Na^+/H^+ Exchange Activity during Phagocytosis in Human Neutrophils: Role of Fcγ Receptors and Tyrosine Kinases

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Abstract. In neutrophils, binding and phagocytosis facilitate subsequent intracellular killing of microorganisms. Activity of Na^+/H^+ exchangers (NHEs) participates in these events, especially in regulation of intracellular pH (pH_i) by compensating for the H^+ load generated by the respiratory burst. Despite the importance of these functions, comparatively little is known regarding the nature and regulation of NHE(s) in neutrophils. The purpose of this study was to identify which NHE(s) are expressed in neutrophils and to elucidate the mechanisms regulating their activity during phagocytosis. Exposure of cells to the phagocytic stimulus opsonized zymosan (OpZ) induced a transient cytosolic acidification followed by a prolonged alkalinization. The latter was inhibited in Na^+-free medium and by amiloride analogues and therefore was due to activation of Na^+/H^+ exchange. Reverse transcriptase PCR and cDNA sequencing demonstrated that mRNA for the NHE-1 but not for NHE-2, 3, or 4 isoforms of the exchanger was expressed. Immunoblotting of purified plasma membranes with isoform-specific antibodies confirmed the presence of NHE-1 protein in neutrophils. Since phagocytosis involves Fcγ (FcγR) and complement receptors such as CR3 (a β_2 integrin) which are linked to pathways involving alterations in intracellular $[Ca^{2+}]_i$ and tyrosine phosphorylation, we studied these pathways in relation to activation of NHE-1. Cross-linking of surface bound antibodies (mAb) directed against FcγRs (FcγRII > FcγRIII) but not β_2 integrins induced an amiloride-sensitive cytosolic alkalinization. However, anti-β_2 integrin mAb diminished OpZ-induced alkalinization suggesting that NHE-1 activation involved cooperation between integrins and FcγRs. The tyrosine kinase inhibitors genistein and herbimycin blocked cytosolic alkalinization after OpZ or FcγR cross-linking suggesting that tyrosine phosphorylation was involved in NHE-1 activation. An increase in $[Ca^{2+}]_i$ was not required for NHE-1 activation because neither removal of extracellular Ca^{2+} nor buffering of changes in $[Ca^{2+}]_i$ inhibited alkalinization after OpZ or FcγR cross-linking. In summary, FcγRs and β_2 integrins cooperate in activation of NHE-1 in neutrophils during phagocytosis by a signaling pathway involving tyrosine phosphorylation.

NEUTROPHILS react to invading microorganisms and mediators present within an inflammatory milieu with a variety of rapid and coordinated responses which include movement of cells out of the vascular space along a gradient of chemotactic molecules followed by phagocytosis and killing of the microorganisms. This bactericidal function is effected by complex processes involving secretion of proteolytic enzymes and reactive oxygen intermediates (produced by the NADPH oxidase) into the phagolysosome (for review see Sha'afi and Molski, 1988). During these processes, dynamic alterations occur in leukocytes including changes in cell volume (Grinstein et al., 1986*b*; Worthen et al., 1994*a*) and intracellular pH (pH_i)[1] (Grinstein and Furuya, 1986*a*,*b*; Grinstein et al., 1986*a*; Nanda et al., 1994), the latter due to a generation of acid equivalents by metabolic pathways involved in phagocytosis and killing. Activity of Na^+/H^+ exchangers (NHEs) is thought to play a major role in many of these functional responses. In particular, NHEs may be important in compensation for the proton load generated by activity of the NADPH oxidase (Simchowitz, 1985*a*,*b*; Grinstein and Fu-

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1. *Abbreviations used in this paper*: BCECF, 2', 7'-bis-(2-carboxyethyl-4-piperidinobenzoyl) guandidine methanesulphonate; $[Ca^{2+}]_i$, intracellular calcium concentration; FcγR, Fcγ receptor; fMLP, formyl-Met-Leu-Phe; MMPA, methyl-methyl-propenyl-amiloride; NHE, Na^+/H^+ exchanger; OpZ, opsonized zymosan; RT, reverse transcriptase.

ruya, 1986*b*; Grinstein et al., 1986a). Despite the importance of these functions, comparatively little is known at the molecular level regarding the nature or regulation of NHE(s) in neutrophils.

Phagocytosis, the process by which microorganisms or particulate matter is engulfed and delivered to a digestive compartment (Wright, 1992), is crucial to the bactericidal function of neutrophils. Phagocytic cells specifically recognize, ingest, and kill potentially pathogenic microorganisms while adjacent host cells are spared. The processes involved in phagocytosis can be arbitrarily divided into two phases: (*1*) recognition and binding of the particle to the surface of the phagocyte, which depends on specific cell surface receptors, and (*2*) engulfment of the particle which is dependent on complex processes involving polymerization and reorganization of the actin cytoskeleton (Axline and Reaven, 1974; Wright, 1992). Binding of particles is enhanced by factors in immune serum including IgG and complement proteins such as C3 which are recognized by specific receptors present on the plasma membrane of phagocytes. In particular, neutrophils express two types of receptors for complement, CR1 and CR3, which recognize surface C3b and C3bi, respectively, both surface-bound fragments of C3 (Wright, 1992; Fällman et al., 1993). CR3, also known as Mac-1, is a member of the β_2 integrin family ($\alpha_m\beta_2$ or CD11b/CD18). Additionally, neutrophils express two receptors for the Fc portions of IgG: FcγRIIA, a 47-kD integral membrane glycoprotein and FcγRIIIB, a heavily glycosylated protein with an apparent molecular mass of 50–80 kD linked by a glycosyl-phosphatidylinositol anchor to the membrane (Huizinga et al., 1990; Selvaraj et al., 1989). There is increasing evidence that in addition to functioning in recognition and binding of microorganisms, ligation of complement and Fc receptors is able to initiate complex transmembrane signaling pathways contributing to the engulfment phase and to activation of other "effector" function of leukocytes such as the oxidative burst (Willis et al., 1988; Huizinga et al., 1989: Zhou and Brown, 1994), degranulation (Huizinga et al., 1990), and phagocytosis (Huizinga et al., 1989; Wright, 1992) which are required for effective intracellular killing.

The Na^+/H^+ exchangers are a family of electroneutral plasma membrane transporters that mediate a one-to-one exchange of extracellular sodium and intracellular protons. These exchangers, present in all mammalian cells, have been postulated to be involved in the regulation of pH_i, cell volume, transcellular absorption and secretion of electrolytes, differentiation and proliferation, and in leukocytes, responses associated with acute inflammation (Boron, 1983; Grinstein et al., 1989; Wakabayashi et al., 1992). These diverse physiological roles attributed to the Na^+/H^+ exchangers may be accounted for by the existence of multiple isoforms. Recently, several isoforms of the mammalian Na^+/H^+ exchanger, termed NHE-1, 2, 3, and 4 have been characterized at the molecular level (Sardet et al., 1989; Tse et al., 1991; Orlowski et al., 1992; Wang et al., 1993).

NHE-1 was the first Na^+/H^+ exchanger isoform cloned and characterized (Sardet et al., 1989) and appears to be expressed ubiquitously. Human NHE-1 is a phosphoglycoprotein of 815 amino acids with an NH_2-terminal domain of ~500 amino acids comprised of alternating hydrophilic and hydrophobic stretches that predicts a structure with 10–12 membrane-spanning segments and a hydrophilic COOH-terminal domain of ~300 amino acids that is thought to extend into the cytosol (Sardet et al., 1989, 1990). Levels of expression of NHE-1 are highest in intestinal epithelial cells where the protein is localized in the baso-lateral membrane (Tse et al., 1991). Additionally, NHE-1 is known to be expressed in HL-60 cells, human leukemic cells of hematopoietic origin (Rao et al., 1993). Other members of the NHE family have closely related structures ranging in size from 717 to 835 amino acids in length. The expression of these isoforms is much more restricted: NHE-3 and NHE-4 transcripts are expressed predominantly in the colon and small intestine with lesser amounts in the kidney and stomach; NHE-2 transcripts are expressed predominantly in the small intestine, colon, stomach, and kidney (Orlowski et al., 1992; Wang et al., 1993).

NHEs can be activated by a variety of stimuli including growth factors, chemotactic agents, extracellular matrix proteins, and osmotic stress (Sardet et al., 1989; Grinstein et al., 1986*a*,*b*; Schwartz et al., 1991*a*). Much of the information on the functional characteristics of specific NHE isoforms is based on studies using Na^+/H^+ exchanger-deficient cells (primarily fibroblasts) stably transfected with the various NHE isoforms (Wakabayashi et al., 1992; Tse et al., 1993; Counillon et al., 1993; Kapus et al., 1994). However, many unanswered questions remain regarding both the identity of NHEs in leukocytes and the mechanism of their regulation during physiological processes such as phagocytosis. Accordingly, the purpose of this study was to investigate the regulation of endogenous NHE activity in neutrophils during phagocytic stimulation and to characterize the identity of the exchangers involved using a combined molecular and pharmacological approach. Our results indicate that NHE-1 is expressed in human neutrophils and that this exchanger is activated during exposure to phagocytic stimuli via a signaling pathway that involves Fc receptors and tyrosine phosphorylation.

Materials and Methods

Materials

Percoll, Dextran T-500, and Ficoll 400 were obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Reagents for Krebs Ringers phosphate dextrose buffer were obtained from Mallinckrodt Chemical Works (Paris, KY). *N*-lauroylsarcosine, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), RPMI 1640, boric acid, EGTA 3-[*N*-Morpholino] propane-sulfonic acid (MOPS), glycerol, isopropanol, EDTA, sodium acetate, 2-mercaptoethanol, trizma-base, bromophenol blue, xylene cyanole, diethyl pyrocarbonate (DEPC), sodium citrate, prostacyclin, apyrase, BSA, zymosan, ATP, poly-L-lysine, ferricytochrome c, superoxide dismutase, and dextrose were obtained from Sigma Chem. Co. (St. Louis, MO). Agarose, sodium dodecyl sulfate, RNA Ladder, DNA Ladder, guanidinium isothiocyanate, CsCl, and the restriction enzymes (HindIII, PstI) were obtained from GIBCO BRL (Gaithersburg, MD). RNAse-inhibitor, NP-40, Hepes, PMSF, leupeptin, and aprotinin were obtained from Boehringer Mannheim Canada (Laval, Quebec). Protein G/A-agarose was obtained from Oncogene Science (Uniondale, NY). Enhanced chemiluminescence (ECL) reagents for Western blotting were obtained from Amersham Life Sciences (Arlington Heights, IL). Prestained SDS-PAGE standards and gelatin were obtained from Bio-Rad Labs. (Hercules, CA). DNA Thermal Cycler 480, MuLV reverse transcriptase, and Ampli Taq DNA poly-

merase were obtained from Perkin Elmer-Cetus (Applied Biosystems, Mississauga, Toronto). The plasmid preparation kit was obtained from Qiagen Inc. (Chatsworth, CA). Primers for human NHE-1 were synthesized by General Synthesis and Diagnosis (Toronto, Ontario). Glycine was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Human stomach total RNA was obtained from Clontech (Palo Alto, CA). ImmunoPure NHS-LC-Biotin and streptavidin-HRP were obtained from Pierce Chemical Co. (Rockford, IL). Sodium pyrophosphate was obtained from Fisher Scientific (Pittsburgh, PA). Diisopropylfluorophosphate, herbimycin A, genistein, erbstatin analogue, and benzamidine were obtained from Calbiochem (La Jolla, CA). Nikkol was obtained from Nikko Chemical Co. Ltd. (Tokyo, Japan). Fura-2 AM, BAPTA-AM, BCECF-AM, FITC-labeled zymozan, and opsonization reagent were obtained from Molecular Probes (Eugene, OR). HOE694 and MMPA were obtained from Dr. J. Pouysségur (Nice, France).

