Anion Channels, Including ClC-3, Are Required for Normal Neutrophil Oxidative Function, Phagocytosis, and Transendothelial Migration*

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NADPH oxidase activity, phagocytosis, and cell migration are essential functions of polymorphonuclear leukocytes (PMNs) in host defense. The cytoskeletal reorganization necessary to perform these functions has been extensively studied, but the role of cell volume regulation, which is likely dependent upon anion channels, has not been defined. Mice lacking the anion channel ClC-3 (Clcn3<sup>−/−</sup>) died from presumed sepsis following intravascular catheter placement, whereas Clcn3<sup>+/−</sup> littermates survived. We hypothesized that ClC-3 has a critical role in host defense and reasoned that PMN function would be compromised in these mice. Clcn3<sup>−/−</sup> PMNs displayed markedly reduced NADPH oxidase activity in response to opsonized zymosan and modestly reduced activity after phorbol 12-myristate 13-acetate. Human PMNs treated with the anion channel inhibitors niflumic acid or 5-nitro-2-(3-phenylpropylamino)benzoic acid had a very similar defect. ClC-3 protein was detected in the secretory vesicles and secondary granules of resting PMNs and was up-regulated to the phagosomal membrane. Clcn3<sup>−/−</sup> PMNs and human PMNs lacking normal anion channel function both exhibited reduced uptake of opsonized zymosan at 1, 5, and 10 min in a synchronized phagocytosis assay. Niflumic acid-treated PMNs also had impaired transendothelial migration in vitro, whereas migration in vivo was not altered in Clcn3<sup>−/−</sup> PMNs. Selective inhibition of the swelling-activated chloride channel with tamoxifen profoundly reduced PMN migration but had no effect on NADPH oxidase activity. In summary, PMNs lacking normal anion channel function exhibited reduced NADPH oxidase activity, diminished phagocytosis, and impaired migration. ClC-3 was specifically involved in the respiratory burst and phagocytosis.

Ingestion and killing of microbial pathogens by polymorphonuclear leukocytes (PMNs)² are essential components of innate host defense and require phagocytosis as well as efficient migration to sites of infection. Both processes require profound changes in cell shape, which occur via rapid mobilization, and reorganization of the actin cytoskeleton. The spherical shape of the unstimulated PMN provides the smallest surface area:volume ratio possible. Any deviation in shape away from spherical requires insertion of additional membrane, loss of cytoplasm, or both. Therefore, tight regulation of PMN volume may be necessary to respond briskly to environmental demands.

Mammalian cells regulate their volume by control of ion flux across the plasma membrane. When exposed to hypoosmotic media, cell swelling occurs secondary to influx of water, but a compensatory regulatory volume decrease is accomplished by loss of potassium (K<sup>+</sup>) and chloride (Cl<sup>−</sup>) through distinct conductances and the associated osmotic loss of water. In PMNs, hypotonic conditions activate a chloride channel that demonstrates electrophysiological properties and pharmacologic sensitivity similar to those of other previously described swelling-induced Cl<sup>−</sup> conductances (ICl<sub>swell</sub>) (1). In addition to the response to hypotonic stress, Cl<sup>−</sup> efflux from PMNs is a component of the response to FMLP, interleukin-8, platelet-activating factor, and C5a, although the specific channel responsible for this conductance is unknown (2). Anion movement has also been implicated in the release of granule contents from certain intracellular vesicles in PMNs (3). However, a role for anion flux in the cell volume regulation accompanying neutrophil migration or phagocytosis has not been previously investigated.

The neutrophil does not have passive distribution of chloride based on resting membrane potential but actively maintains a very high intracellular Cl<sup>−</sup> concentration through inward active transport (4). The anion conductance(s) and transporters responsible for maintenance of a steady-state intracellular Cl<sup>−</sup> concentration and/or the modulation of Cl<sup>−</sup> flux across plasma and phagosomal membranes during cell activation have not been well defined. Neutrophils have several distinct potential transport mechanisms for Cl<sup>−</sup>. In addition to the ICl<sub>swell</sub> described above (1), a Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>−</sup> co-transporter (4) and a calcium-activated, voltage-independent Cl<sup>−</sup> current (5) have been defined electrophysiologically in neutrophils. mRNA for several members of the CIC family of Cl<sup>−</sup> channels, including CIC-3, has also been identified by reverse transcription-PCR, although the presence of any of the proteins have not been demonstrated (6, 7).

Mice deficient in CIC-3, a member of the CIC family of voltage-sensitive anion channels, have a complex phenotype, including growth retardation, blindness, kyphoscoliosis, seizures, and premature death (8). Notably, CIC-3-deficient mice developed signs of sepsis within 24–72 h after placement of intravascular blood pressure monitoring devices using aseptic technique. The vast majority of knock-out mice succumbed following this procedure, whereas almost all heterozygotes and wild-type littermates survived. Based on these observations, we reasoned that the knock-out mice might be prone to sepsis because of a defect in one or more elements of innate immunity.

To test the hypothesis that CIC-3 is important in host defense, we explored the role of CIC-3 in PMN function. Because of inherent differ-
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ences between human and murine PMNs, we complemented studies of murine PMNs with an examination of human PMNs in the presence of several structurally distinct, well characterized pharmacologic inhibitors of anion channels to mimic the CIC-3 knock-out phenotype. The current studies demonstrate that NADPH oxidase activity, phagocytosis, and transendothelial migration were significantly impaired in PMNs lacking normal anion channel activity. These data suggest a critical and previously unappreciated role for anion channels during a number of essential PMN functions including two functions (phagocytosis and transendothelial migration) that are linked by the requirement for PMN shape change. In addition, a specific requirement for CIC-3 for normal NADPH oxidase activity and phagocytosis is demonstrated.

