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Research Article

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Proton Secretion by the Sodium/Hydrogen Ion Antiporter in the Human Neutrophil

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Abstract

The reducing equivalents used by the human neutrophil respiratory burst oxidase are derived from NADPH generated by the hexose monophosphate shunt. The CO_2 generated by the HMP shunt is spontaneously hydrated and the protons (H^+) are secreted upon the dissociation of carbonic acid. The mechanism and significance of H^+ secretion by the resting and stimulated neutrophil was investigated. A basal rate of H^+ secretion by resting neutrophils observed in a choline buffer was augmented with the addition of sodium (Na^+) (K_m for Na^+ was 3.22±0.32 mM). Amiloride, a Na^+/H^+ antiporter inhibitor, reduced H^+ secretion in Na^+-containing buffers with a K_i = 1.02 μM. This Na^+/H^+ exchange mechanism was also operative in cells stimulated with a variety of agonists, and an increased H^+ flux, relative to resting cells, was observed at higher Na^+ concentrations.

Cytoplasts incorporating acridine orange were also used to assess Na^+-H^+ flux. Cytoplasts were used to avoid alteration of the fluorescent pH probe by HOCl formed in intact neutrophils. Alkalinization of the cytoplasm was dependent on extracellular Na^+ in concentrations similar to that found to augment H^+ secretion in intact cells. Also, amiloride competitively inhibited H^+ secretion by the cytoplasts.

Both superoxide (O_2^-) production and lysozyme release in cells stimulated with opsonized zymosan or concanavalin A was significantly inhibited in the absence of Na^+, restored to normal with the addition of Na^+ in low concentrations, and inhibited again in the presence of amiloride. A Na^+/H^+ antiporter similar to that found in other cell types is present in the human neutrophil and appears linked to activation of the respiratory burst and degranulation.

Introduction

The human neutrophil, when stimulated by various agonists, reduces molecular oxygen in a cyanide-insensitive respiratory burst to form reactive oxygen species (1). The respiratory burst oxidase has been characterized as a membrane-bound NADPH-oxidase, which generates the one electron reduction product, superoxide (O_2^-) (2). The reducing equivalents are derived from the reduced pyridine nucleotide, NADPH, which is generated in the concomitant activation of the hexose monophosphate (HMP)[1] shunt (2). Previous studies from this laboratory have demonstrated that the activity of the HMP shunt may be ascertained by the quantitation of CO_2 production, and that the ratio of CO_2 formed to oxygen consumed is near unity (3). The CO_2 is spontaneously hydrated and the protons (H^+) are secreted upon the dissociation of carbonic acid. While resting cells secrete protons at a lower rate, the source in this instance is lactate and not the HMP shunt (3, 4).

In this study, the mechanism of H^+ secretion in the human neutrophil was examined. The importance of cation exchange was suggested by the previous findings that substitution of isotonic glucose for sodium (Na^+) markedly depressed O_2^- production in neutrophils stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP) (5). Further, replacement of Na^+ with K^+ or choline depressed O_2^- production, lysosomal enzyme release, and the hyperpolarization response in concanavalin A (Con A) or immune complex-stimulated neutrophils (6). A recent report has demonstrated that extracellular Na^+ regulates the respiratory and secretory threshold response to FMLP (7), which is likely due to modulation of receptor sensitivity (8). A ouabain-sensitive Na^+ electrogenic pump has been reported in the neutrophil, which correlates with neutrophil membrane hyperpolarization and computed membrane conductance, but the role of this pump in neutrophil stimulus-coupled activation pathways is undefined (9). A Na^+-coupled mechanism for active H^+ secretion has been suggested in studies with rabbit neutrophils, but the mechanism has not been further elucidated (10). Amiloride-sensitive Na^+/H^+ exchange has been reported in HL-60 cells and has been implicated as important for cellular differentiation (11). Na^+/H^+ exchange has also been reported as important in neutrophils for the normalization of intracellular pH in acid-loaded cells (12); however, the mechanism of exchange has not been characterized, nor has its role in the respiratory burst activation been investigated.