Antibodies

Anti-integrin antibodies: 60.3 was obtained from Ms. Cathy Cambrian (Bristol-Meyers Squibb, Seattle, WA), IB4 whole molecule and Fab from Dr. David Chambers (San Diego Regional Cancer Center, San Diego, CA) and 6.5E and KIM 185 from Dr. Martyn Robinson (Cell Tech Biologics, Slough, UK). Antibodies against FcγR III (3G8) and FcγRII (IV.3) were obtained from Medarex Inc. (Annandale, NJ). Peroxidase-conjugated affinity-purified goat anti–rabbit IgG (whole molecule) and goat anti–mouse (GAM) $F(ab')_2$ were obtained from Cappel, Organon Teknika Corporation (Durham, NC). Cy3-labeled donkey anti–mouse $F(ab')_2$, Texas red–labeled donkey anti–rabbit $F(ab')_2$ were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Polyclonal antibodies to human NHE-1 were raised by injecting rabbits with a fusion protein constructed with *Escherichia coli* β-galactosidase and the last 157 amino acids of the carboxy-terminal domain of the human NHE and affinity purified as described (Sardet et al., 1990).

Cell Isolation

Human neutrophils (>98% pure) were isolated from citrated whole blood obtained by venipuncture, using dextran sedimentation and discontinuous plasma-Percoll gradients as previously described (Haslett et al., 1985; Downey et al., 1992). Platelets were isolated from acid citrate dextrose (ACD) anticoagulated whole blood obtained by venipuncture, using prostacyclin and apyrase as previously described (Crawford et al., 1992).

Phagocytosis Assay

Phagocytosis of opsonized zymosan particles was assayed according to the method of Hed and Stenhahl (1982). Briefly, neutrophils suspended in Hanks' buffered salt solution containing calcium and magnesium were allowed to settle on glass coverslips placed in 6-well plates (Costar, Cambridge, MA). Where indicated, 24 mM HCO_3^- was added to the medium which was equilibrated with 5% CO_2 or *N*-methyl-D-glucammonium replaced Na^+ as the predominant cation. FITC-labeled particles (Zymosan A Bioparticles, Molecular Probes) were opsonized, added to the coverslips in a ratio of 50 particles per neutrophil, and allowed to interact for 30 min at 37°C in a humidified atmosphere (in the presence of 5% CO_2 where indicated). Phagocytosis was stopped by fixing the cells in 1.5% paraformaldehyde for 15 min. Cells were washed three times with buffer and 0.4 mg/ml trypan blue added to quench the fluorescence of extracellular FITC-labeled zymosan particles that remained adherent to neutrophils. The coverslips were mounted using Immu-mount (Shandon, Pittsburgh, PA) and viewed under a fluorescence microscope (Leitz) using a 63× PlanApo oil immersion objective. A total of 200 cells were counted per coverslip and the number of cells containing intracellular particles was enumerated. In experiments using methyl-methyl-propenyl-amiloride (MMPA), the compound was added 5 min before the addition of the zymosan and the specified concentration was maintained throughout the assay.

Oxidative Burst

Activation of the NADPH oxidase was quantitated using the superoxide dismutase inhibitable reduction of cytochrome c as described in detail previously (Waddell et al., 1994). Briefly, treated cells were incubated in Hanks' buffered salt solution (with or without added calcium as indicated) containing 75 μM cytochrome C at 37°C for 20 min. Reference tubes were treated identically except that superoxide dismutase (30 U/ml) was added before the stimulus. The reaction was terminated by placing the tubes on ice followed by centrifugation, and the absorbance of the supernatant was measured at 550 nM in a spectrophotometer (Spectronic 1201, Milton Roy, Rochester, NY). The amount of O_2^- generated was calculated by using a reduced-oxidized extinction coefficient of 21.1 mM^{-1} cm^{-1}.

Cell Culture

HL-60 cells were purchased from the American Type Tissue Culture Collection (Rockville, MD) and PLB-985 cells human myeloid leukemia cells were obtained from Dr. Mary Dinauer (Riley Hospital for Children, Indianapolis, IN). Both cell lines were cultured in RPMI 1640 media containing 10% heat-inactivated FBS. AP-1 cells are NHE-deficient Chinese hamster ovary cells isolated by the H^+ suicide technique (Pouysségur et al., 1984). AP-$1^{rat\ NHE\text{-}1}$ and AP-$1^{rat\ NHE\text{-}3}$ cells were obtained by stable transfection of AP-1 cells with the complete coding region of the rat NHE-1 or NHE-3, respectively, as described in detail elsewhere (Orlowski, 1993).

RNA Isolation

Total RNA was isolated from neutrophils, PLB-985 cells, and HL 60 cells by the guanidinium isothiocyanate-cesium chloride protocol (Chirgwin et al., 1979; Groppe and Morse, 1993). Human stomach total RNA was obtained from Clontech.

Primers Used for PCR

Amplification primers for NHE isoforms were synthesized based on published nucleotide sequences obtained from GenBank. Base pairs (bp) are reported with reference to the site at which the oligonucleotide sequence was located in genomic maps in Sardet et al. (1989), Orlowski et al. (1992), and Wang et al. (1993). AP-1 cells transfected with the cDNA encoding rat NHE-1 were used as a positive control for NHE-1 detection. Digested cDNA plasmids encoding rat NHE-2 and 4 were used as positive controls for NHE-2 and 4 primers, respectively. Total RNA from human stomach was used as a positive control for NHE-3 primers. Primer sequences were as follows: rat NHE-1 (5′-3′), CCT ACG TGG AGG CCA AC (bp 1867-1883); (3′-5′), GAG CCA ACA GGT CTA CC (bp 2279-2295); transmembrane domain of human NHE-1 (5′-3′), CCT ATG TCG AGG CCA AC (bp 1147-1163); (3′-5′), CAG CCA ACA GGT CTA CC (bp 1559-1575); COOH-terminal domain of human NHE-1 (5′-3), CAC ACG CTG GTG GCA GAC (bp 2007-2024); (3′-5′), CTC ACA GAC TCT TCC ACC (bp 2531-2548); rat NHE-2 (5′-3′), GCT GTC TCT GCA GGT GG (bp 497-513); (3′-5′), CGT TGA GCA GAG ACT CG (bp 1150-1176); rat NHE-3 (5′-3′), CTT CTT CTA CCT GCT GC (bp 424-440); (3′-5′), CAA GGA CAG CAT CTC GG (bp 981-997); rat NHE-4 (5′-3′), CTG AGC TCT GTG GCT TC (bp 2156-2172); (3′-5′), GGA GGA AAT GCA GCA GC (BP 2521-2537).

Reverse Transcriptase PCR

Total RNA (1 μg) from the sources as specified was transcribed to cDNA using murine leukemia virus (MuLV) reverse transcriptase and random hexamers using the Gene Amp RNA PCR kit in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Emeryville, CA) according to manufacturer's instructions. All components of the amplification mixtures were tested for contamination by 35 PCR cycles in the absence of RNA template. Contamination of RNA preparations with genomic DNA was assessed by reverse transcription in the absence of MuLV reverse transcriptase followed by PCR as described above. Amplified products were analyzed by ethidium bromide staining on agarose gel electrophoresis.

DNA Sequencing

The amplified cDNA fragments from RT-PCR using primers from the transmembrane and COOH-terminal domains of human NHE-1 were cloned into the TA vector (Invitrogen, San Diego, CA). The resultant clones were sequenced by the dideoxy method, using a commercially available kit (Pharmacia). In addition, selected clones were sequenced using an automated fluorescence sequencer (Pharmacia ALF) at the Biotechnology Center at the University of Toronto.

SDS-PAGE, Immunoblotting, and Membrane Preparation

Electrophoresis was carried out as previously described (Fialkow et al., 1993). Briefly, neutrophils were incubated 2.5 mM diisopropylfluorophosphate (DFP), sedimented, resuspended in boiling 2% SDS sample buffer, and boiled for an additional 15 min. Samples (the equivalent of 8×10^5 cells) and molecular weight standards were subjected to electrophoresis in the presence of SDS on a 4–20% polyacrylamide gradient gel (Novex Experimental Technology, San Diego, CA) according to the method of Laemmli (1970). After electrophoresis the proteins were transferred to a nitrocellulose membrane, incubated in a blocking solution containing 0.25% gelatin, incubated with the primary antibody, washed, and incubated with horseradish peroxidase–conjugated goat anti–rabbit IgG. The Western blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham).

Neutrophil membranes were prepared according to the method of Kjeldsen et al. (1994) in the laboratory of Dr. Niels Borregaard. In brief, cells were pretreated with diisopropylfluorophosphate, washed, and resuspended in disruption buffer (in mM: 100 KCl, 3 NaCl, 1 $ATPNa_2$, 3.5 $MgCl_2$, 10 Pipes, pH 7.2) containing 0.5 mM PMSF and disrupted by nitrogen cavitation. The postnuclear supernatant was applied to a three-layer Percoll gradient and centrifuged for 30 min at 37,000 *g*. The γ-band containing secretory vesicles and plasma membranes was collected, solubilized in SDS sample buffer, and separated on a 4–20% polyacrylamide gradient gel as described above.

Measurement of pH_i and $[Ca^{2+}]_i$

pH_i was determined by fluorescence spectrophotometry in a thermostated stirred cuvette in a fluorescence spectrophotometer (Hitachi F-2000, Tokyo, Japan) using the pH-sensitive dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) as described previously (Downey and Grinstein, 1989). The nigericin/K^+ method (Thomas et al., 1979) was used to calibrate pH_i. For acid loading, cells were preincubated in Na^+-buffer containing 50 mM NH_4Cl for 10 min (NH_4Cl prepulse technique; Boron, 1983), sedimented, and resuspended in *N*-methyl-D-glucammonium$^+$-rich media. NaCl was then added (20 mM final concentration) and rates of pH_i change were determined by measuring the slopes of pH_i change during the first 1 min of sodium-induced cytoplasmic alkalinization of NH_4Cl-loaded cells. Nominally bicarbonate-free media was used throughout to minimize interference with other potential pH_i regulatory systems. The Na^+, *N*-methyl-D-glucammonium, and K^+-rich media were similar in osmolality because of isosmotic replacement of NaCl for the chloride salt of *N*-methyl-D-glucammonium$^+$ and K^+, respectively.

For measurement of calcium, cells were loaded by incubation for 20 min at 37°C in KRPD containing 2 μM Fura-2 AM. Cells were washed and resuspended in Ca^{2+} measuring buffer (in mM: NaCl, 140; KCl, 5; Hepes, 10; glucose, 10; $MgCl_2$, 1, pH 7.4, and either $CaCl_2$, 1; or EGTA, 1 as indicated). Fura-2 fluorescence was measured in a Hitachi F2000 fluorometer using an excitation wavelength of 335 nm and an emission wavelength of 495 nm. The fluorescence was calibrated using the ionomycin/Mn^{2+} technique (Nasmith and Grinstein, 1987).

Confocal Immunofluorescence Microscopy

For detection of capping of CD11b/CD18 and FcγRII, cells were incubated with 1° antibody (IB4 or IV.3) for 20 min at 4°C, washed, and resuspended in Na^+ buffer and incubated with Cy3-labeled donkey-anti–mouse $F(ab')_2$ for 10 min at 37°C. Cells were washed once in ice cold Na^+ buffer and fixed with 1.5% paraformaldehyde for 10 min at room temperature, washed three times with Na^+ buffer, and adhered to poly-L-lysine–coated coverslips and mounted using Immu-Mount (Shandon, Pittsburgh, PA). Polyclonal antibodies to human NHE-1 were used in our attempts to detect NHE-1. After fixation with 1.5% paraformaldehyde for 10 min, cells were allowed to adhere to glass coverslips, and then permeabilized with 0.5% NP-40 in PBS containing 1% BSA. Cells were preblocked with 5% goat serum in PBS containing 0.5% NP-40, washed three times with PBS containing 1% BSA, incubated with a 1:100 dilution of primary antibody in PBS containing 1% BSA overnight at 4°C, washed three times in PBS, incubated with Texas red–labeled donkey anti–rabbit $F(ab')_2$ secondary antibody for 4 h at 4°C, washed three times and mounted as above. The samples were viewed using a BioRad MRC 600 laser scanning confocal imaging system mounted on a Leitz Metallux-3 microscope using a ×100 (1.32 NA) oil immersion objective (Leitz) as described in detail previously (Downey et al., 1993). Digital images were imported into Adobe Photoshop, arranged, and labeled and printed on a Kodak XL 7700 digital printer. Prints are representative of multiple cells observed on each coverslip from at least three separate cell preparations done on different days.