EXPERIMENTAL PROCEDURES

Materials

Hanks’ Balanced Salt Solution (HBSS), Hepes-RPMI, L-glutamine, and Dulbecco’s phosphate-buffered saline (DPBS) were obtained from BioWhittaker (Walkersville, MD). Fetal bovine serum was obtained from HyClone (Logan, UT), and horse serum was obtained from American Biosciences. Round glass coverslips (12-mm diameter) were from Fisher (Pittsburgh, PA). PMA was from LC Laboratories (Woburn, MA). Zymosan particles were from ICN Biochemicals (Aurora, OH). Monoclonal antibodies to gp91phox (54.1) and p22phox (44.1) were the generous gift of Drs. A. J. Jesaitis, M. Quinn, and J. Burritt (Montana State University, Bozeman, MT). Polyclonal antibody to CIC-3 was from Sigma. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG+IgM and fluorescein isothiocyanate-conjugated donkey anti-rabbit F(ab’)2 were from Jackson ImmunoResearch Laboratories (West Grove, PA). NFA and NPPB were purchased from Sigma and dissolved in Me2SO at a concentration of 10 mM, and used at final concentrations of 10−3 to 10−7 M as described. The appropriate concentration of Me2SO was used for control PMNs in all experiments. Additional reagents were all obtained from Sigma. Hanks’ buffers made with alternative anions for studies of anion selectivity were made in sterile water as follows (concentrations in mM): NaCl, NaBr, or NaI (138 mM); KCl 5 mM, CaCl2 (1 mM), MgSO4·7H2O (0.4 mM), Na2HPO4·2H2O (0.4 mM), K2HPO4·2H2O (0.4 mM) MgCl2·6H2O (0.5 mM), and NaHCO3 (4.2 mM). 1% human serum albumin and 0.1% dextrose were added, and pH was adjusted to 7.4 with HCl. In preliminary studies, this HBSS buffer was compared with commercial HBSS (BioWhittaker) with albumin and dextrose added, as used in all other studies of NADPH oxidase activity, and demonstrated identical results.

Cln3−/− Mice and Murine Leukocyte Isolation

Generation of the Cln3−/− mice has been previously described (8). All animals had free access to food and water. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa. Pairs of mice were injected with 1 ml of 3% thioglycollate intraperitoneally 16 h prior to leukocyte isolation. Migrated cells were harvested by peritoneal lavage using 3 ml of 3% thioglycollate intraperitoneally and used at final concentrations of 10−3 to 10−7 M as described. The appropriate concentration of Me2SO was used for control PMNs in all experiments. Additional reagents were all obtained from Sigma. Hanks’ buffers made with alternative anions for studies of anion selectivity were made in sterile water as follows (concentrations in mM): NaCl, NaBr, or NaI (138 mM); KCl 5 mM, CaCl2 (1 mM), MgSO4·7H2O (0.4 mM), Na2HPO4·2H2O (0.4 mM), K2HPO4·2H2O (0.4 mM) MgCl2·6H2O (0.5 mM), and NaHCO3 (4.2 mM). 1% human serum albumin and 0.1% dextrose were added, and pH was adjusted to 7.4 with HCl. In preliminary studies, this HBSS buffer was compared with commercial HBSS (BioWhittaker) with albumin and dextrose added, as used in all other studies of NADPH oxidase activity, and demonstrated identical results.

Human PMN Purification

Human PMNs were isolated according to standard techniques from hepatic anti-coagulated venous blood from healthy consenting adults in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. PMNs were isolated using dextran sedimentation and Hypaque-Ficoll density-gradient separation, followed by hypotonic lysis of erythrocytes as previously described (9). Purified PMNs were resuspended in 0.9% saline prior to use in migration experiments.

Cell Viability

Exclusion of trypan blue was used to assess cell viability following treatment with the anion channel inhibitors NFA and NPPB. Human PMNs were diluted to 2 × 106 cells/ml and incubated with the inhibitor for time points up to 1 h. Equal volumes of the treated cells and trypan blue were mixed and incubated for 3 min before counting on a hemacytometer to determine the percentage of cells stained with trypan blue. A minimum of 400 cells per condition was counted. Tamoxifen-treated cells were assayed for cell viability using propidium iodide staining followed by flow cytometry. Cells were incubated with HBSS buffer or tamoxifen (5–10 μM) for 1 h, then washed and resuspended in HBSS containing propidium iodide.

Subcellular Fractionation of Human PMN

Neutrophils were treated with the serine protease inhibitor diisopropafluorophosphate (1 mM) for 20 min at room temperature and centrifuged at 200 × g for 5 min. Cells were resuspended in relaxation buffer (10 mM PIPES, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2) with ATP (1 mM ATP(αβ)2) at 50 × 106 PMN/ml with added phenylmethylsulfonyl fluoride (1 mM). Cells were placed in an ice-cold cavitation apparatus and closed tightly. The bomb was pressurized to 350 p.s.i. with nitrogen and allowed to equilibrate for 20 min at 4 °C. Cavitates were expelled dropwise into a tube containing EGTA (final concentration, 1.25 mM) and then centrifuged 200 × g for 20 min to remove unbroken cells and nuclei. These postnuclear supernatants were placed on top of either one-layer (isolation of phagosomes) or three-layer (isolation of granule fractions) Percoll gradients made as described previously (10) and centrifuged at 48,400 × g for 15 min at 4 °C without a brake. Subcellular fractions were carefully removed from the gradient, and Percoll was removed from each fraction by centrifugation. Fractions were then washed with relaxation buffer. Samples were resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2.3% SDS) and heated at 50 °C for 1 h prior to electrophoresis.

Protein Electrophoresis and Immunoblotting

Samples were resolved in 5–20% gradient gel by SDS-PAGE and then transferred to nitrocellulose. Immunoblots were processed using polyclonal antibody specific for CIC-3, or murine monoclonal antibodies to gp91phox (54.1) and/or p22phox (44.1) (11) and horseradish peroxidase-labeled donkey anti-rabbit or goat anti-mouse antibody (Bio-Rad) followed by enhanced chemiluminescence (ECL) detection (Super Signal Substrate, Pierce). Immunoblots were scanned using the Typhoon imager (Amersham Biosciences), and relative abundances were quantitated using ImageQuanNT software for the PhosphorImager (Amersham Biosciences).

Free-flow Electrophoresis

Free-flow electrophoresis was used to separate secretory vesicles from plasma membrane using the γ-fraction isolated by three-layer Percoll density centrifugation as starting material (12). To reduce the surface charge on plasma membrane vesicles, the fraction was treated with neuraminidase (10 units/ml) for 30 min at 37 °C at a final concentration of 0.2 unit/ml. Following neuraminidase treatment, samples were centrifuged at 120,000 × g for 45 min and resuspended in 1 ml of buffer (6 mM triethanolamine, pH 7.4, sucrose 270 mM with conductivity adjusted to 420 μS/cm) by mixing 15 times with a 21-gauge needle. The electrode buffer was 50 mM triethanolamine and 50 mM acetic acid, pH
7.4, and free-flow electrophoresis was performed on an Elphor VaP 22 (Bender & Hobein, Munich) at 5 °C using a flow rate of 3.12 ml/h/fraction and current of 120 mA giving a voltage of 1120 V. Latent alkaline phosphatase activity assay was used to confirm separation of secretory vesicles from plasma membrane vesicles (12). Briefly, 100 μl of each fraction collected by free-flow electrophoresis was plated into duplicate 96-well microplates. 25 μl of 2% Triton X-100 was added to each well of one plate, and the mixture was incubated for 15 min at room temperature. 200 μl of 2 mg/ml p-nitrophenol phosphate was added to each well, and the mixture was incubated for 30 min in the dark followed by reading on Spectramax at 405 nm. Fractions containing secretory vesicles demonstrate additional alkaline phosphatase activity after Triton permeabilization, i.e., latent activity, whereas plasma membrane vesicles do not demonstrate additional latent alkaline phosphatase activity.