In our study of augmented H^+ secretion of stimulated neutrophils, we have endeavored to determine the transport mechanism, and here propose that it involves the same Na^+/H^+ antiporter characterized in the renal microvillus membrane (13). In the microvillus preparation the antiporter is electroneutral, with a Na^+/H^+ coupling ratio of 1.0, and mediates no net fluxes of Na^+ and H^+ whenever the respective transmembrane gradients are balanced. The K_m for Na^+ in this preparation is 6–13 mM, and the exchange is specifically inhibited by amiloride at low concentration. The secretion of H^+ associated with the respiratory burst of the human neutrophil is here examined to de-

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1. *Abbreviations used in this paper:* Con A, concanavalin A; Con A FITC, Con A fluorescein isothiocyanate; FMLP, formyl-methionyl-leucyl-phenylalanine; HMP, hexose monophosphate; OZ, opsonized zymosan; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene; SOD, superoxide dismutase.

termine whether electroneutral exchange with Na^+ is a significant mechanism of H^+ flux in the activated cell. Further, we have examined the importance of this exchange in the function of the stimulated neutrophil.

Methods

Ouabain, choline chloride, α-methylmannoside, phorbol myristate acetate, trypan blue, superoxide dismutase (SOD), Con A, Con A fluorescein isothiocyanate (Con A FITC), cytochalasin B, acridine orange, zymosan and monensin were purchased from Sigma Chemical Co. (St. Louis, MO), and the other reagents, 5,6-carboxyfluorescein (Molecular Probes, Junction City, OR), 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS), and valinomycin (Calbiochem-Behring Corp., La Jolla, CA), were obtained as listed. Amiloride HCl and dimethylamiloride were kindly provided by Merck Sharp & Dohme Div., Merck & Co., Inc., West Point, PA.

Neutrophils were isolated from healthy volunteers by red cell sedimentation in Dextran-citrate, followed by hypotonic lysis and Ficoll-Hypaque density centrifugation as previously described (3). For studies in Na^+-free buffers, the neutrophils were suspended in 145 mM choline chloride, 5 mM glucose, 1 mM K_2HPO_4, 0.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$, pH 7.42. Sodium chloride (NaCl) was added as indicated. The physiological control buffer (phosphate-buffered saline [PBS]) consisted of 145 mM NaCl and like concentrations of phosphate, calcium, and magnesium as used above (2). The Na^+-free buffers were checked for Na^+ content by flame photometry, and Na^+ was never detectable.

Proton secretion. Cells at a concentration of 5×10^6 cells/ml were added to 8 ml of indicated buffer and were incubated at 37°C with constant stirring in a YSI model 5301 bath stirrer assembly (Yellow Springs Instruments, Yellow Springs, CO) connected to a Haake Water circulator (Haake Instruments, Rochelle Park, NJ). A pH titration unit with a model PHM 62 pH-meter (Radiometer, Copenhagen, Denmark) maintained pH at 7.42 by automatic titration with 0.015 N NaOH delivered from an autoburette unit. H^+ secretion was quantitated from the amount of NaOH titrated to maintain the pH at 7.42 as previously described (3). Proton secretion in stimulated cells was measured after 3 min of preincubation in designated buffers with opsonized zymosan (OZ) (1.5 mg/ml), or Con A (100 μg/ml). In cells stimulated with Con A, the cells were preincubated with 5 μg/ml cytochalasin B.

Cytoplasts. Cytoplasts were made exactly as described by Roos et al. (14), which necessitated minimal modification of our usual procedure for isolation of neutrophils. Alkaline phosphatase and myeloperoxidase were assessed as previously described (15), with results similar to those reported (14). Cytoplasts from 2 U of blood were incubated for 1 h at 4°C in PBS, pH 6.0, washed three times in a solution of 200 mM sucrose, 10 mM Hepes, and 10 mM Tris base, pH 6.0, and then resuspended in the same buffer to a protein concentration of 25 mg/ml. Acridine orange was added to a final concentration of 0.6 μM in 2 ml of the same sucrose buffer but adjusted to pH 7.5. Fluorescence was measured at 37°C in a Perkin-Elmer fluorescence spectrophotometer (model 650-10S), using an excitation wavelength of 493 nm and an emission wavelength of 530 nm (16). After equilibration of the signal, 25 μl of the cytoplast preparation were added under constant stirring and an immediate decline in the signal was observed. Again, after equilibration, various concentrations of NaCl were added and the initial slope of the subsequent increase in emission (signifying alkalinization) was recorded. The experiments were done with and without sodium azide at 10^{-4} M to eliminate any possible change in the dye signal due to the effects of HOCl produced by the myeloperoxidase system (17). Experiments were also done with various Na^+ concentrations in samples preincubated with 50 μM amiloride. To rule out the possible contribution of Cl^--dependent H^+ secretion, the change in fluorescence was noted after addition of 25 mM sodium cyclamate to the same buffer, and compared with addition of the same concentration of NaCl. To insure that the process observed was electroneutral, cytoplasts were suspended in a high K^+ buffer (100 mM KCl, 10 mM Hepes, and 10 mM Tris base, pH 7.5) and alkalinization was observed with addition of 25 mM NaCl in the presence and absence of 5 μg/ml valinomycin.