Results

Exposure of Neutrophils to Opsonized Zymosan Induces Cytosolic Alkalinization Consequent to an Increase in Na^+/H^+ Exchange Activity

An increase in Na^+/H^+ exchange activity is known to occur after exposure of neutrophils to soluble agents such as fMLP (Grinstein et al., 1986*b*) and tumor necrosis factor (Yuo et al., 1993) and appears to be essential for full expression of some functional responses including chemotaxis and the oxidative burst (Worthen et al., 1994*a*; Simchowitz, 1985*a*,*b*). By contrast to activation by soluble agonists, little is known about the involvement of Na^+/H^+ exchange during phagocytosis. Accordingly, changes in pH_i were studied during exposure to the phagocytic stimulus opsonized zymosan (OpZ), yeast particles coated with IgG and complement fragments (Zalavary et al., 1994). Fig. 1 *a* illustrates that phagocytosis of OpZ particles induced a transient cytosolic acidification followed by a more prolonged alkalinization. Similar changes occurred in HCO_3^--containing medium, although the kinetics of cytosolic alkalinization were somewhat slower (Fig. 1 *a*, *second trace*). The alkalinization was absent in Na^+-free medium and was inhibited by the amiloride analogue MMPA, an inhibitor of the Na^+/H^+ exchanger (Simchowitz and Cragoe, 1986). It is noteworthy that in the presence of MMPA, cytosolic acidification ensued after exposure to OpZ (Fig. 1 *a*) likely due to a generation of acid equivalents by metabolic processes activated during phagocytosis. In MMPA-treated neutrophils exposed to OpZ in HCO_3-containing buffer, progressive cytosolic acidification was prevented and pH_i tended to return to near resting values (not illustrated but see c.f. Fig. 5 *c*). This correction of pH_i was likely due to concomitant Cl^-/HCO_3^- exchange since it was absent in HCO_3^--free medium. Taken together, these data provide evidence that activation of Na^+/H^+ exchange occurs during phagocytosis (Simchowitz and Cragoe, 1986; Swallow et al., 1990) and contributes to the maintenance of pH_i.

Fcγ and Complement Receptors Cooperate in Activation of Na^+/H^+ Exchange Activity

Several types of plasma membrane receptors are involved in binding and phagocytosis of opsonized particles by phagocytes: complement receptors type 1 (CR1) and type 3 (CR3) (Wright, 1992), and Fc receptors FcγRII and FcγRIII (for review see Lin et al., 1994). In addition to functioning in recognition and binding of particles, these receptors also participate in initiation of transmembrane signaling cascades (Wright, 1992). Spatial clustering of these receptors, achieved by multivalent ligands or "cross-linking" by antibodies, is usually required for activation of cellular responses. It is noteworthy that there is a high degree of cooperativity among these phagocytic receptors (Zhou and Brown, 1994). To determine which of these receptors is involved in signaling activation of NHE in neu-

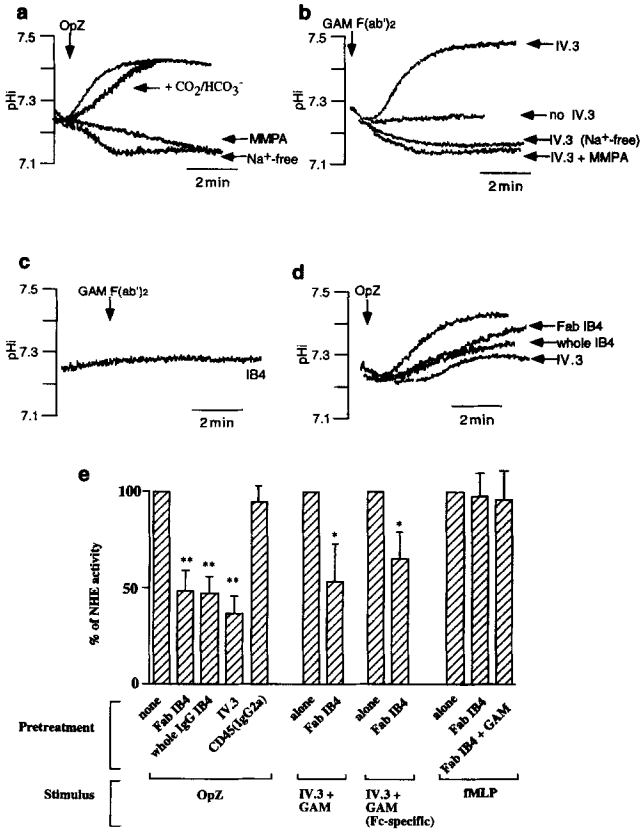

Figure 1. NHE activation by opsonized zymosan (OpZ) and Fcγ receptor cross-linking. Intracellular pH (pH_i) was measured using BCECF in a thermostated stirred cuvette in a fluorescence spectrophotometer as described in Materials and Methods. The nigericin/K^+ method was used to calibrate pH_i. (*a*) (*Top trace*) Neutrophils were suspended in Na^+ medium. OpZ was added at the time indicated by the arrow. (*Second trace*) (labeled CO_2/HCO_3^-) Neutrophils were suspended in Na^+ medium containing 24 mM $NaHCO_3^-$ equilibrated with 5% CO_2 and OpZ added at the time indicated by the arrow. (*Third trace*) 2 μM methyl-methyl-propenyl-amiloride (MMPA), an inhibitor of NHE activity was added immediately before addition of OpZ in Na^+ medium. (*Bottom trace*) Cells were resuspended in Na^+-free medium (containing NMG^+) and OpZ was added at the indicated time. Each trace is representative of experiments done from cells isolated from four different donors. (*b*) Cells were incubated with (*top trace*) or without (*second trace*) primary antibody against FcγRII (IV.3, 1 μg/ml) for 10 min at 4°C, washed, and resuspended in Na^+ medium. Secondary (cross-linking) antibody, GAM $F(ab')_2$, 10 μg/ml, was added at the indicated time. (*Third trace*) Cells were incubated with IV.3 for 10 min at 4°C, washed and resuspended in NMG^+ medium (Na^+-free medium). GAM $F(ab')_2$ was added at the indicated time. (*Bottom trace*) Cells were incubated with IV.3 for 10 min at 4°C, washed and resuspended in Na^+ medium. MMPA, an inhibitor of NHE activity, was added immediately before addition of GAM $F(ab')_2$ at the time indicated by the arrow. Each trace is representative of experiments done from cells isolated from four different donors. (*c*) Cells were incubated with primary antibody against β_2 integrins (IB4; directed against the common β chain, 29 μg/ml) for 10 min at 4°C, washed and resuspended in Na^+ medium. Secondary antibody (GAM $F(ab')_2$) was added at the indicated time. (*d*) Cells were incubated without (*top trace*) or after pretreatment with either primary antibody against β_2 integrins Fab IB4, 30 μg/ml (*second trace*) or whole molecule IB4, 29 μg/ml (*third trace*) or anti-FcγRII (IV.3), 10 μg/ml (*fourth trace*) before addition of OpZ at the time indicated by the arrow. Experiments were done in Na^+ medium and are representative of experiments done from cells isolated from four different donors. (*e*) Assessment of activation of NHE by measurement of the rate of pH_i change (determined by measuring the slopes of pH_i change) during the first minute after stimulation. (*Group 1*) Cells were incubated without (none) or after pretreatment with antibody (Fab IB4 or whole IgG IB4, IV.3, or anti-CD45) before addition of OpZ. (*Group 2*) Cells were incubated without (alone) or after pretreatment with antibody (Fab IB4) before stimulation with IV.3 + GAM $F(ab')_2$ or IV.3 + IV.3 + GAM $F(ab')_2$ (Fc-specific). (*Group 3*) Cells were incubated without (none) or after pretreatment with antibody (Fab IB4 + Fab IB4 + GAM $F(ab')_2$) before stimulation with fMLP. *($P < 0.05$), **($P < 0.01$), statistical significance as compared with the untreated group. Each data point represents the mean value ± SEM.

trophils, we used the strategy of cross-linking monoclonal antibodies (mAb) bound to phagocytic receptors with secondary goat anti-mouse (GAM) antibodies. Fig. 1 *b* illustrates that similar to phagocytosis of OpZ, cross-linking FcγRII with the mAb IV.3 resulted in a transient cytosolic acidification followed by a prolonged alkalinization. This alkalinization was amiloride-sensitive and was inhibited in the absence of extracellular Na^+ providing evidence that it was due to activation of Na^+/H^+ exchange. To determine if our method of antibody "cross-linking" resulted in clustering of the receptors, a fluorescently labeled donkey anti-mouse secondary antibody was used and the cells were visualized by confocal fluorescence microscopy. Fig. 2 *a* illustrates that in the absence of a secondary cross-linking antibody, FcγRII is distributed diffusely over the plasma membrane in small patches or aggregates. This patchy distribution may be accounted for by our technique of incubation of the cells with the primary antibody at 4°C which allows patching of surface receptors. Fig. 2 *b* illustrates that after cross-linking with secondary antibodies, the distribution of FcγRII receptors was altered dramatically with formation of large patches and caps.

In contrast to FcγRII, cross-linking of FcγRIII resulted in a much smaller (0.1 pH unit) and variable cytosolic alkalinization (data not shown) suggesting that FcγRII was the primary Fc receptor involved in activation of Na^+/H^+ exchange during phagocytosis. However, since the primary antibodies used for these experiments were whole molecules, it is not possible to rigorously exclude that part of the alkalinization observed after cross-linking FcγRIII was mediated via binding of anti-FcγRIII antibodies to FcγRII via their Fc portions.