Bacterial Cell Cultures

Streptococcus pneumoniae (serotype 2, strain D39) were stored at −80 °C, and an aliquot was streaked on blood agar plates 16 h prior to use. Bacteria were resuspended in endothelial basal medium, and bacterial concentration was determined by measuring optical density (A520). Staphylococcus aureus (strain RN450) and Escherichia coli (strain MC4100) were grown overnight from frozen stocks in tryptic soy broth (S. aureus) or nutrient broth (E. coli) at 37 °C with continuous shaking. Fresh subcultures were set up at A0.1 and grown for 2 h to mid-log phase. Bacterial concentration was determined by measuring A520 and confirmed by measuring colony forming units (CFU) in tryptic soy agar or nutrient broth agar plates. Bacteria were added to the Transwell lower chambers at known optical density, and samples from T = 0 to T = 4 h (just prior to the addition of the PMNs) were plated to determine the bacterial growth during the initial 4-h incubation with endothelial cells.

Measurement of NADPH Oxidase Activity

Chemiluminescence—Lucigenin- and isoluminol-enhanced chemiluminescence assays of NADPH oxidase activity were performed in a 96-well plate using the Wallac Victor3 luminometer (PerkinElmer Life Sciences). 200 μl of a PMN suspension containing 2.5 × 10⁶ PMNs/ml in HBSS with 1% human serum albumin and 0.1% dextrose was added to each well with final concentration of either lucigenin (100 μM) or isoluminol (100 μM) plus horseradish peroxidase (20 units/ml) as specified. Cells were stimulated by addition of either serum-opsonized zymosan (OpZ, 5 particles per cell) or PMA (10 ng/ml final concentration). Submaximal concentrations of agonists were used to investigate differences between the cells in the presence or absence of anion channel inhibition. Chemiluminescence was quantitated as relative luminescence units using a kinetic assay with readings every minute for 1 h. The leukocyte populations isolated after chemical induction of peritonitis in the mice were quantified according to cell type for every experiment by cytospins followed by Diff Quick staining and microscopy. PMN percentages ranged from 57 to 78%, with the remainder of cells from the macrophage lineage. NADPH oxidase activity of murine PMNs was normalized to the percentage of PMNs in each sample, to account for differences in the PMN percentage between knock-out and wild-type mice in each paired experiment. Use of this correction method yielded data that was not different from that obtained after PMNs were separated by density centrifugation before testing (data not shown).

Reduction of Ferricytochrome c—Extracellular O₂⁻ generation was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c in a 96-well microplate using SPECTRmax plus (Molecular Devices). PMN suspensions were diluted and added to the microplate as described above. Cytochrome c (100 μM) was added to the suspension just prior to loading in the microplate. In duplicate wells superoxide dismutase was added at a final concentration of 50 μg/ml. The maximum rate (Vmax) of O₂⁻ generation and the total nanomoles of O₂⁻/10 min was calculated as the superoxide dismutase-inhibitable reduction of ferricytochrome c, with readings at absorbance 550 nm every 15 s for 30 min following injection of PMA (10 ng/ml).

Oxygen Consumption—After calibration of the Clark electrode, PMNs (1 ml, 5.0 × 10⁷/ml) were added to the sample chamber with 1.4 ml of HBSS and allowed to warm for 2 min. The probe was inserted, and the recorder was set to 100%. The samples were then measured for 5 min to determine the resting oxygen consumption rate. The stimulus was added in a 10-μl volume, OpZ, 50 × 10⁶ particles/10 μl, or PMA (10 ng/ml), and measurements were continued for 20 min. The maximum rate of O₂ consumption (Vmax) and total O₂ consumed/10 min were calculated for each sample.

Analysis of Cell Swelling by Flow Cytometry

PMNs at a concentration of 1 × 10⁶/ml were analyzed for forward scatter (FSC) profiles using a FACSCalibur flow cytometer (BD Biosciences). Baseline forward scatter histograms were obtained for control PMNs in HBSS with Ca²⁺ and Mg²⁺. PMNs treated with tamoxifen (10 μM) in HBSS were analyzed for changes in forward and side scatter profiles over a 30-min period following addition of the tamoxifen to the PMN suspension to determine if tamoxifen had any effect on PMN size in isotonic buffer. Both tamoxifen and control PMNs were subjected to hypotonic stress by addition of sterile water to the isotonic buffer containing the PMNs. Cells were stressed with sterile water at 10 and 25% of the final volume. Forward and side scatter histograms were obtained at time 0 (immediately after addition of the sterile water) and at 30 s, and then every 1 min for 10 min. Additional histograms were obtained at 10-min intervals up to 40 min.

PMN Microbicidal Activity

S. aureus were subcultured for 2 h, diluted to 5 × 10⁶ CFU/ml, and incubated for 30 min at 37 °C in 100% normal human serum for opsonization. Bacteria were centrifuged and resuspended in HBSS with 1% human serum albumin and 0.1% dextrose and were mixed 1:1 (v/v) with PMNs to yield a final multiplicity of infection ranging from 2 to 3. Bacteria/PMN suspensions were coincubated for 10 min at 37 °C and centrifuged at 1500 rpm at 4 °C for 5 min. The supernatant was assayed to enumerate bacterial colonies for quantitation of the percentage of the initial inoculum that was not phagocytosed. Cell suspensions were washed and resuspended, and PMN with adherent/ingested bacteria were incubated for an additional 30–60 min. 10-μl aliquots of each cell suspension was added to a 1% saponin solution to lyse the PMN. (This concentration of saponin had no effect on S. aureus viability, data not shown.) Samples were serially diluted and aliquoted into plates with tryptic soy broth agar poured on top. CFU were counted after overnight incubation.

Synchronized Phagocytosis Assay

Zymosan particles were opsonized with 100% normal human serum or 100% pooled murine serum for 30 min at 37 °C, washed with DPBS, and then resuspended in Hepes-RPMI. For phagocytosis assays, PMNs were plated onto acid-washed glass coverslips precoated with 10% normal human serum to obtain ~1 to 2 × 10⁶ cells/coverslip. Phagocytosis of OpZ, by adherent PMNs was synchronized, by centrifugation of cold OpZ particles onto PMNs (2 min, 400 × g, 15 °C), and internalization of the bound particles was stimulated by rapidly warming the cells to 37 °C.
After 0–10 min at 37 °C, coverslips were processed for microscopy as described below (13). In some experiments PMNs in suspension were treated with NFA (10−3 M) for 10 min at 37 °C prior to plating on glass coverslips.

