer layered on 600 μl of a mixture of (ratio 4:1; density 1.005 ± 0.001 g/ml) of silicone oil (density 1.041 g/ml; silicone AR 200 fluid, 200 centistokes; Serva Electrophoresis) and paraflin oil (density: 0.873 g/ml; 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 intercellular spaces in the cell pellet was measured by centrifuging 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−. 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− movements were 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 merciopic 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 sticking to the tube walls. Where indicated, neutrophils suspensions were preincubated with chloride transport blockers, or control buffer, for 15 min at 37°C.


FIGURE 1. Determination of standard chloride concentrations by means of Chlorofix reagent. The assay was performed as detailed in Materials and Methods.
can be read at 460 nm. Fig. 1 shows a calibration curve obtained with different concentrations of NaCl. The Chloroxim 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 Chloroxim 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 (5–10 × 10⁶/ml in HBS-BSA) were collected and centrifuged for 4 min at 400 × g. The resulting pellets were suspended in 2 ml of isotonic 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 incubation at 37°C, the cell lysates were stored on ice for 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 Chloroxim 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). Cl⁻ measured in neutrophils at the selected incubation time was expressed as a percentage of Cl⁻ at t = 0. Mean basal Cl⁻ concentration (Cl⁻₀, at t = 0), as assessed by Chloroxim 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. C. albicans-associated Cl⁻ was found to account for <1.5% of the total Cl⁻ content, and therefore, it was neglected in the calculations of Cl⁻₀.

**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 pelleted by centrifugation at 2000 × g for 5 min, transferred into a microtubе, 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 measurements 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 × 10⁶ cells/ml and 6 × 10⁵ 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 Physiology and Pathology, University of Trieste) and prepared by immunoabsorption on protein G-Sepharose column (Pharmacia LKB Biotechnology). Depletion of IgG from the eluted serum was ascertained by means of ELISA performed with rabbit anti-human IgG polyclonal Abs (Sigma-Aldrich) (data not shown). Zymosan was suspended in 0.9% NaCl and heated in a boiling water bath for 15 min. After centrifugation, the pellet was resuspended in Ca²⁺/Mg²⁺-HBS and opsonized with 10% pooled human serum for 30 min at 37°C. Serum-treated zymosan (STZ) was then washed with HBS, suspended in the same medium and counted electronically (Coulter Counter Mod ZBI; Coulter Electronics).

**Labeling of *C. albicans***

Labeling of *C. albicans* blastospores was performed according to the method described by Dri et al. (29), with slight modifications. Serum-opsonized yeast particles were diluted in NaHCO₃ buffer (0.1 M, pH 8.3), washed and suspended in the same medium (300 × 10⁶/ml) containing 1.5 μg/ml 5(6)-FAM-SE to start labeling. After 30 min of incubation at 4°C in the dark under continuous stirring, the reaction was stopped by adding an equal volume of 50 mM glycine in 0.9% NaCl, pH 8.5, to remove and quench weakly bound 5(6)-FAM-SE molecules. After a further 30 min of incubation at 4°C in the dark under stirring, *C. albicans* particles were washed twice at 2000 × g for 7 min in PBS, suspended in the same medium, and stored at −20°C.

**Assay of phagocytosis**

Neutrophils suspended at 2.5 × 10⁶/ml in Ca²⁺/Mg²⁺-HBS were incubated at 37°C in a shaking water bath with opsonized, 5(6)-FAM-SE-labeled *C. albicans* (1.3 neutrophil to yeast ratio). Unless otherwise stated, the mixtures were incubated for 30 min at 37°C. To stop phagocytosis, aliquots of the incubation mixtures were withdrawn into the tubes used for fluorescence flow cytometry (FCM) analysis containing an equal volume of 250 μg/ml of TB dissolved in ice-cold 0.1 M citrate buffer (pH 4.0). In this way, sample pH was lowered to nearly 4.0, thereby optimizing the TB quenching effect (30). After 1 min of incubation in ice, the samples were analyzed by FCM.

**Quantitative evaluation of recognition and ingestion by FCM**

FCM analysis of neutrophils incubated with fluorescence-labeled yeasts was conducted according to the method described by Bussetto et al. (31). FCM was performed with a FACS Calibur (BD Biosciences), equipped with an air-cooled 15-mW argon-ion laser, operating at 488 nm, 5(6)-FAM-SE green fluorescence (FL1) was collected using a 530 ± 30 band-pass filter, red fluorescence emitted from TB and to 560-FAM-SE-labeled *C. albicans* particles (FL3) was collected using a 650 ± 15 bandpass filter. The data were collected using linear amplification for 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 yeasts 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′-tetramethylbenzidine (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 was dissolved 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 Cesla 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, 500 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 at 405 nm.