Complement receptors are also involved in phagocytosis of opsonized particles and neutrophils express two types of complement receptors, CR1 and CR3. Zymosan that is opsonized with human serum under the conditions used here is coated with both IgG and fragments of complement, predominantly iC3b, a ligand for CR3. As CR3 is a β_2 integrin (CD11b/CD18), and since ligation and spatial clustering of integrins can activate Na^+/H^+ exchange in other cell types such as fibroblasts (Schwartz et al., 1989), endothelial cells (Ingber et al., 1990; Schwartz et al., 1991*a*), and lymphocytes (Schwartz et al., 1991*b*), we explored whether CR3 (CD11b/CD18) was involved in NHE activation during phagocytosis by cross-linking these receptors with the mAb IB4. Fig. 1 *c* illustrates that in con-

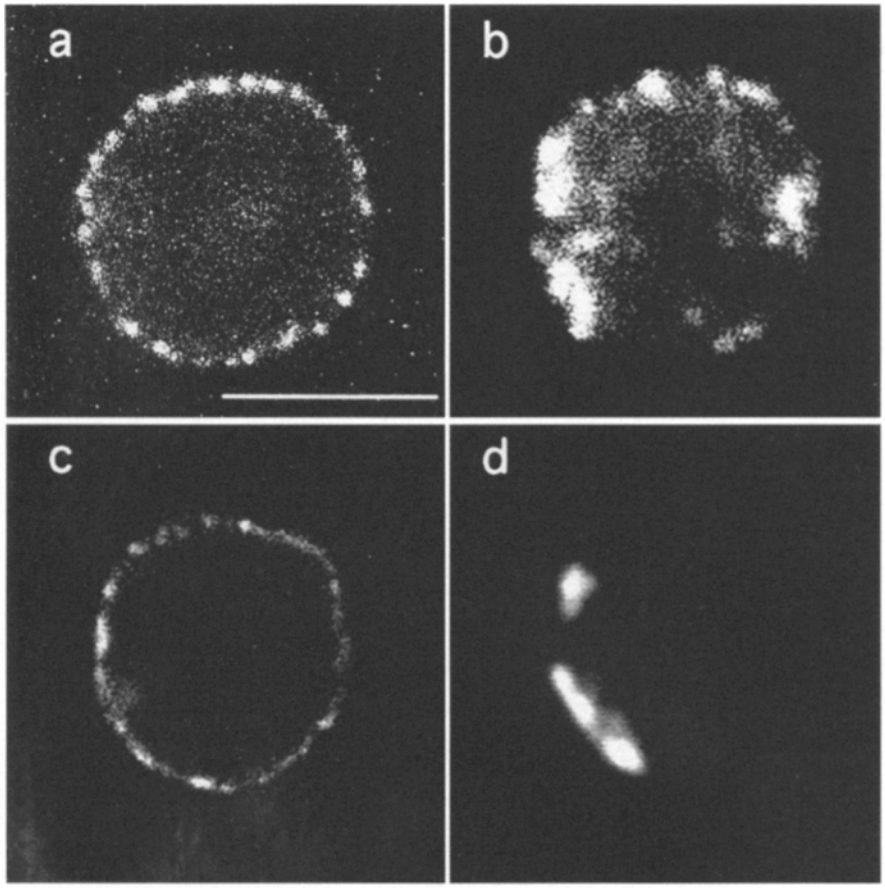

Figure 2. Immunofluorescence imaging of the distribution of FcγRII and CD11b/CD18. (*a*) Neutrophils were incubated with primary antibody directed against FcγRII (IV.3) for 10 min at 4°C, washed, and fixed with 1.5% paraformaldehyde. Cy3-labeled donkey anti–mouse $F(ab')_2$ was added for 20 min at 4°C. The cells were washed, allowed to adhere to poly-L-lysine–coated coverslips and mounted. The bar is 5 μm in length. (*b*) Neutrophils were incubated with primary antibody directed against FcγRII (IV.3) for 10 min at 4°C, washed, and Cy3-labeled donkey anti–mouse $F(ab')_2$ was added for 10 min at 37°C. Cells were washed, fixed with 1.5% paraformaldehyde, allowed to adhere to poly-L-lysine–coated coverslips, and mounted. (*c*) Neutrophils were incubated with primary antibody directed against β_2 integrins (IB4) for 10 min at 4°C, washed, and fixed with 1.5% paraformaldehyde. Cy3-labeled donkey anti–mouse $F(ab')_2$ was added for 20 min at 4°C. The cells were washed, allowed to adhere to poly-L-lysine–coated coverslips, and mounted. (*d*) Neutrophils were incubated with primary antibody directed against β_2 integrins (IB4) for 10 min at 4°C, washed, and Cy3-labeled donkey anti–mouse $F(ab')_2$ was added for 10 min at 37°C. Cells were washed, fixed with 1.5% paraformaldehyde, allowed to adhere to poly-L-lysine coated coverslips, and mounted. The samples were viewed using a BioRad 600 laser scanning confocal imaging system mounted on a Leitz Metallux-3 microscope using a ×100 (1.32 NA) oil immersion objective. Digital images were imported into Adobe Photoshop running on an Apple Macintosh computer, arranged, labeled, and printed using a Kodak XL 7700 digital printer. Prints are representative of multiple cells observed on each coverslip from at least three separate cell preparations done on different days.

trast to cross-linking integrins in other cell types, cross-linking of β_2 integrins in neutrophils did not activate Na^+/H^+ exchange. The mAb IB4 recognizes the common β_2 chain, thus in theory should cross-link any of the members of this family (CD11a/CD18, CD11b/CD18, CD11c/CD18). To ensure that the lack of effect of IB4 cross-linking on Na^+/H^+ exchange activity was not due to the epitope specificity of the antibody, we tested a battery of additional anti–β_2-integrin antibodies including 60.3, 6.5E, and KIM 185 (an anti–β-chain antibody that activates integrin adhesive function; Andrew et al., 1993). None of these antibodies, either alone or after cross-linking with GAM, activated of Na^+/H^+ exchange (data not shown). To ensure that the lack of effect was not due to failure to induce spatial clustering of these receptors, a fluorescently labeled donkey anti–mouse secondary antibody was again used and the cells visualized by confocal fluorescence microscopy. Fig. 2, *c* and *d* illustrate that these conditions resulted in patching and capping of CR3 (CD11b/CD18). Additionally, cross-linking of CR3 (CD11b/CD18) under these conditions was capable of inducing a transient increase in intracellular Ca^{2+} as we (Waddell et al, 1994) and others (Ng-Sikorski et al., 1991) have reported.

Nearly twenty years ago, it was proposed that complement receptors increased the efficiency of presentation of IgG to Fc receptors on phagocytes (Ehlenberger and Nussenzweig, 1977). To determine if CR3 (CD11b/CD18) functioned in a cooperative manner with Fcγ receptors, neutrophils were first incubated with saturating amounts of IB4 followed by exposure to OpZ. Fig. 1, *d* and *e* illustrate that these conditions diminished OpZ-induced alkalinization by ~50%. This inhibition occurred when either whole molecule or Fab fragments of IB4 were used and was specific for antibodies to β_2 integrins because preincubation of cells with an isotype-matched (IgG2a) anti-CD45 antibody had no effect on OpZ-induced alkalinization (Fig. 1 *e*). By comparison, incubation of neutrophils with saturating amounts of antibody to FcγRII before exposure to OpZ was more effective in preventing OpZ-induced alkalinization (Fig. 1, *d* and *e*). This inhibition ranged from 50–90% among individual blood donors.

The observed inhibition of OpZ-induced Na^+/H^+ exchange activity by β_2-integrin antibodies could potentially be explained by two different mechanisms. First, β_2 integrins could function by facilitating presentation of the opsonized zymosan particles to Fc receptors by interacting with complement fragments (iC3b) bound to zymosan ("binding function"). Antibodies to β_2 integrins, either

whole or Fab fragments, would block such an interaction. Alternatively, β_2 integrins could participate as actual signaling molecules which would affect the cellular response to signals transmitted via Fc receptors ("signaling function"). Fab fragments of IB4 which are univalent would be less likely than bivalent intact IgG molecules to initiate such a signal. The data presented in Fig. 1 *d* demonstrate that Fab fragments of IB4 can diminish OpZ-induced Na^+/H^+ exchange activity, providing evidence in favor of a mechanism involving a binding function of β_2 integrins.

To distinguish more definitively between binding and signaling functions, several additional experiments were conducted. First, the effect of Fab fragments of IB4 on the response to cross-linking FcγRII with IV.3 and goat anti–mouse (GAM) secondary antibodies (a "pure" Fc stimulus) was examined. Fig. 1 *e* illustrates that Fab fragments of IB4 partially inhibited this Fc-mediated activation of Na^+/H^+ exchange. To examine the possibility that this inhibitory effect might be due to interference by surface-bound IB4 with efficient Fc receptor aggregation (e.g., by formation of FcγR11-CR3 heterodimers), additional experiments were conducted by pretreating cells with Fab fragments of IB4 followed by cross-linking whole molecule IV.3 (anti-FcγRII) with Fc-specific GAM antibody (which should not bind to Fab fragments of IB4). Under these conditions, Fab fragments of IB4 were still able to partially diminish (~30%) Fc-induced activation of Na^+/H^+ exchange (Fig. 1 *e*). This effect could be explained by IB4 prevention of a positive signal transduced by activated β_2 integrins or by a negative signal initiated by binding of IB4 to β_2 integrins. To examine this latter possibility, we studied the effects of IB4 pretreatment on activation of Na^+/H^+ exchange by fMLP, an agonist that does not use CR3 or Fc receptors. Fig. 1 *e* illustrates that fMLP-induced activation of Na^+/H^+ exchange was not affected by IB4 pretreatment. Taken together, these data suggest that while β_2 integrins are not primarily involved in activation of Na^+/H^+ exchange activity, they function in a cooperative manner with Fc receptors.

Molecular Characterization of NHEs: Neutrophils Express NHE-1 but Not NHE-2, 3, or 4

The results above indicate that activation of Na^+/H^+ exchange occurs after exposure to phagocytic stimuli. To date, four isoforms of the Na^+/H^+ exchanger (NHE), termed NHE-1, 2, 3, and 4, have been cloned and sequenced (Sardet et al., 1989; Orlowski et al., 1992; Wang et al., 1993; Tse et al., 1993). To determine which isoforms were expressed in neutrophils, reverse transcriptase PCR (RT-PCR) analysis was employed. Total RNA was used as the template for the RT reaction. Fig. 3 *a* illustrates that messenger RNA for NHE-1 was expressed in neutrophils as determined using primers based on either rat or human sequence information. To confirm the presence of mRNA for NHE-1, additional experiments were carried out. RT-PCR was conducted using additional sets of primers directed at other regions of the molecule. Fig. 3 *e* illustrates that RT-PCR using primers directed at the transmembrane (TM) and carboxy-terminal portions or NHE-1 generated products of the predicted size. The identity each of these PCR products was confirmed by sequence analysis of the cDNA which demonstrated >98% homology with human NHE-1 (data not shown) in the regions amplified. In contrast, this analysis failed to detect expression of NHE-2, 3, or 4 (Fig. 3, *b–d*). To date, human NHE-2 and 4 have not been cloned and our RT-PCR analysis for these isoforms was based on rat sequence information. Thus, it is not possible to exclude that failure to detect NHE-2 and 4 was due to sequence divergence between the human and rat isoforms. However, this seems unlikely because even though there is over 95% sequence homology between rat and human NHE-1 and between rat and human NHE-3 in the region delimited by the primers, no product was obtained from the RT-PCR reaction even at low stringency (annealing temperatures between 45 and 50°C).

To determine if NHE-1 protein was expressed in neutrophils, anti–NHE-1 antibodies were raised against a fusion protein comprising the carboxy-terminal 157 amino acids of the human NHE-1. Fig. 4 *a* illustrates the specificity of these antibodies: a prominent immunoreactive band (denoted by the arrow) of ~110 kD was noted in whole cell extracts from antiport-deficient hamster cells expressing NHE-1 (AP-1 NHE-1: lane *1*) but not in the same cell line expressing NHE-3 (AP-1 NHE-3: lane *2*) or in the nontransfected parental cell line (AP-1: lane *3*). These antibodies failed to detect immunoreactive material of the appropriate molecular weight in whole cell extracts of human neutrophils (not illustrated). To increase the sensitivity of the detection system, plasma membranes were purified from human neutrophils followed by SDS-PAGE and immunoblotting. Fig. 4 *b* illustrates that under these conditions, a prominent immunoreactive band of appropriate molecular weight could be detected in neutrophil plasma membranes (lane *1*). This band could be competed off by incubation of the 1° antibody with excess NHE-1 fusion protein proving the specificity of this detection system (lane *2*). Isoform-specific antibodies for NHE-2 and NHE-4 are currently not available.