**Immunofluorescence Microscopy**

PMNs on glass coverslips were prepared for immunofluorescence microscopy by fixation for 15 min at room temperature in 10% neutral buffered formalin and then permeabilized in −20 °C acetone for 5 min. Fixed cells were rinsed with DPBS and then blocked in DPBS supplemented with 0.5 g/liter sodium azide, 5 g/liter bovine serum albumin, and 10% horse serum (blocking buffer) for 1 h at 25 °C or overnight at 4 °C. Fixed and permeabilized cells were incubated with primary antibodies (diluted in blocking buffer) for 1 h at 25 °C in a humidified chamber and then washed six times in DPBS-azide-bovine serum albumin. After incubation with secondary antibodies or rhodamine-phalloidin for an additional hour, coverslips were washed six times in DPBS-azide-bovine serum albumin, once with ddH2O, and then mounted onto glass slides in Gelvatol. Specificity of staining was assessed by omission of primary antibodies and by the use of mouse and rabbit isotype control antibodies (Zymed Laboratories Inc., South San Francisco, CA). Samples were viewed using a Zeiss Axioplan2 photomicroscope (Carl Zeiss, Inc., Thornwood, NY), and digital images were obtained using a Zeiss AxioCam and AxioVision 3.1 software.

**Endothelial Cell Culture**

Human dermal microvascular endothelial cells (HMVEC-Ds) were purchased from Clonetics (San Diego, CA) and cultured on collagen-coated flasks (Type VI, human placental collagen, Sigma) using endothelial growth medium-2 (Clonetics) with added bovine brain extract, endothelial cell basal medium-2 (Clonetics) with added bovine brain extract, epithelial growth medium-2 (Clonetics) with added bovine brain extract, Gentamicin, and hydrocortisone according to manufacturer’s specifications. Cells were received from Clonetics at passage 4 and used between passages 5 and 8. Cells were passed from T-75 flasks at 70–80% confluence. HMVEC-Ds were detached using trypsin-EDTA and cultured on collagen-coated Transwell (Costar, Cambridge, MA) 12-mm filters for continuous blood pressure and heart rate recording. In the first several days following placement, Clcn3−/− mice developed signs of sepsis, including lethargy, shivering, and progressive hypotension followed by death. Approximately 90% of the CIC-3-deficient mice died following monitor placement, whereas only 6% (1 mouse) of wild-type littermate controls died after the same procedure. Pretreatment of the Clcn3−/− mice with antibiotics (Enrofloxacin) improved their survival to 50% (n = 10, data not shown). Both the appearance of the mice after monitor placement and the improved survival following pretreatment with antimicrobial therapy suggested that bacterial sepsis was likely the cause of death.

**RESULTS**

**Clcn3−/− Mice—Clcn3−/− and Clcn3+/+ mice underwent placement of implantable telemetry devices using aseptic technique for continuous blood pressure and heart rate recording. In the first several days following placement, Clcn3−/− mice developed signs of sepsis, including lethargy, shivering, and progressive hypotension followed by death. Approximately 90% of the CIC-3-deficient mice died following monitor placement, whereas only 6% (1 mouse) of wild-type littermate controls died after the same procedure. Pretreatment of the Clcn3−/− mice with antibiotics (Enrofloxacin) improved their survival to 50% (n = 10, data not shown). Both the appearance of the mice after monitor placement and the improved survival following pretreatment with antimicrobial therapy suggested that bacterial sepsis was likely the cause of death.**

**ROS Generation by Clcn3−/− PMNs—To determine if this phenotype of the Clcn3−/− mice reflected a defect in cellular elements of innate immunity, neutrophil function was assessed. Thioglycollate-elicted leukocytes were isolated from the peritoneal cavity of Clcn3−/− and Clcn3+/+ mice, and lucigenin-enhanced chemiluminescence following stimulation with opsonized zymosan (OpZ) at a particle:cell ratio of 5:1 or PMA (10 ng/ml) was assessed. Clcn3−/− PMNs had a significant reduction in the Vmax as well as the total ROS generated in response to the particulate stimulus OpZ (Fig. 1A). Total ROS was also diminished following stimulation with PMA, although the Vmax was unaltered (Fig. 1B). As a substrate for chemiluminescence, lucigenin detects multiple intracellular and extracellular ROS. To assess differences in extracellular superoxide generation we used the cytochrome c.
assay. In contrast to our results with lucigenin-enhanced chemiluminescence, following stimulation with PMA there was no significant difference in the total extracellular superoxide generated in the CIC-3-deficient PMNs, with 13.4 ± 0.7 nmol of O$_2^\cdot$/10$^6$ PMNs generated by Clcn3$^{-/-}$ PMNs and 13.5 ± 1.4 nmol of O$_2^\cdot$/10$^6$ PMNs by Clcn3$^{+/+}$ PMNs, n = 3. Taken together, these data suggest that the diminished ROS production by CIC-3-deficient PMNs in response to PMA occurs in an intracellular location.

**Anion Channel Inhibition in Human PMN Oxidative Function**—To extend our findings of abnormal NADPH oxidase activity in murine PMNs lacking CIC-3 to human PMNs, anion channel inhibitors were used. Because there are no selective inhibitors of CIC-3, several structurally distinct anion channel inhibitors were used, including 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), niflumic acid (NFA), 4,4'-disothiocyanoethylbene-2,2'-disulfonic acid (DIDS), and tamoxifen. The concentrations used for each of the inhibitors are within the pharmacologic range known to inhibit Cl$^-$ flux, including anion movement across CIC family channels (6, 15). DIDS treatment elicited a small burst of lucigenin-enhanced chemiluminescence in resting PMNs (data not shown), as has been previously described (16, 17), but the other agents did not. Control PMNs samples were treated with the appropriate concentration of Me$_2$SO, and there was no effect of the Me$_2$SO control on the respiratory burst. There was no effect of NFA, NPPB, or Me$_2$SO at concentrations used on cell viability as determined by trypan blue assay. Pretreatment of human PMNs with NFA (10$^{-3}$ M) markedly inhibited the generation of ROS in response to both OpZ (Fig. 2A) and PMA (Fig. 2B), as measured using lucigenin-enhanced chemiluminescence. Of note, the kinetics of inhibition in human PMNs pretreated with NFA closely resembled that seen in Clcn3$^{-/-}$ PMNs: diminished V$_{max}$ and reduced total ROS following OpZ, and a reduction in total ROS generated with no alteration in V$_{max}$ in response to PMA. NPPB pretreatment of PMNs (5 × 10$^{-4}$ to 10$^{-4}$ M) caused a similar concentration-dependent inhibition of the respiratory burst in response to OpZ and PMA (Fig. 2, C and D). The NFA- and NPPB-mediated inhibition of NADPH oxidase activity in response to PMA was completely reversible upon washout (data not shown).