Neutrophil functional assays. The generation of O_2^- by the SOD-inhibitable reduction of cytochrome *c* was measured by the continuous method previously described (2). Stimuli employed were OZ (1.5 mg/ml) and Con A (100 μg/ml) under conditions identical to those employed for the proton secretion studies. The respiratory burst NADPH-oxidase was harvested from PMA-stimulated neutrophils and activity measured as detailed previously (2). Oxygen consumption studies were concomitantly performed to verify O_2^- results, according to our described methods (2, 3).

For lysozyme release studies, 5×10^6 cells/ml were preincubated at 37°C with 5 μg/ml cytochalasin B for 5 min and then stimulated with Con A (100 μg/ml). After 15 min at 37°C, the cells were pelleted at 200 *g* for 6 min and lysozyme content was assayed in the supernatant and pellet as previously described (18).

The effect of varying Na^+ concentrations on Con A binding was determined. Con A FITC was dialyzed exhaustively against a Na^+-free choline buffer. Neutrophils (10^7 cells/ml) were incubated in PBS, 0- and 10-mM NaCl choline buffers, pH 7.5, at 37°C for 10 min, after addition of Con A FITC (100 μg/ml). The cells were pelleted, washed once with buffer, resuspended, and counted again. Fluorescence was observed to be quenched when the Con A FITC bound, hence the cells were then sonicated, solubilized with Triton X-100 (0.2% vol/vol) for 5 min and incubated with 220 mM α-methylmannoside for 15 min. This preparation was centrifuged at 10,000 *g* for 5 min and the fluorescence of the supernatant measured with excitation 495 nm and emission 520 nm.

Monensin studies. Neutrophils were suspended to 5×10^7 cells/ml in PBS, pH 7.2, containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. 5,6-carboxyfluorescein was dissolved in dimethyl sulfoxide (DMSO) and then added to the cell suspension to a concentration of 50 μM and the mixture incubated for 30 min at 22°C. The cells were pelleted at 200 *g* for 6 min, resuspended in PBS, and stored at 4°C. Using a Perkin-Elmer 650-10S fluorescence spectrophotometer, emission at 520 nm was measured using 494 nm for excitation. Monensin (10 mM in ethanol) was added to the saline suspension to a final concentration of 10 μM (6, 19), and the increase in emission fluorescence (signifying cell alkalinization) was recorded. Using the same method as described above, O_2^- generation was measured with addition of monensin (10 μM) to cells in the saline buffer. Control experiments using ethanol were also performed.

Results are expressed as means±SEM, and differences were analyzed by the paired *t* test and concluded to be statistically different at $P < 0.05$. Intercepts of regression lines were compared by analysis of covariance using the method suggested by Zerbe et al. (20).

Results

Proton secretion. In Na^+-free media, resting neutrophils secrete H^+ at a constant rate of 0.60±0.10 fmol/cell per min. When Na^+ (20 mM) is added to the media, the rate of H^+ secretion increased by 1.10±0.12 fmol/cell per min ($n = 6$, $P < 0.01$). To further characterize the Na^+ dependency of H^+ secretion by neutrophils we examined the effect of progressive increments in media Na^+ concentrations (1–10 mM) on the augmentation of H^+ secretion. These results are presented in Fig. 1 as a double-reciprocal plot (Na^+-independent effects subtracted). The enhancement of H^+ secretion by Na^+ follows first order kinetics. The calculated K_m for Na^+ in resting cells is 3.22±0.32 mM, and the V_{max} for H^+ secretion is 1.14±0.10 fmol/cell per min.