Pharmacological Characterization of NHE Activity: Recovery from Acid Loading

The results above indicate that NHE-1 mRNA and protein are expressed in neutrophils, but give no functional information. We therefore used pharmacological means to ascertain the functional significance of NHE-1 expression. Recently, a competitive inhibitor of NHE designated HOE694 was described which displays a much higher affinity for NHE-1 than for NHE-2 and NHE-3 (Counillon et al., 1993). To characterize Na^+/H^+ exchange pharmacologically, the ability of neutrophils to recover from an acid load was studied. Cells were acid-loaded by the NH_4Cl prepulse technique (Pouysségur et al., 1984). After removal of NH_4Cl, cells were suspended in *N*-methyl-D-glucammonium$^+$-rich solution; the resultant pH_i was ~6.8 (Fig. 5 *a*). After addition of 20 mM Na^+ to the medium, recovery of pH_i occurred rapidly, ostensibly through Na^+/H^+ exchange (Pouysségur et al., 1984). In Fig. 5 *a* it is apparent that recovery of pH_i was inhibited by submicromolar concentrations of HOE694. Fig. 5 *b* illustrates that the IC_{50} for inhibition of recovery from an acid load by HOE694 was ~0.01 μM. This value corresponds most closely to the IC_{50} value of 0.16 μM for NHE-1 as com-

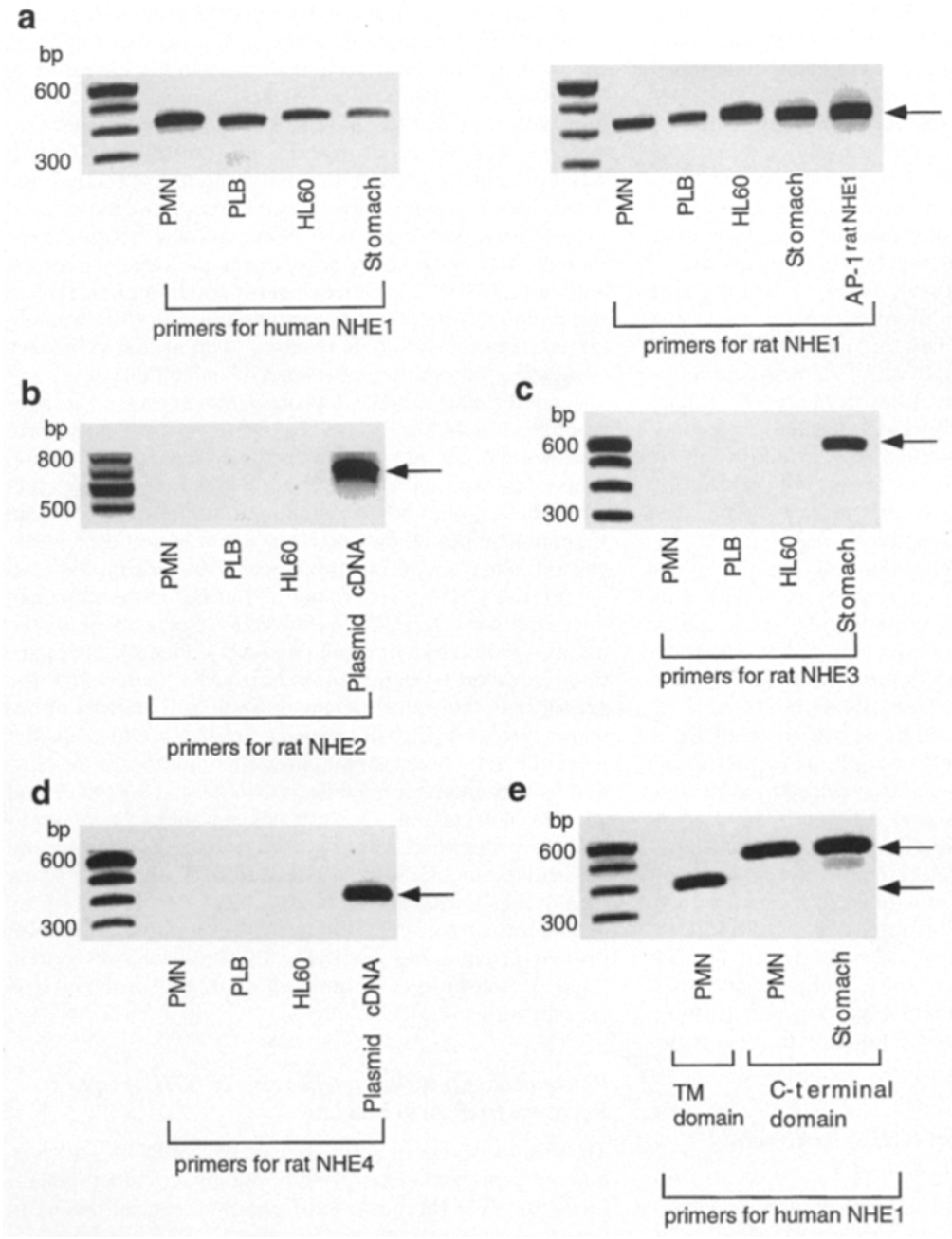

Figure 3. Reverse transcription PCR (RT-PCR) analysis of Na^+/H^+ exchanger (NHE) isoforms. Total RNA from human neutrophils (*PMN*), PLB cells (*PLB*), HL-60 cells (HL60), human stomach (*Stomach*) and chinese hamster ovary cells overexpressing rat NHE-1 cDNA (AP-$1^{rat\ NHE\text{-}1}$ cells) was reverse transcribed into cDNA as described in Materials and Methods. The cDNA samples or digested plasmid cDNA for NHE-2 and NHE-4 (plasmid cDNA) were amplified by PCR for 35 cycles with the primers as specified. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining and captured by a CCD camera. The digital image was imported into Canvas running on a Macintosh computer, labeled and printed with a Linotronic printer. The position of DNA size marks (*left*, in bp) and of the NHE PCR product is indicated by the arrow. Electrophoresis of the PCR products for NHE-1 revealed the expected band of 429 (*TM domain*) or 542 bp (*COOH-terminal domain*) from human neutrophils, PLB cells, HL-60 cells, and human stomach using either rat or human primers. (*a*) RT-PCR analysis with PCR primers for the transmembrane domain (TM) of human NHE-1(*left*) and rat NHE-1(*right*). The expected molecular size of PCR products is indicated (429 bp). (*b*) RT-PCR analysis with PCR primers for rat NHE-2. The expected molecular size of PCR products is indicated (680 bp). (*c*) RT-PCR analysis with PCR primers for rat NHE-3. The expected molecular size of PCR products is indicated (574 bp). (*d*) RT-PCR analysis with PCR primers for rat NHE-4. The expected molecular size of PCR products is indicated (382 bp). (*e*) RT-PCR analysis with PCR primers for the transmembrane domain (TM) of human NHE-1(*left*) and COOH-terminal domain of human NHE-1 from neutrophil (*middle*) and stomach (*right*) total RNA. The expected molecular size of PCR products is indicated (429 or 542 bp).

pared to an IC_{50} of 5 μM for NHE2 and 650 μM for NHE-3 when expressed in transfected fibroblast cell lines (Counillon et al., 1993). HOE694 also inhibited cytosolic alkalinization after exposure to OpZ or after FcγRII cross-linking at submicromolar concentrations (Fig. 5, *c–e*). In cells treated with HOE694 in HCO_3^--containing medium, progressive cytosolic acidification was prevented and pH_i eventually returned to near resting values (Fig. 5 *c*, *third trace*), presumably due to concomitant Cl^-/HCO_3^- exchange. These studies are most consistent with NHE-1 as the predominant isoform responsible for pH recovery from an acid load and for the cytosolic alkalinization after exposure to the phagocytic stimulus or after cross-linking of Fcγ receptors. However, we cannot exclude the possibility that an alternate isoform, as yet undescribed and with an HOE694 inhibitory profile similar to NHE-1, might be expressed in neutrophils and contribute to regulation of pH_i under these conditions.

Role of NHE in Phagocytosis

To determine if NHE activity was required for phagocyto-

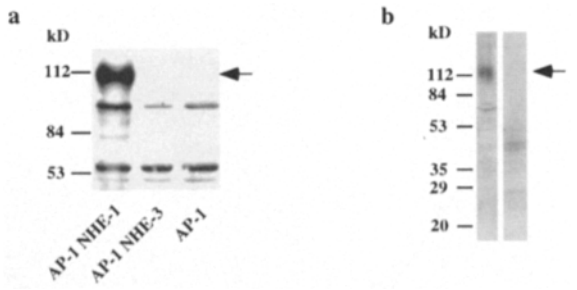

Figure 4. Immunological detection of NHE-1 protein. Plasma membranes from human peripheral blood neutrophils were purified according to the method of Kjeldsen et al. (1996), proteins separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti–NHE-1 polyclonal antibodies. Immunoreactive bands were visualized using ECL (Amersham) with goat anti–rabbit antibody coupled to horseradish peroxidase. (*a*) (Lane *1*) Whole cell lysates from AP-1 cells transfected with the cDNA for rat NHE-1; (lane *2*) whole cell lysates from AP-1 cells transfected with the cDNA for rat NHE-3; and (lane *3*) whole cell lysates from untransfected AP-1 cells. (*b*) (Lane *1*) Purified plasma membranes from human peripheral blood neutrophils blotted with anti–NHE-1 antibody; (lane *2*) purified plasma membranes from human peripheral blood neutrophils blotted with anti–NHE-1 antibody that was incubated with excess NHE-1 fusion protein. The arrow indicates the expected molecular mass of NHE-1 (~110–115 kD).

sis, this process was compared under several conditions: in the presence or absence of extracellular Na^+, in the presence of inhibitors of NHE including the amiloride analogue MMPA, HOE694 (not illustrated), or in HCO_3^--containing buffer. Fig. 5 *f* illustrates that phagocytosis was the same under all of these conditions. These data suggest that Na^+/H^+ exchange activity is not required for phagocytosis although associated processes such as the oxidative burst and intracellular killing are greatly attenuated in the absence of NHE activity (Swallow et al., 1990).

Signaling Pathways Involved in NHE Activation

Role of Alterations in $[Ca^{2+}]_i$. Recent studies have demonstrated that NHE-1 is a calmodulin-binding protein and the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) can regulate the activity of NHE-1 in fibroblasts (Bertrand et al., 1994). It is also noteworthy that many of the conditions leading to NHE activation including phagocytosis (Rosales and Brown, 1991), Fcγ receptor cross-linking (Huizinga et al., 1990; Brunkhorst et al., 1992; Della Bianca et al., 1993; Graham et al., 1993; Naziruddin et al., 1992; Rosales and Brown, 1992; and Fig. 6 *e*), and stimulation with fMLP also result in an increase in $[Ca^{2+}]_i$. To examine the effects of alterations in $[Ca^{2+}]_i$ on Na^+/H^+ exchange activity, cells were exposed to thapsigargin, an agent that releases Ca^{2+} from intracellular stores (Demaurex et al., 1994). To separate the effects of Ca^{2+} released from intracellular stores and that entering from the extracellular medium, cells were suspended in Ca^{2+}-free medium. Fig. 6 *a* illustrates that treatment with thapsigargin induced cytosolic acidification, followed by sustained alkalinization that was accompanied by a transient increase in $[Ca^{2+}]_i$ (Fig. 7 *d*). These effects were prevented by preincubation of cells with the intracellular Ca^{2+} buffering agent BAPTA-AM (Fig. 7 *d*), confirming that they were a consequence of the increase in $[Ca^{2+}]_i$. Additionally, HOE694 prevented the cytosolic alkalinization (Fig. 6 *a*) providing evidence that it was due to activation of NHE-1. Treatment of cells with ionomycin, a Ca^{2+} ionophore also capable of releasing Ca^{2+} from intracellular stores, had similar effects on pH_i (data not shown). However, the interpretation of these effects is complicated by the fact that ionomycin can exchange H^+ for Ca^{2+}, potentially inducing a Ca^{2+}-dependent cytosolic alkalinization independent of NHE activation. Taken together, these data provide evidence that an increase in $[Ca^{2+}]_i$ was capable of activating NHE-1.