**TABLE 1**

**Oxygen consumption by control and NFA-treated PMNs using the Clark electrode**

In response to OpZ (particle:cell ratio 5:1) control PMNs consumed significantly more O$_2$ (nmol of O$_2$/5 × 10$^6$ PMN/10 min) and had a greater maximum rate of O$_2$ consumption (V$_{max}$, nmol of O$_2$/5 × 10$^6$ PMN/10 min) than did NFA-treated PMNs. In response to PMA (10 ng/ml), control PMNs consumed more O$_2$ over the 10-min period than did NFA-treated PMNs, but the maximum rate of O$_2$ consumption was not significantly different between the two groups.

| Condition | O$_2$ consumed | V$_{max}$ |
|-----------|----------------|-----------|
| Control   | 151.8 (±5.7)   | 8.7 (±8.7) |
| NFA       | 69.4 (±4.6)    | 8.68 (±1.2) |

*p < 0.05 as compared with control PMNs.

Oxygen consumption was also used as a measure of PMN NADPH oxidase activity (intracellular and extracellular). After stimulation with OpZ, control PMNs consumed significantly more O$_2$ than did PMNs pretreated with NFA (151.8 ± 5.7 nmol of O$_2$/5 × 10$^6$ PMN/10 min versus 84.7 ± 8.7 nmol of O$_2$/5 × 10$^6$ PMN/10 min) and had a more rapid rate of O$_2$ consumption as well (V$_{max}$ 27.1 ± 1.1 versus 13.2 ± 1.0 nmol of O$_2$/5 × 10$^6$ PMN/min control versus NFA, n = 3). Using PMA as an agonist, control PMNs consumed more total O$_2$ than did NFA-treated PMNs but had similar maximal rates of O$_2$ consumption (Table 1).

To determine if the reduction in oxidase activity after anion channel inhibition reflected alterations in both intracellular and extracellular ROS production, we used the membrane-impermeable substrate isoluminol to selectively monitor extracellular ROS. Following stimulation with OpZ, NFA-treated PMNs generated less ROS in comparison to control PMNs (Fig. 3A), similar to the results seen with lucigenin. In contrast, NFA-treated PMNs had similar levels of extracellular ROS generated in comparison to control PMNs when stimulated with PMA and, in fact, demonstrated a reproducible increase in V$_{max}$ (Fig. 3B). Pretreatment of PMNs with NPPB inhibited extracellular generation of ROS in response both to OpZ and PMA (Fig. 3, C and D) measured with isoluminol-enhanced chemiluminescence.
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FIGURE 3. Effect of anion channel inhibitors on the NADPH oxidase activity of human PMNs using isoluminol-enhanced chemiluminescence to detect extracellular ROS. NFA-treated PMNs (filled circles) demonstrated markedly reduced ROS generation in comparison to control PMNs (open squares) following stimulation with OpZ (particle:cell ratio of 5:1) (A). In contrast, extracellular ROS generation following PMA (10 ng/ml) was very similar in control and NFA-treated PMNs, although the maximum rate of ROS generation appeared to be greater in the NFA-treated PMNs (B). n = 6 separate assays with triplicate wells. NPPB pretreatment of PMNs led to a concentration-dependent inhibition of ROS generation in response to both OpZ (C) and PMA (D) as measured with isoluminol-enhanced chemiluminescence (n = 4). Control (open squares), NPPB 10^{-4} M (filled triangles), NFA 5 	imes 10^{-4} M (open squares).

In view of the differential effects of NFA and NPPB treatment on extracellular ROS production as measured with isoluminol, reduction of ferricytochrome c was used to quantitatively assess the extracellular release of O_2^- following stimulation with PMA. There was no significant difference in the total amount of extracellular superoxide generated following PMA stimulation by control versus NFA-treated PMNs (46.8 ± 1.36 versus 46.6 ± 0.3 nmol of O_2^-/10^6 PMNs, n = 3), but NFA-treated PMNs had a greater maximum rate of O_2^- production (V_{max} 67.4 ± 6.4 [NFA] versus 47.5 ± 6.2 [control] milliunits/min). In contrast, pretreatment of PMNs with NPPB led to diminished total extracellular O_2^- generation (32.7 ± 0.92 nmol of O_2^-/10^6 PMNs, n = 3), as well as a slight reduction in V_{max}. These data were consistent with our findings using isoluminol chemiluminescence (Fig. 3, B and D). Taken together these data demonstrate that anion channel blockade with either NFA or NPPB significantly reduces intraphagosomal ROS formation following ingestion of a particulate stimulus. In addition, NFA pretreatment diminishes intracellular generation of oxidant species following PMA, similar to the alteration in NADPH oxidase activity exhibited by CIC-3^-/- PMNs, whereas NPPB-treated PMNs have a reduction in both intracellular and extracellular ROS following PMA stimulation. The differential effect of NPPB versus NFA on extracellular NADPH oxidase activity in response to PMA may result from blockade of anion channels in addition to CIC-3 by NPPB.

In addition to anion channel blockade, NFA has cyclooxygenase inhibitory effects. Because inhibition of the cyclooxygenase pathway may affect NADPH oxidase activity (18), we tested the effects of indomethacin, a non-steroidal anti-inflammatory compound with well described cyclooxygenase inhibitory effects (19) on ROS generation in PMNs. Indomethacin (100 nm) had no effect on ROS generation following stimulation with either OpZ or PMA as measured by lucigenin-enhanced chemiluminescence (data not shown) suggesting that the inhibitory effects of NFA on the respiratory burst are not secondary to cyclooxygenase inhibition.