β-glucuronidase was assayed according to Gianetto and de Duve (34), using phenolphthalein 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 in the da 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 integrins) (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 (Fab′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 cytoplasmic 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 36Cl− 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 Na36Cl (see above). For each assay, 1 ml of the cell suspension was put in a thermostatted cuvette in a 650-105 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 Cl− transport inhibition on changes in cytosolic Ca2+, neutrophils were preincubated with the selected inhibitors, or otherwise results from activation of Cl−/Cl− 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 36Cl− 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 (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-
opsonization with purified IgGs or IgG-depleted serum, neutrophils were washed twice with prewarmed HBS-BSA buffer to remove the tracer and suspended at 2.5 $\times$ 10$^6$/ml in HBS-BSA containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$. After 20 min of incubation at 37°C in a shaking water bath, 800-$\mu$l aliquots of cell suspensions were collected from duplicate tubes and centrifuged for 4 min at 400 $\times$ g. 750-$\mu$l aliquots of the supernatants of cells at rest (rest) or exposed to serum-opsonized C. albicans were subsequently transferred in duplicate wells of a microtiter plate. After addition of fluorescence-labeled, serum opsonized-C. albicans particles (cell-to-particle ratio, 1:3), the incubation was prolonged for additional 30 min. Aliquots of the incubation mixtures were then withdrawn for radioactivity counting. 

Data represent the mean $\pm$ SD of three to eleven experiments. B, Cl$_i$ was measured in neutrophils at rest or stimulated with either C. albicans (cell-to-particle ratio: 1:3), 10 nM fMLP or 0.6 nM TNF by using the Chlorofix chemical assay (for details see Materials and Methods). At the selected incubation time, aliquots of neutrophil suspensions were collected and centrifuged for 4 min at 400 $\times$ g. The resulting pellets were suspended in isotonic sucrose and subsequently centrifuged for 30 s at 3500 $\times$ g to wash out extracellular Cl$^-$. The pellets were suspended in distilled water to lyse the cells and release intracellular Cl$^-$. After 20 min of incubation at room temperature followed by a freeze-and-thaw cycle, the samples were centrifuged for 15 s at 12,000 $\times$ g and aliquots of the supernatants (25–75 $\mu$l) were subsequently transferred in duplicate wells of a microtiter plate. After addition of the Chlorofix reagent (200 $\mu$l), the plate was incubated for 2–3 min at room temperature and absorbance was read at 460 nm in a microplate reader. Cl$_i$, measured in neutrophils at the selected incubation times was expressed as a percentage of Cl$_i$, at $t = 0$. Neutrophil mean basal Cl$^-$ concentration (Cl$_i$, at $t = 0$) was 82.5 $\pm$ 19.8 nM (mean $\pm$ SD, n = 5). Data represent the mean $\pm$ SEM of three to five experiments.

FIGURE 3. Recognition, but not ingestion, is required to trigger 36Cl$^-$ efflux in neutrophils during phagocytosis of serum-opsonized C. albicans. 36Cl$^-$-loaded neutrophils (2.5 $\times$ 10$^6$/ml in HBS-BSA containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$) were incubated for 5 min at 37°C in a shaking water bath without or with 2.5 $\mu$g/ml cytochalasin D. After addition of fluorescence-labeled, serum opsonized-C. albicans particles (cell-to-particle ratio, 1:3), the incubation was prolonged for additional 30 min. Aliquots of the incubation mixtures were then withdrawn for radioactivity counting. Data represent the mean $\pm$ SEM of three to six experiments.

Relationship between Cl$^-$ efflux and phagocytosis-induced degranulation

The regulatory role of Cl$^-$ movements in several neutrophil functions is well established. Accordingly, fluctuations of Cl$^-$ have been shown to promote conformational changes and to increase expression of $\beta_2$ integrins, TNF- and $\beta_2$ integrin-mediated spreading and metabolic activation, as well as cytoplasmic alkalization (5–9). However, little is known concerning the relationships between 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 degranulation (44–46) and Cl$^-$ ions efflux occur. First, several structurally unrelated chloride transport blockers (1, 47–52) were screened for their effect on particle recognition and ingestion. We performed these experiments to identify drugs that did not affect phagocytosis. Based on our results (Table I), we excluded MA and CHC inasmuch as these compounds altered phagocytosis at any concentration tested. DIDS, 9-AC, and EA could be used at concentrations not exceeding 30 $\mu$M, 1 mM, and 150 $\mu$M, respectively. In contrast, NA, NPPB, and FLX were usable at all the concentrations tested.

derived opsonins are required to induce Cl$^-$ efflux by C. albicans. Accordingly, nonopsonized yeasts did not elicit Cl$^-$ efflux. Additionally, opsonization with purified IgGs or IgG-depleted serum, respectively. In contrast, NA, NPPB, and FLX were usable at all the concentrations tested.
lowered Cl− concentrations. In fact, suspension of resting cells for 30 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 subsequently 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%).