Recent studies have demonstrated that emptying of intracellular Ca^{2+} stores in neutrophils leads to the opening of membrane Ca^{2+} channels with resultant influx of Ca^{2+} (Demaurex et al., 1994). To study a possible contribution of an influx of extracellular Ca^{2+} to activation of Na^+/H^+ exchange by phagocytic stimuli, cytosolic alkalinization in response to OpZ or Fcγ receptor cross-linking was compared in the presence and absence of extracellular Ca^{2+}. Fig. 6, *b* and *c* illustrate that the magnitude of cytosolic alkalinization was identical under these two conditions, establishing that extracellular Ca^{2+} influx was not required for NHE activation under these circumstances. To determine whether an increase in $[Ca^{2+}]_i$ resulting from release from intracellular stores was required for NHE activation by OpZ and Fc cross-linking, cells were loaded with BAPTA-AM, resuspended in calcium-free medium, and stimulated with either OpZ or by FcγR cross-linking with the mAb IV.3. Under these conditions, although the resultant increase in $[Ca^{2+}]_i$ was prevented (Fig. 6 *d*: *bottom trace*), the cytosolic alkalinization, while slightly slower, was of similar magnitude (Fig. 6 *e*). To ensure that the conditions used for BAPTA-loading were sufficient to prevent a response known to be calcium-dependent (Morel et al., 1991), activation of the NADPH oxidase by fMLP was measured using superoxide-dismutase inhibitable reduction of cytochrome c. Fig. 6 *f* illustrates that under these conditions, fMLP-induced activation of the NADPH oxidase was completely abrogated. These studies indicate that activation of NHE in response to a variety of physiological stimuli (OpZ, FcγR cross-linking, fMLP) can proceed in the absence of an increase in $[Ca^{2+}]_i$.

Role of Tyrosine Phosphorylation

As our results indicated that activation of Na^+/H^+ exchange by phagocytic stimuli could proceed by a pathway that was calcium-independent, we investigated the contribution of alternate signaling pathways. Recent studies have linked Fcγ receptors with signaling pathways involving tyrosine phosphorylation in neutrophils (Dusi et al., 1994*a*; Zhou and Brown, 1994). Additionally, the tyrosine kinase *fgr* is known to be associated with FcγRII in neutrophils and to become activated after cross-linking this receptor (Hamada et al., 1993). To determine whether activation of NHE-1 by OpZ or after FcγR cross-linking involved similar pathways, we studied tyrosine phosphorylation of cellular proteins in neutrophils subject to these treatments (Fig. 7 *a*). Both OpZ and cross-linking of FcγRII induced tyrosine phosphorylation of multiple poly-

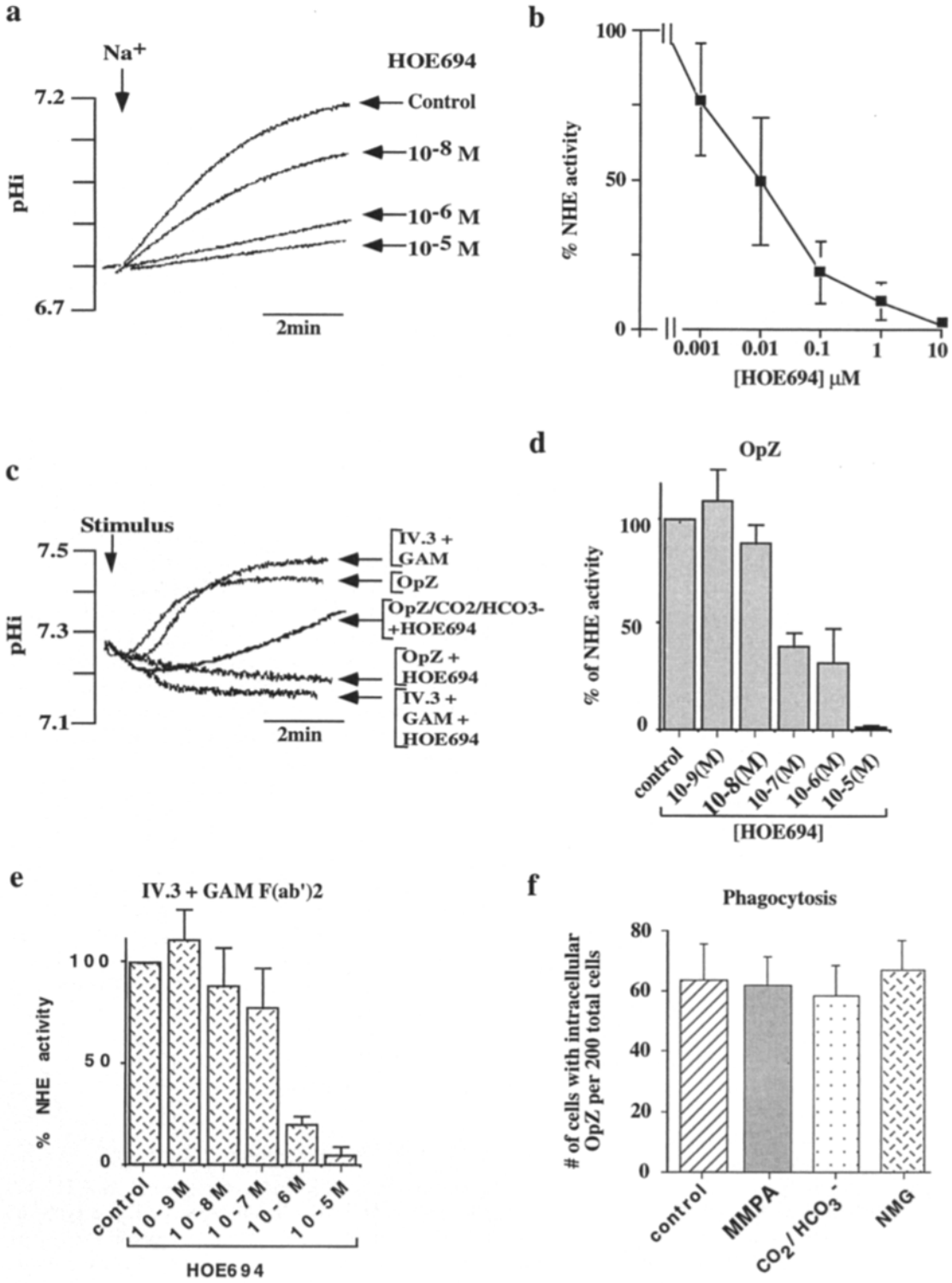

Figure 5. Inhibition of NHE-1 activity by the amiloride analogue HOE694. (*a*) Cells were acid loaded by preincubation in Na^+-buffer containing 50 mM NH_4Cl for 10 min (NH_4Cl prepulse technique; Boron, 1983), sedimented and resuspended in *N*-methyl-D-glucammonium$^+$-rich media. NaCl was then added (final concentration 20 mM) and rates of pH_i change were determined by measuring the slopes of pH_i change during the first minute of sodium-induced cytoplasmic alkalinization of NH_4Cl-loaded cells. (*b*) Dose-response curve for inhibition of initial rate (first minute) of acid-induced NHE activation neutrophils. (*c*) OpZ and FcγR cross-linking induced alkalinization were inhibited by HOE694. Cells were incubated with OpZ (*second trace*) or with primary antibody against FcγRII (IV.3) for 10 min at 4°C, followed by secondary (cross-linking) antibody, GAM $F(ab')_2$ (*top trace*). The third trace represents cells suspended in Na^+ medium containing 24 mM $NaHCO_3^-$ equilibrated with 5% CO_2 and preincubated 10 μM HOE694. OpZ was added at the time indicated by the arrow. The fourth trace represents OpZ-treated cells preincubated 10 μM HOE694. The fifth trace represents FcγRII cross-linked cells treated cells preincubated 10 μM HOE694. Each trace is representative of experiments done with cells isolated from four different donors. (*d* and *e*) Dose-dependent inhibition by HOE694 of cytosolic alkalinization induced by OpZ or Fc-cross-linking.

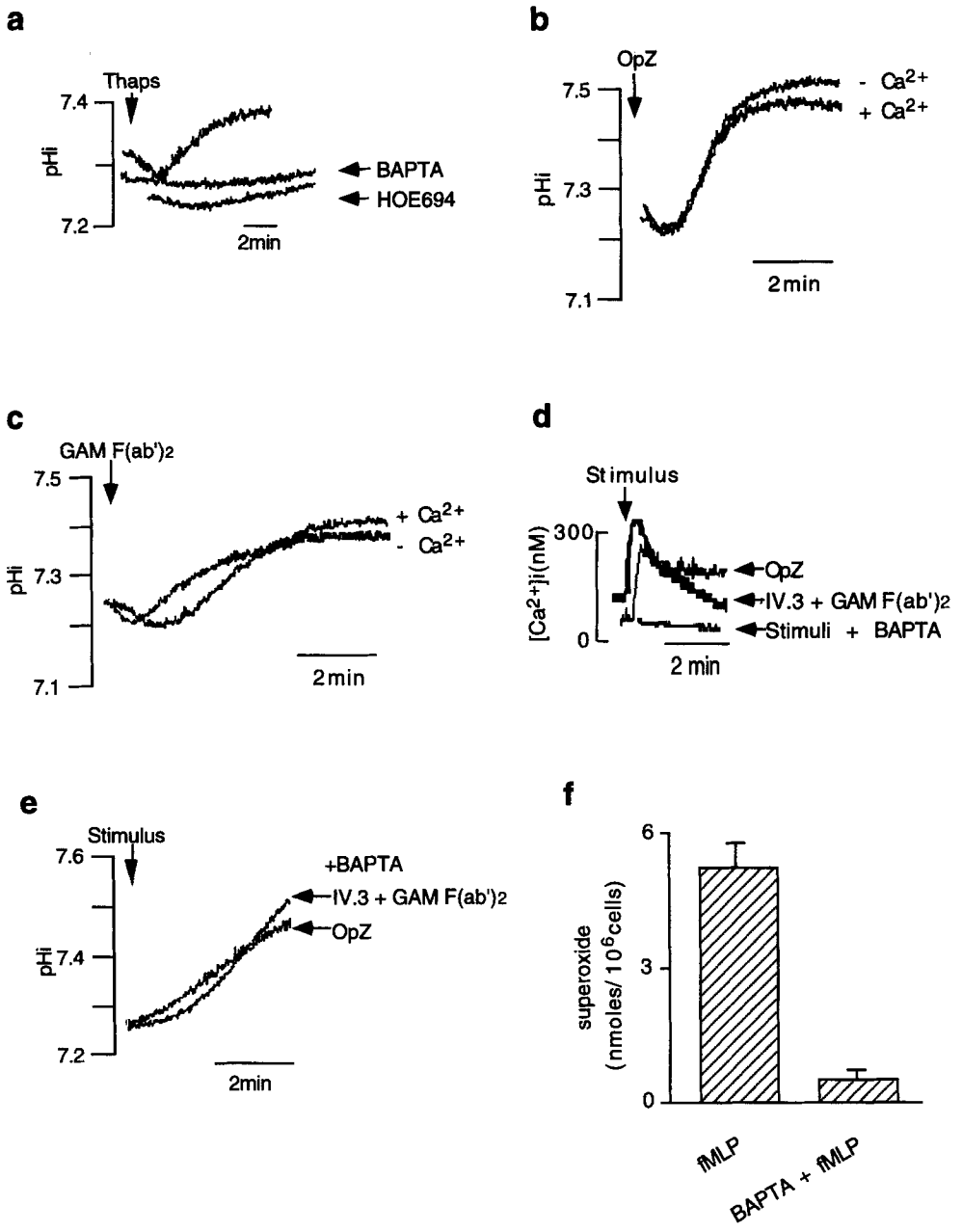

Figure 6. Changes in intracellular calcium are not required for activation of NHE. Intracellular pH (pH_i) and $[Ca^{2+}]_i$ were measured fluorimetrically using BCECF and Fura 2 AM, respectively, as described in Materials and Methods. (*a*) pH_i. Thapsigargin (50 nM) was added to neutrophils suspended in calcium-free medium where indicated in the absence (*top trace*) or presence of 20 μM BAPTA (*middle*) or in the presence of 10 μM HOE694 (*bottom trace*). (*b* and *c*) pH_i. Neutrophils were resuspended in Na^+ medium with 1 mM $CaCl_2$ (+ Ca^{2+}) or in the same medium without added calcium but with 1 mM EGTA (calcium-free: $-Ca^{2+}$) followed by addition of OpZ (*b*) or FcγR cross-linking (*c*) as indicated. (*d*) $[Ca^{2+}]_i$. Neutrophils were resuspended in Na^+ medium without added calcium but with 1 mM EGTA (calcium-free) and treated with either OpZ (*top trace*) or FcγR cross-linking (*middle trace*). The bottom trace represents neutrophils preincubated with 20 μM BAPTA and then exposed to either OpZ or FcγR cross-linking. (*e*) pH_i. Neutrophils preincubated with 20 μM BAPTA were resuspended in Na^+ medium without added calcium but with 1 mM EGTA (calcium-free) followed by FcγR cross-linking (*top trace*) or OpZ (*bottom trace*) as indicated. (*f*) Superoxide production was quantitated using the superoxide dismutase inhibitable reduction of cytochrome c in response to 10^{-7} M fMLP. Where indicated, cells were preincubated with 20 μM BAPTA. Data represent the superoxide production in nmol/10^6 cells/20 min. Each data point represents the mean value ± SEM of $n = 4$ experiments.