At the concentrations used, both NFA and NPPB might have blocking effects on the swelling-activated Cl^- channels (ICl_{swell}) in human PMNs, and the reduction in NADPH oxidase activity could have resulted from an effect of NFA and NPPB on ICl_{swell}. The pharmacology of the swelling-induced chloride channel has been rigorously tested in many cell types, and tamoxifen strongly inhibits ICl_{swell} at a concentration of 10 \mu M but has relatively little effect on the conductance at 1 \mu M and does not inhibit other anion channels at that concentration (20, 21). Although the ability of tamoxifen to block ICl_{swell} has not been studied in PMNs, the swelling-induced conductance in PMNs has been demonstrated to have similar electrophysiologic properties and pharmacologic sensitivity to a number of other agents as compared with other cell types (1). First, we explored a role for a tamoxifen-sensitive channel in the response to hypotonic stress in PMNs. Using flow cytometry we observed onset of PMN swelling immediately following hypotonic stress (25% sterile water added to isotonic buffer) by forward light scatter with a peak in cell size at 2 min, followed by the expected regulatory volume decrease (Fig. 4A). Tamoxifen treatment of PMNs had no effect on the cell volume in isotonic buffer and elicited no increase in propidium iodide staining of the PMNs, suggesting that there was no direct toxicity of the inhibitor. However, tamoxifen-treated PMNs subjected to hypotonic stress immediately showed a broadened curve by forward light scatter followed by an apparent decrease in cell size within 30 s (Fig. 4B). By microscopic examination, this “decrease” in cell size reflected rupture of all PMNs, with PMN nuclei as the gated “cell” by flow cytometry. Use of 10% sterile water as the hypotonic stress also caused rupture of the tamoxifen-treated PMNs (data not shown.) Considered in combination with the published data on ICl_{swell}, these data strongly suggest that tamoxifen also inhibited the swelling-activated conductance in PMNs and that this conductance is necessary for cells to survive hypotonic challenge. Next, we utilized tamoxifen to determine if ICl_{swell} activity is necessary for normal ROS generation. Pretreatment of human PMNs with tamoxifen (1–10 \mu M) did not reduce ROS generation in response to either OpZ or PMA (Fig. 4, C and D). These data suggest that inhibition of intracellular ROS generation by NFA and NPPB was not secondary to blockade of the swelling-induced chloride conductance.

Ion Selectivity of the Anion Channel(s) Involved in PMN Oxidative Function—The swelling-induced chloride conductance is an outwardly rectifying current, which exhibits an anion selectivity of 1^- > Br^- > Cl^- (reviewed in Ref. 21). Although the one or more specific
genes or protein(s) responsible for this conductance remain controversial, this anion selectivity is distinct from that generally demonstrated for channels of the ClC family (15, 22). We investigated the anion selectivity of the respiratory burst response using buffers made with alternative anions. In human PMNs stimulated with PMA, NADPH oxidase activity as measured by lucigenin-enhanced chemiluminescence was markedly sensitive to the anion used with a selectivity of Cl− > Br− > I− (Fig. 5A). This pattern was identical following stimulation with OpZ (data not shown). Murine Clcn3(+/−) PMNs displayed the same ion selectivity during NADPH oxidase activation as did human PMNs (Fig. 5B), but the anion selectivity was altered in the Clcn3(−/−) PMNs stimulated with PMA with Cl− > Br− > I− (Fig. 5C), suggesting that in the absence of ClC-3 a distinct anion conductance functions in a compensatory role.
Role of the Anion Channel CIC-3 in Neutrophil Function

Expression of CIC-3 Protein in Human PMN—Based on the significant alteration in NAPD oxidase activity in murine PMNs lacking CIC-3 and in human PMNs treated with anion channel inhibitors, and on the similar ion sensitivity displayed by human and murine Clcn3<sup>+/+<sup> PMNs, we investigated the subcellular localization of CIC-3 protein in human PMNs. mRNA for CIC-3 had previously been demonstrated in human PMNs (7), but expression of CIC-3 protein in PMNs had not been documented. Subcellular fractions of resting PMNs were isolated and probed for CIC-3 protein. The majority of CIC-3 protein in resting PMNs was detected in the γ-fraction (74.7% ± 5.7%), which contains both plasma membrane vesicles and secretory vesicles, with the remainder in the β-fractions that are comprised of secondary and tertiary granules. No CIC-3 protein was detected in the α-fraction containing azurophilic granules (Fig. 6A). Free-flow electrophoresis was used to further fractionate the γ-fraction and demonstrated that 90.7 ± 0.8% of CIC-3 was detected in the secretory vesicle fraction in resting PMNs by free-flow electrophoresis (Fig. 6B).

Based on the localization of CIC-3 in resting cells to secretory vesicles and secondary granules, and on the impairment of ROS generation in response to a particulate stimulus in the absence of CIC-3, we investigated recruitment of CIC-3 to the phagosomal membrane. Human PMNs were coincubated with OpZ followed by nitrogen cavitation and fractionation using a one-layer Percoll gradient. The percentage of total CIC-3 protein in the upper layer (layer 1) containing the OpZ phagosomes was enhanced in cells that had been exposed to a particulate stimulus as compared with unstimulated PMNs suggesting redistribution of CIC-3 to the phagosome (Fig. 6C). Taken together, these data are consistent with localization of CIC-3 in secretory vesicles and secondary granules in resting PMNs with mobilization during phagocytosis.

Anion Channel Involvement in PMN Microbicidal Activity—Killing of <i>S. aureus</i> by PMNs occurs in an oxygen-dependent manner and requires a functional NADPH oxidase. However, female carriers of chronic granulomatous disease who are obligate heterozygotes for the membrane component of the NADPH oxidase demonstrate reduced levels of NADPH oxidase activity in <i>vitro</i>, but have no increased susceptibility to infection as long as they have ~10% normal phagocytes (31). We reasoned that, despite the reduction in NADPH oxidase activity in NFA-treated human PMNs and in Clcn3<sup>−/−<sup> PMNs, levels of ROS would still be adequate for bacterial killing in <i>vitro</i>. NFA-treated PMNs had similar levels of microbicidal activity versus <i>S. aureus</i> in comparison to control PMNs (Fig. 7A) when controlled for uptake of bacteria. Although the phagocytic assay was not synchronized, NFA-treated PMNs had fewer ingested or adherent bacteria after the initial 10-min incubation when compared with control PMNs, suggesting an alteration in phagocytosis. Clcn3<sup>−/−<sup> and Clcn3<sup>+/−<sup> PMNs also displayed very similar levels of microbicidal activity versus <i>S. aureus</i> in <i>vitro</i> (Fig. 7B).