Relationship between Cl− movements and cytosolic Ca2+ elevation

Ca2+ 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 Ca2+ concentrations. In this regard, azurophilic granules display the highest Ca2+ threshold. In fact, proteins contained within these granules are released only when cytosolic Ca2+ 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 Ca2+ levels. To address this issue, the effects of chloride channels

<table>
<thead>
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<th>Cell Treatment</th>
<th>Recognition (%)</th>
<th>Ingestion (%)</th>
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<tr>
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<td>NPPB (μM)</td>
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<td>96.3</td>
</tr>
</tbody>
</table>

a Neutrophils (2.5 × 106/ml) were incubated for 15 min at 37°C in a shaking water bath with the indicated substances and subsequently mixed with serum-opsonized, S(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 HBSS 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.
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 either 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 spectrophotofluorometer. 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 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 inhibitors 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 either in the presence (Fig. 8, B and C) or in the absence (Fig. 8D) 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\(^{2+}\) buffering 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-
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 Ca2+ 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 Ca2+ increase, one of the earliest events in the signaling pathways elicited by FcγR(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 protein (55, 60–71). To determine whether these regulatory proteins could play a role in the activation of Cl− movements, 36Cl−-loaded neutrophils were pretreated with specific inhibitors and subsequently exposed to opsonized 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 protein but upstream of Ca2+ 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 36Cl− efflux from neutrophils exposed to opsonized candida (top panel or fMLP (bottom panel). As expected, wortmannin markedly inhibited Ca2+ elevation induced by engagement of either FcγR(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 PLCγ2 and PLCβ2, respectively (55, 69, 70). Both PLC isoforms catalyze the breakdown of PtdIns(4,5)P2 to generate Ins(1,4,5)P3, 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)P3-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 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 Ca\(^{2+}\) 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 G\(_i\) protein dissociates into a GTP-bound \(\alpha\)-subunit (G\(_{\alpha}\)-GTP) and a \(\beta\)-\(\gamma\)-subunit (reviewed in Ref. 68). Several reports have clearly demonstrated that Ptx ribosylates G\(_i\), thereby preventing G\(_i\) 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 G\(_i\) 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 \(\beta\)-chain (CD18) or the individual \(\alpha\)-chains (CD11a, CD11b, CD11c) of \(\beta\)_2 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, fluorescein-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 \(\beta\)-glucan receptors (Dec21) or mannose receptors (82, 83) in the stimulation of Cl\(^{-}\) efflux. Cl\(^{-}\) fluxes induced by candida particles coated with either IgG or C3/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 Fc\(\gamma\)R(s)/CR3 cooperation in the induction of neutrophil activation (84), because particles opsonized with whole serum, IgG or C3/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 Fc\(\gamma\)R(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⁻ efflux. Neutrophils were loaded with 36Cl⁻ and fura-2 AM, washed in HBS buffer, and suspeded 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⁻ 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⁻ efflux as described in Fig. 2. Data of intracellular Ca²⁺/H11001 and 36Cl⁻ efflux are expressed as percentage of control fura-2 fluorescence and 36Cl⁻ 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⁻ - 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⁻ 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).
BAPTA/EGTA abolished Ca²⁺ response (Fig. 9A) but did not significantly influence phagocytosis-induced ³⁶Cl⁻ 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 the Src family protein TK p56lck elicits Cl⁻ fluxes evoked by fMLP. Because other signal transducers downstream of Gₛ protein (TK, PI3K, PLC) do not appear to be involved in fMLP-induced Cl⁻ efflux (Fig. 10, bottom panel), it remains to be established whether Gₛ-derived subunits may serve by engagement of immune receptors and fMLP-R depend on TK and Gₛ 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 Gₛ protein activation is required to elicit Cl⁻ fluxes evoked by fMLP. Because other signal transducers downstream of Gₛ protein (TK, PI3K, PLC) do not appear to be involved in fMLP-induced Cl⁻ efflux (Fig. 10, bottom panel), it remains to be established whether Gₛ-derived subunits may serve

![FIGURE 8.](image_url)

**Table III. Effect of Cl⁻ transport inhibitors on fMLP-induced degranulation**

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<th>Cell Treatment</th>
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<th>Inhibition (%)</th>
<th>LF Release (%)</th>
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<td>50 μM FLX</td>
<td>19.7 ± 5.6</td>
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</table>

⁶ Neutrophils (2.5 x 10⁶/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 3 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 x 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.

⁶ 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 Gt 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 volume-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 (t1/2 < 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 also been 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⁻, 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 CIC-3 (Cldn3<sup>−/−</sup> PMN) 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 Ca<sup>2+</sup>-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 Ca<sup>2+</sup>-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 [³⁶Cl⁻] efflux occurs normally in Ca<sup>2+</sup>-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 perimean anions, particularly chloride, in phagocyte activation.

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Disclosures
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