peptides including prominent bands at 42–44, 60, 72–80, and 145 kD. This enhanced tyrosine phosphorylation was largely inhibited by both genistein and herbimycin, suggesting that activation of tyrosine kinases induced by both OpZ and cross-linking of FcγRII contributed to the increase in tyrosine phosphorylation. Importantly, treatment of cells with tyrosine kinase inhibitors (genistein, herbimycin, or erbstatin analog) abrogated cytosolic alkalinization after OpZ (Fig. 7 *b*) or after FcγRII cross-linking with the mAb IV.3 (Fig. 7 *c*), suggesting that the signaling pathway leading to NHE activation involved tyrosine phosphorylation.

(*f*) Effect of inhibition of NHE on phagocytosis. Phagocytosis of opsonized zymosan particles was assayed as described in Materials and Methods. In experiments using methyl-methyl-propenyl-amiloride (MMPA), the compound was added 5 min before addition of the zymosan and the specified concentration was maintained throughout the assay. HOE694 did not inhibit phagocytosis (not illustrated). Where indicated, 24 mM HCO_3^- was added to the medium which was equilibrated with 5% CO_2 or *N*-methyl-D-glucammonium (NMG) replaced Na^+ as the predominant cation. Each value represents the mean value ± SEM of $n = 4$ experiments with cells from different donors. No significant differences existed between groups.

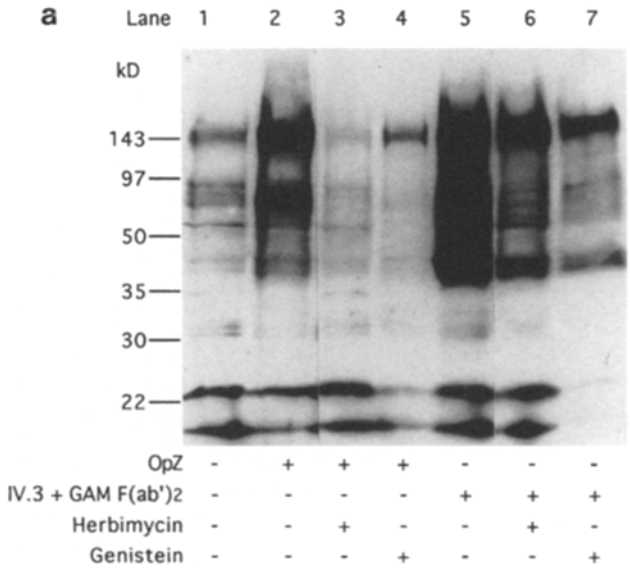

Figure 7. Inhibitors of tyrosine kinases prevent OpZ and FcR cross-linking induced NHE activation. (*a*) OpZ and FcγR cross-linking induces tyrosine phosphorylation of multiple cellular polypeptides. Autoradiogram of an immunoblot of whole cell tyrosine phosphorylation after exposure to either OpZ (lanes *2–4*) or FcγR cross-linking (lanes *5–7*). Polyclonal anti-phosphotyrosine was used as the primary antibody for immunoblotting. Lane *1* represents unstimulated neutrophils. Lanes *2–4* represent neutrophils exposed to OpZ for 1 min in the absence (lane *2*) or presence of 5 μM herbimycin for 4 h at 37°C (lane *3*) or 100 μM genistein for 30 min at 37°C (lane *4*). Lanes *5–7* represent neutrophils treated with primary antibody against FcγRII (IV.3) for 10 min at 4°C, followed by secondary (cross-linking) antibody, GAM $F(ab')_2$ in the absence (lane *5*) or presence of 5 μM herbimycin for 4 h at 37°C (lane *6*) or 100 μM genistein for 30 min at 37°C (lane *7*). (*b*) Inhibition of OpZ-induced alkalinization by tyrosine kinase inhibitors. Intracellular pH (pH_i) was measured fluorometrically using BCECF-AM. Neutrophils were incubated with 100 μM genistein for 30 min at 37°C, 5 μM erbstatin analogue for 1 h at 37°C, or 5 μM herbimycin for 4 h at 37°C, washed and resuspended in Na^+ medium. OpZ was added at the indicated time. Each trace is representative of experiments done with cells isolated from four different donors. (*c*) Inhibition of FcγR cross-linking–induced alkalinization by tyrosine kinase inhibitors. Cells were incubated with 100 μM genistein for 30 min at 37°C, 5 μM erbstatin analogue for 1 h at 37°C, or 5 μM herbimycin for 4 h at 37°C and incubated with IV.3 for 10 min at 4°C. Cells were washed and resuspended in Na^+ medium. GAM $F(ab')_2$ was added at the indicated time. (*d*) $[Ca^{2+}]_i$ was measured fluorimetrically using Fura 2 AM as described in Materials and Methods. Where indicated, thapsigargin (50 nM) was added to neutrophils suspended in calcium-free medium in the absence (−) or presence of 100 μM genistein (+) or after preincubation with 20 μM BAPTA-AM (*bottom trace*). (*e*) Inhibition of thapsigargin-induced alkalinization by genistein. Cells were incubated in the absence or presence (+) of 100 μM genistein for 30 min at 37°C. Where indicated, thapsigargin (50 nM) was added to the cells suspended in calcium-free medium and pH_i measured fluorimetrically using BCECF-AM.

The results above indicate that phagocytic stimuli (OpZ and Fc cross-linking) can induce NHE activity by a pathway that is dependent on tyrosine phosphorylation and apparently independent of increases in $[Ca^{2+}]_i$. On the other hand, thapsigargin can induce NHE activity by a calcium-dependent pathway. These seemingly incompatible results can be reconciled by recent studies that demonstrated that increases in $[Ca^{2+}]_i$ contribute to enhanced tyrosine phosphorylation in neutrophils (Gomez-Cambronero et al., 1991). We therefore considered the possibility that thapsigargin-induced activation of NHE might be mediated secondarily by tyrosine phosphorylation. To this end, Na^+/H^+ exchange activity in response to thapsigargin was compared in the presence and absence of tyrosine kinase inhibitors. Pretreatment of cells with genistein (Fig. 7 *e*) or herbimycin (not shown) prevented cytosolic alkalinization but not the increase in $[Ca^{2+}]_i$ induced by thapsigargin (Fig. 7 *d*). These data are in accord with the notion that thapsigargin-induced activation of Na^+/H^+ exchange activity may be indirect and a consequence of activation of a tyrosine kinase or inhibition of a tyrosine phosphatase. Fig. 8 summarizes the experiments relating $[Ca^{2+}]_i$ and tyrosine phosphorylation to activation of Na^+/H^+ exchange. Several points merit consideration. First, Ca^{2+} mobilization induced by the phagocytic stimuli OpZ and Fc cross-linking is markedly diminished by tyrosine kinase inhibitors such as genistein (Fig. 8 *a*), compatible with the occurrence of tyrosine phosphorylation and activation of PLCγ (Dusi et al., 1994*b*). Second, while Ca^{2+} mobilization induced by OpZ and Fc cross-linking is prevented by BAPTA (Fig. 8 *a*), activation of NHE is not (Fig. 8 *b*). Third, while Ca^{2+} mobilization in response to thapsigargin is not affected by tyrosine kinase inhibitors (Fig. 8 *a*), Na^+/H^+ exchange is completely abrogated (Fig. 8 *b*). A proposed model for the regulation of NHE activity in neutrophils to account for these observations is discussed below and illustrated in Fig. 9.

Discussion

In this study, the molecular identity and functional aspects of Na^+/H^+ exchange activity in human neutrophils during phagocytosis were investigated. Our results indicate that mature human neutrophils express messenger RNA and protein for the NHE-1 isoform of the exchanger. This conclusion is based on studies using RT-PCR with isoform specific primers and cDNA sequencing that detected the presence only of NHE-1 transcripts and by the demonstration of the presence of NHE-1 protein in purified plasma membranes using isoform-specific antibodies. The pharmacological profile of the neutrophil exchanger is also consistent with the presence of functional NHE-1 (see below).

In neutrophils, phagocytic cells whose primary function is in host defense, one of the main functions of NHE-1 may be protection from an acid load as is known to occur in acidic environments such as abscesses (Swallow et al., 1990) or during the respiratory burst where large amounts of H^+ are released into the cytosol during superoxide synthesis (Simchowitz, 1985*a*,*b*; Grinstein et al., 1986*a*,*b*). In fact, phagocytosis of OpZ is known to induce a large and sustained respiratory burst (Borregaard et al., 1984), and it follows that NHE activation under these conditions may play an important role. The importance of pH compensation during these conditions can be appreciated by inspection of the pH_i traces during phagocytosis in the presence

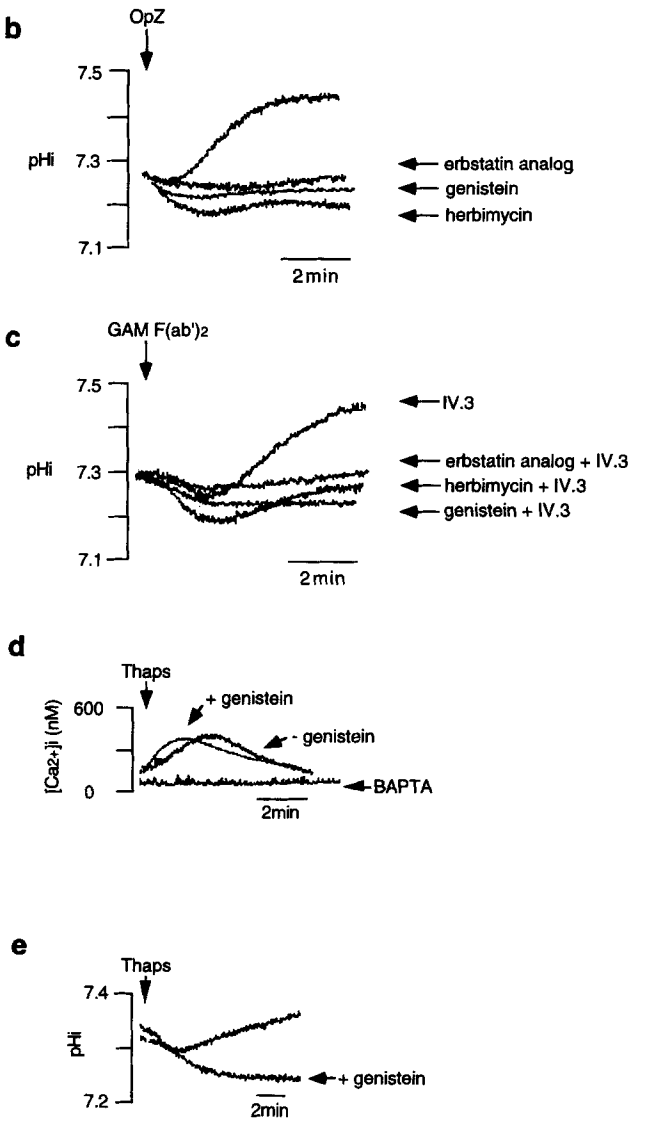

Figure 7.