Role of Anion Channels during Phagocytosis—Both Clcn3<sup>−/−<sup> PMNs and human PMNs treated with the anion channel inhibitors NFA or NPPB produced less ROS following stimulation with OpZ. This reduction in oxidant species could result from diminished particle uptake or reduced production inside each phagosome, or both. Taken together with our findings of reduced cell associated <i>S. aureus</i> in NFA-treated human PMNs, we explored the role of anion channels, including CIC-3, in phagocytosis of particulate stimuli using a synchronized assay for quantitative assessment. The number of bound and internalized zymosan particles was counted using both phase-contrast microscopy and staining of the phagosome with antibody to gp91<sub>phox</sub> as has been previously described (23). Murine Clcn3<sup>+/+<sup> and Clcn3<sup>−/−<sup> PMNs had equal numbers of surface-bound extracellular particles at 1, 5, and 10 min after initiation of synchronized phagocytosis (Fig. 8A). In contrast, Clcn3<sup>−/−<sup> PMNs formed fewer phagosomes in comparison to wild-type control PMNs at all time points (Fig. 8B). These data suggest that
Role of the Anion Channel ClC-3 in Neutrophil Function

Anion Channel Involvement in PMN Transendothelial Migration—Reasoning that cell volume regulation or shape change might provide a link for some of the processes disrupted by deletion of ClC-3 and/or inhibition of anion channel function, we hypothesized that PMN transendothelial migration would also be impaired in cells following anion channel inhibition. Using an in vitro model of PMN transendothelial migration in response to intact, live bacteria, we studied the effect of anion channel inhibition on PMN migration. Pretreatment of PMNs with NFA markedly impaired transendothelial migration in response to both the bacterial chemoattractant fMLP as well as intact S. aureus, S. pneumoniae, and E. coli (Fig. 9). Indomethacin was used as a control for the cyclooxygenase inhibitory effects of NFA and had no effect on migration (data not shown). Murine PMN transendothelial migration was studied in vivo due to the lack of availability of murine endothelial cells to study using the in vitro model. In marked contrast to the human binding of OpZ to the PMN membrane was not impaired, but rather uptake into the cell was diminished. NFA was used to study the effect of anion channel inhibition on phagocytosis in human PMNs as the pattern of ROS inhibition in NFA-treated human PMNs most closely resembled the Clcn3−/− PMN phenotype. NFA pretreatment decreased the number of OpZ phagosomes inside human PMNs in a similar manner to Clcn3−/− PMNs (Fig. 8C). The number of extracellular-bound particles was also similar in both groups (data not shown). Taken together, the findings of reduced phagocytosis, redistribution of ClC-3 protein to the phagosomal membrane, and impairment of the respiratory burst in response to OpZ suggest a role for ClC-3 in phagocytosis.

DISCUSSION

The role of ion flux across the phagosomal membrane of human PMNs has been a topic for significant discussion recently, although the PMNs pretreated with NFA, there were no differences in the total number of leukocytes that migrated into the peritoneal space or in the differential counts of these migrated cells in the Clcn3−/− and Clcn3+/+ mice, following induction of inflammatory peritonitis.

We reasoned that the effects of NFA pretreatment of PMN transendothelial migration might be secondary to inhibition of the swelling-activated conductance and investigated a specific role for IClswell in PMN transendothelial migration using tamoxifen-treated PMNs. These PMNs were found to have virtually no migration in response to any of the stimuli tested (Fig. 10). The impairment of PMN migration by tamoxifen was concentration-dependent and was maximal at a concentration of 10 μM. Because tamoxifen also has concentration-dependent antagonist/partial agonist effects on the estrogen receptors ERα and ERβ, we used a pure ER antagonist as a control in our migration model. ICI 182,780 used at 1 μM had no significant effect on migration, and tamoxifen (5 μM) was intermediate, * p ≤ 0.05 as compared with migration of control PMNs, n = 4. This finding is suggestive of a role for ClC-3 in normal neutrophil transendothelial migration such that IClswell is essential for normal neutrophil transendothelial migration and that additional anion channels may also play a role in this process. In view of the complexity of the data presented, we have included a summary table to facilitate comparison of the findings in the murine and human systems (Table 2).
controversy has focused on the relative contribution of specific cation conductances (K+ versus H+) across the forming phagosomal membrane (25). A number of distinct anion channels are likely present in PMNs, and the requirement for chloride ions for normal function of the myeloperoxidase-halide system is well established (26). An adequate concentration of intraphagosomal chloride for MPO-dependent reactions is likely to be present based on high resting intracellular Cl− (4) and additional uptake of Cl− during phagocytosis. However, although a number of reports demonstrate the importance of anion flux during the proximal reactions of the respiratory burst that generate superoxide and subsequently hydrogen peroxide (27, 28), the specific channels required are not well defined.

The current investigation suggests that anion channels in general, and ClC-3 in particular, are critical regulators of several PMN responses. Based on our initial observation that Clcn3−/− mice had dramatically altered survival following intravascular catheter placement, we explored a role for ClC-3 in specific PMN functions pertinent to host defense. Although we acknowledge that the data presented do not offer a definitive explanation for the abnormal immune phenotype of the Clcn3−/− mice, we have defined several elements of the cell biology of neutrophil function that require anion channels. Both murine PMNs lacking ClC-3 protein and human PMNs treated with the anion channel inhibitors NFA or NPPB demonstrated markedly reduced NADPH oxidase activity following stimulation with opsonized zymosan. In response to OpZ, phagocytosis begins almost immediately, and NADPH oxidase activity subsequently hydrogen peroxide (27, 28), the specific channels required are not well defined.

Clinical experience with X-linked CGD carriers who have a mosaic population of neutrophils, some of which have functional NADPH oxidase activity and some without, has suggested that humans with ≳10% of normal levels of ROS production have no increased susceptibility to infection (30). Although we have no direct evidence that the observed phenotype of markedly diminished survival in the Clcn3−/− mice resulted from abnormal PMN function, it is clear that PMNs lacking ClC-3 still had significantly greater than 10% of the oxidants generated by the wild-type controls. As expected from the quantity of ROS generated, both NFA-treated and CIC-3-deficient PMNs had no alteration in microbicidal activity versus S. aureus suggesting adequate oxidant species to support oxygen-dependent killing. However, the impairment in phagocytosis we show here could account for the reduction in ROS following stimulation with opsonized zymosan and diminished phagocytosis of pathogenic bacteria would provide one potential explanation for the phenotype observed. In addition, by cellular fractionation we know that the CIC-3 protein was redistributed to the phagosome after stimulation with OpZ. The reduction in phagocytosis observed may be caused by a delay in one or many of the steps of phagosome formation. Phagocytosis inherently requires profound alterations in cell shape that are likely to be accompanied by transient changes in cell volume. Thus, it is tempting to speculate that, in the absence of CIC-3, cell volume regulation may be impaired thereby slowing the ability of the cell to change shape and impairing particle engulfment.