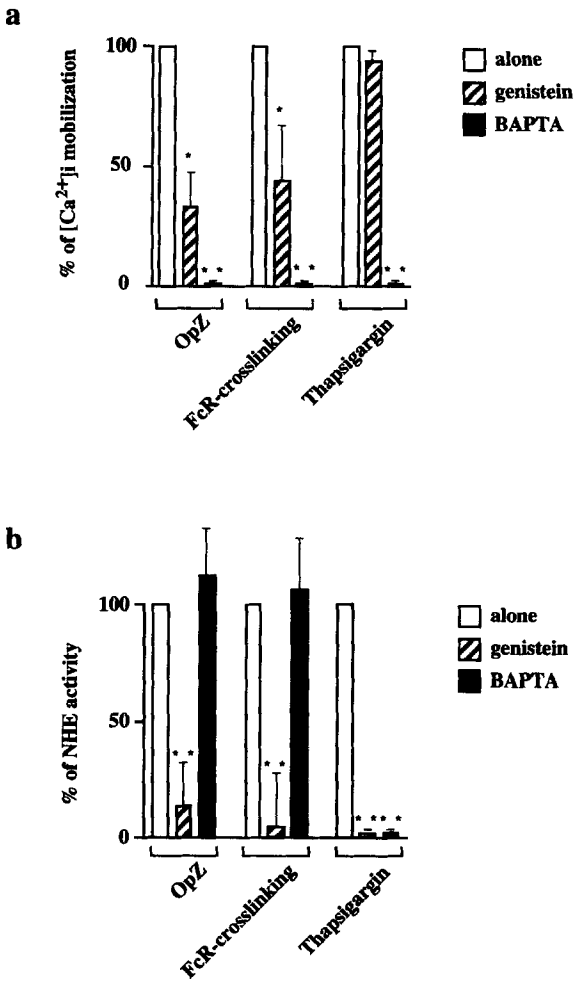

Figure 8. Summary of the experiments demonstrating the relationship between $[Ca^{2+}]_i$ mobilization, tyrosine phosphorylation, and NHE activity. *a* illustrates $[Ca^{2+}]_i$ mobilization in response to OpZ, FcγRII cross-linking or thapsigargin. Open bars indicate no pretreatment, striped bars indicate pretreatment with 100 μM genistein and solid bars indicate pretreatment with 20 μM BAPTA-AM. 100% represents the magnitude of $[Ca^{2+}]_i$ mobilization stimulated cells without genestein or BAPTA-AM pretreatment. *b* illustrates NHE activity in response to OpZ, FcγRII cross-linking or thapsigargin. Open bars indicate no pretreatment, striped bars indicate pretreatment with 100 μM genistein, and solid bars indicate pretreatment with 25 μM BAPTA-AM. 100% represents the magnitude of cytosolic alkalinization in stimulated cells without genestein or BAPTA-AM pretreatment. *($P < 0.05$), **($P < 0.01$), statistical significance compared with the group receiving no pretreatment. Each data point represents the mean value ± SEM of $n = 4$ experiments.

of inhibitors of NHE or in the absence of extracellular Na^+ (Fig. 1). Under these conditions, the intracellular pH fell by almost 0.2 pH units which could potentially affect the functioning of certain enzyme systems. The findings that inhibitors of Na^+/H^+ exchange did not inhibit phagocytosis suggest that NHE activity was not required for phagocytosis, perhaps due to the presence of alternate pH compensating systems (vide infra). However, at least two other phagocytic functions associated with phagocytosis including the oxidative burst, required for effective intracellular killing, and motility, important for emigration of leukocytes to the site of infection, are known to be markedly diminished by inhibitors of Na^+/H^+ exchange (Swallow et al., 1990; Worthen et al., 1994*a*). Thus, NHE activation during phagocytosis might be envisioned to participate in these functions that are crucial for the microbicidal function of neutrophils.

It is noteworthy that Na^+/H^+ exchange is but one of the mechanisms available for the extrusion of H^+ equivalents in leukocytes, which is likely the reason that pH_i did not fall even further. Examples of other systems include proton pumps (Nanda et al., 1992) and HCO_3^- transporters (Simchowitz and Roos, 1985). The latter system would be expected to contribute negligibly in experiments carried out in nominally HCO_3^--free buffers. However, Fig. 5 *c* illustrates that in HCO_3^--containing buffers, progressive cytosolic acidification is prevented in HCO_3^--containing medium, likely by HCO_3^-/Cl^- exchange. Na^+/H^+ exchange activity is also involved in the regulation of cell volume, including the increase in volume (RVI) that occurs after osmotic shrinkage and the cell swelling that occurs after leukocyte activation (Grinstein et al., 1986*b*, 1992). The

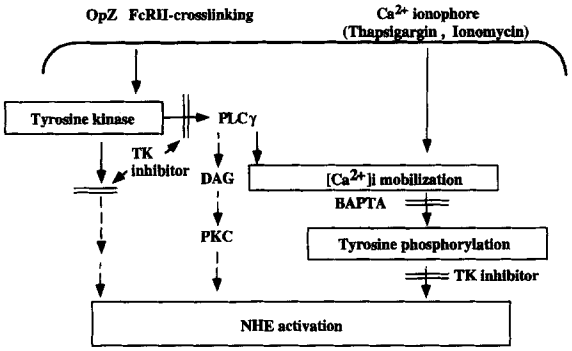

Figure 9. Proposed model for the regulation of NHE activity in neutrophils. OpZ and Fc-cross-linking induced NHE activation requires activation of a tyrosine kinase(s) but not $[Ca^{2+}]_i$ mobilization. The signaling pathway may involve activation of PLCγ with formation of diacyl glycerol (DAG) and subsequent activation of an isoform of protein kinase C that does not require $[Ca^{2+}]_i$ mobilization. An alternate pathway used by thapsigargin or ionomycin involves $[Ca^{2+}]_i$ mobilization with subsequent tyrosine phosphorylation. Solid lines indicate pathways for which there is experimental evidence and dashed lines indicate speculative pathways.

importance of these changes in cell volume in the context of phagocytosis is unknown but may be crucial for directed cell movement (Worthen et al., 1994*a*), which in the case of neutrophils, is involved in emigration from the vascular space and chemotaxis toward the site of infection.

The current studies indicate that activation of the exchanger can occur in the absence of an influx of extracellular Ca^{2+} or release of Ca^{2+} from internal stores. This applies to activation by multiple agents including phagocytic stimuli, FcγR cross-linking, the formyl peptide fMLP, and PMA. On the other hand, an increase in $[Ca^{2+}]_i$ resulting from thapsigargin or ionomycin resulted in activation of Na^+/H^+ exchange activity. The latter results are consistent with the recent report that ionomycin activated NHE-1 expressed in exchanger-deficient fibroblasts (Wakabayashi et al., 1994). These investigators suggested that the mechanism for this calcium-dependent activation involved Ca^{2+}-calmodulin binding to a high affinity-binding region of NHE-1 that functions as an autoinhibitory domain at basal $[Ca^{2+}]_i$. Our studies provide evidence that Ca^{2+} may alternatively induce activation of NHE indirectly, through pathways involving tyrosine phosphorylation. Calcium could conceivably activate tyrosine kinases and/or inhibit tyrosine phosphatases. It should be noted that the magnitude, pattern, or spatial localization of the increase in $[Ca^{2+}]_i$ resulting from different stimuli may be important in signaling activation of NHE. This might explain why cross-linking of β_2 integrins, despite inducing an increase in $[Ca^{2+}]_i$ (Ng-Sikorski et al., 1991; Waddell et al., 1994), was unable to activate NHE (Fig. 1 *c*).

Our results link activation of NHE-1 during phagocytosis to signaling pathways dependent on tyrosine phosphorylation. Evidence in support of the importance of tyrosine phosphorylation in signaling pathways in neutrophils has been provided by several recent studies (Naccache et al., 1990; Connelly et al., 1991; Dusi et al., 1994*a*; Gaudry et al., 1992). It is important to note that a recent report has suggested that tyrosine kinases regulate Na^+/H^+ exchange in intestinal cells (Donowitz et al., 1994). However, our results are the first to link tyrosine kinases to NHE activation initiated by ligation of FcγRII receptors in neutrophils. Evidence supporting this notion includes (*a*) the enhanced tyrosine phosphorylation of cellular proteins during phagocytosis or after FcγRII cross-linking, and (*b*) the observed inhibition of tyrosine phosphorylation and cytosolic alkalinization by the tyrosine kinase inhibitors, genistein and herbimycin. Tyrosine phosphorylation of the exchanger itself could not be studied because of our inability to immunoprecipitate NHE-1. It is possible that a tyrosine kinase(s) may be situated upstream of the exchanger in the signaling pathway. One attractive possibility is that a member of the *src* family of tyrosine kinases such as *fgr*, which is known to be associated with FcγRII in neutrophils and to be phosphorylated on tyrosine and activated after cross-linking this receptor (Hamada et al., 1993), is involved in activation of the exchanger. Other possible intermediates that are tyrosine phosphorylated include the adaptor protein Shc (Cutler et al., 1993) and MAP kinase (Grinstein and Furuya, 1992; Worthen et al., 1994*b*). The intermediates involved in this signaling pathway are the subject of current investigations.

The current studies suggest that β_2 integrins do not play a primary role in activation of Na^+/H^+ exchange activity during phagocytosis. Rather in neutrophils, β_2 integrins appear to function in a cooperative manner with Fcγ receptors by two different mechanisms: by increasing the efficiency of presentation of IgG bound to the surface of the phagocytic particle and perhaps by provision of a positive signal. Such a cooperative effect between members of the β_2 integrin family such as CR3 and Fcγ receptors has been the subject of recent reports (Sehgal et al., 1993; Zhou et al., 1993; Zhou and Brown, 1994). The failure of β_2 integrins to directly activate Na^+/H^+ exchange is in contrast to other cell types where β_1 and β_2 integrins have been demonstrated to activate this function. For example, studies in fibroblasts (Schwartz et al., 1989) and endothelial cells (Ingber et al., 1990) indicate that pH_i was higher when cells were adherent as compared to those grown in suspension. Subsequent studies revealed that cross-linking of integrins was central to the alkalinization response, specifically, that pH_i changes were attributed to clustering of the β_1 integrin $\alpha_5\beta_1$ induced by fibronectin (Schwartz et al., 1991*a*) or by ligation of CD11a/CD18 in T-lymphocytes (Schwartz et al., 1991*b*). The reason for this difference in behavior between the cell types is not known but may be due to differences in the intracellular signaling pathways used in different cell types.

In conclusion, we have demonstrated that the NHE-1 isoform of Na^+/H^+ exchangers is expressed in mature human neutrophils. However, alternate isoforms may be expressed and their characterization is the subject of ongoing investigations. NHE-1 appears to be responsible for maintenance of intracellular pH in two circumstances of physiological importance: compensation from an external acid load and during phagocytosis where endogenously generated acid equivalents are generated during the oxidative burst. Activation of the exchanger during phagocytosis occurs by a pathway involving FcγRII receptors and tyrosine phosphorylation of unknown intermediates. While FcγRII

is the receptor primarily responsible for initiation of the signaling pathways leading to NHE activation, β_2 integrins (CD11/CD18 including CR3) appear to participate in a cooperative manner, perhaps by facilitating presentation of surface bound IgG to the Fcγ receptor. These observations highlight the importance of this exchanger in these physiological processes of phagocytic cells and begin to decipher the pathways involved in its regulation.

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