After demonstrating quantitative abnormalities in phagocytosis, we studied transendothelial migration of PMNs as an additional end point requiring rapid and repeated cellular shape change. NFA-treated PMNs displayed marked impairment in transendothelial migration to multiple diverse stimuli. PMN migration in vitro appears to have similarities to the process in vivo in that there are variable requirements for neutrophil adhesion molecules (14). NFA-treated PMNs did not have selective inhibition to one stimulus but displayed a more globally impaired phenotype. Inhibitory effects of niflumic acid on chemotaxis of rat PMNs have previously been demonstrated using Boyden chamber measurements (31). The processes of chemotaxis and transendothelial migration inherently require significant shape change as a PMN that is near spherical in the resting state elongates to move along a directional gradient and move between adjacent endothelial cells. In contrast, there were no quantitative differences in PMN migration to the peritoneal space in Clcn3+/+ versus Clcn3−/− mice following induction of inflammatory peritonitis. Taken together, these data suggest that inhibition of transendothelial migration in human PMNs by NFA may reflect blockade of a cell-surface anion channel distinct from CIC-3, possibly IClswell.

The swelling-induced chloride conductance, known to mediate the regulatory volume decrease following hypotonic stress, is also inhibited by tamoxifen. Tamoxifen completely inhibited cells from migrating across the endothelial monolayer to any of the stimuli tested. This conductance has been demonstrated to be essential for invasive migration of human glioma cells (32, 33) using an in vitro chemotaxis assay. Collectively, these data suggest that this chloride conductance facilitates cell volume changes and, consequently, the cell shape changes required for migration. The anion conductance implicated in this process is presumably a cell-surface channel that is involved in the maintenance of steady-state volume in unstimulated cells and the rapid changes in volume following stimulation.

Whether CIC-3 is responsible for the swelling-activated chloride conductance (IClswell) in other cell types has engendered great debate (34–36). CIC-3 has been proposed to be a cell-surface channel regulated by: 1) swelling (34), 2) calcium-calmodulin-dependent protein kinase II (35), or 3) the cystic fibrosis transmembrane regulator (36). Localization of CIC-3 protein to intracellular vesicles has supported an alternative hypothesis that CIC-3 is predominantly an intracellular anion channel.

### TABLE 2

Summary table comparing results from murine and human PMNs

|           | Total NADPH oxidase activity | Extracellular NADPH oxidase activity | Microbicidal activity | Phagocytosis | TEM |
|-----------|-----------------------------|-------------------------------------|-----------------------|--------------|-----|
| Murine    |                             |                                     |                       |              |     |
| Clcn3−/−  | ↓                           | ↓                                   |                       | ↑            | ↓   |
| Human     |                             |                                     |                       | ↑            |     |
| NFA       | ↓                           | ↓                                   |                       | ND*          |     |
| NPPB      | ↓                           | ↓                                   |                       | ND           | ND  |
| Tamoxifen |                             |                                     |                       | ND           | ND  |

* ND, not determined.
located in vesicular membranes, that regulates acidification of synaptic vesicles and lysosomes (37, 38). Most recently, ClC-3 has been suggested to be a chloride/proton antiporter rather than an ion channel. This notion is based on the structural similarity of ClC-3 to subfamily members ClC-4 and ClC-5. These two endosomal proteins may function as electrogenic Cl−/H+ exchangers that display a steep voltage dependence, activated only by positive voltages (39, 40).

The current investigation is the first report of ClC-3 protein expression in human PMNs. In our studies, subcellular fractionation demonstrates that ClC-3 is in the secretory vesicle and secondary granule compartments of resting cells and not enriched at the plasma membrane. Although this does not preclude the possibility that ClC-3 is rapidly trafficked to the membrane in stimulated cells, it suggests that ClC-3 itself is not the primary swelling-induced conductance in PMNs. In addition, our data provide significant further evidence that ClC-3 is not the IC1swell in PMNs, and that ClC-3 and not IC1swell is involved in regulation of the oxidative burst. First, the order and magnitude of ion selectivity for the anion conductance involved in the generation of the respiratory burst is identical in human and murine wild-type PMNs (Cl− > Br− > 1). This pattern of ion selectivity is distinct from that seen in the reduced respiratory burst generated by Clcn3−/−/− PMNs and distinct from the well established selectivity of the IC1swell (15). Second, NADPH oxidase activity is reduced in Clcn3−/−/− PMNs but not in tamoxifen-treated PMNs with functional inhibition of IC1swell. Rather, the Clcn3−/−/− PMNs seem phenotypically most similar to the NFA-treated human PMNs in all assays studied except for transendothelial migration.

Both Clcn3−/−/− PMNs and NFA-treated PMNs had alterations in PMA-stimulated NADPH oxidase activity, with reduced intracellular ROS detected. This finding cannot be easily explained by impaired shape change or phagocytosis. If ClC-3 is actually an antiporter, rather than a channel, the initiation of the respiratory burst may generate sufficient membrane depolarization to drive Cl−/H+ exchange. The NADPH oxidase generates a rapid depolarization of ~100 mV, and proton movement is required to balance this charge (41, 42). The positive voltages required to initiate activity of ClC-4 and ClC-5 antiporter activity (and by analogy ClC-3) may exist in the setting of the phagosome upon activation of the NADPH oxidase. It therefore seems possible that ClC-3 participates in charge neutralization during the oxidative burst. The existence of an intracellular vesicular pool for ROS generation has also been a topic of ongoing controversy in the phagocyte field (43). There are data to suggest that NADPH oxidase assembly and ROS generation following stimulation with PMA do occur in an intracellular compartment that is not generated from plasma membrane, and our data would support this interpretation of others’ results (44).

In summary, murine PMNs lacking ClC-3 and human PMNs treated with pharmacologic inhibitors of anion channels appear to have a proximal functional defect as evidenced by the diverse functions affected, including abnormal NADPH oxidase activity to both soluble and particulate stimuli, diminished phagocytosis, and impaired transendothelial migration. Regulation of shape change provides a potential link between many of the processes disrupted in the absence of normal anion channel function. One explanation for the spectrum of abnormalities observed is that ClC-3 is required for a proximal signaling event that activates a variety of these downstream functions, including activation of the IC1swell. Disruption of ClC-3 function might result in impaired control of IC1swell and subsequent abnormalities in the control of cell size and shape. If ClC-3 is required for normal levels of intracellular ROS generation and H2O2 production as our data suggest, the recently reported requirement for H2O2 in the activation of IC1swell could link these processes (45). This hypothesis might reconcile a number of the apparently conflicting and controversial observations regarding the role of ClC-3 as an IC1swell.

We have defined specific roles for two individual anion conductances in PMNs. The swelling-induced chloride conductance is necessary for transendothelial migration of PMNs. In addition, a distinct anion conductance is necessary for several unique and diverse PMN functions necessary for host defense, including the respiratory burst and phagocytosis. We speculate that this channel is ClC-3 and that its localization in secretory vesicles positions it ideally to participate in the intracellular signaling processes that are likely to be ongoing during phagocytosis and transendothelial migration.

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