When neutrophils in Na^+-free media are incubated with OZ, H^+ secretion is increased from 0.60±0.10 to 2.20±0.11 fmol/cell per min ($n = 5$, $P < 0.001$). In the presence of physiological concentrations of Na^+ (140 mM), zymosan had even a greater stimulatory effect on H^+ secretion. It increased from 1.56±0.12 to 4.24±0.12 fmol/cell per min ($n = 6$, $P < 0.01$). This obser-

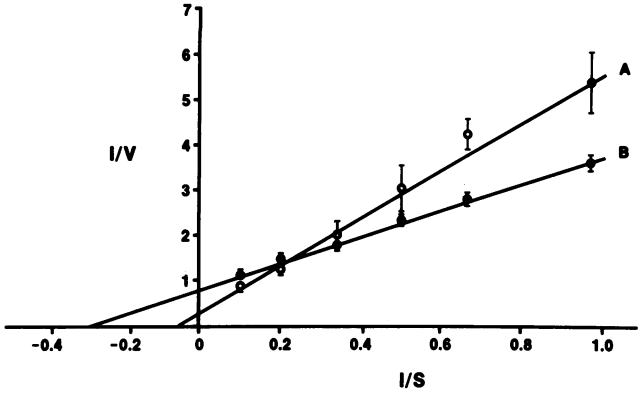

Figure 1. Sodium dependence of neutrophil proton secretion. Double reciprocal plot of the rate of Na^+-dependent media acidification by (*A*) zymosan-stimulated or (*B*) resting neutrophils vs. media Na^+ concentration. *V* is expressed as femtomoles H^+ per cell per minute and *S* as Na^+ in meq. The lines were drawn by linear regression analysis. The equation for line *A* is: $Y = (0.33\pm0.04) + (5.23\pm0.07)$ (*X*); ($r = 0.84$, $n = 47$, $P < 0.01$), and for line *B*: $Y = (0.88\pm0.01) + (2.83\pm0.02)$ (*X*); ($r = 0.93$, $n = 47$, $P < 0.01$).

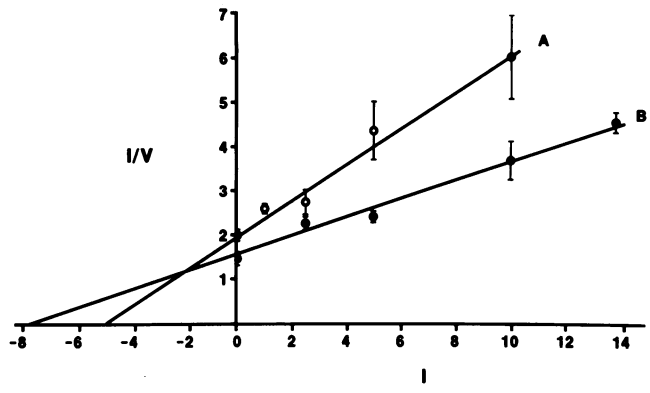

Figure 2. Amiloride inhibition of neutrophil proton secretion. The inverse of the rate of Na^+-dependent media acidification by resting neutrophils vs. media amiloride concentration, at two concentrations of Na^+. *V* is expressed as femtomoles H^+ per cell per minute and *I* as micromoles of amiloride. The lines were drawn by linear regression analysis. Line *A* represents 4 mM NaCl-containing buffer and line *B* 8 mM NaCl. The equation for line *A* is: $Y = (2.03\pm0.09) + (0.41\pm0.02)$ (*X*); ($r = 0.76$, $n = 23$, $P < 0.01$), and for line *B*: $Y = (1.54\pm0.03) + (0.21\pm0.01)$ (*X*); ($r = 0.92$, $n = 23$, $P < 0.01$).

vation suggested to us that zymosan not only stimulates Na^+-independent but also Na^+-dependent H^+ secretion. To characterize the kinetics of Na^+-dependent portion of H^+ secretion in zymosan-treated cells, we also determined the effect of progressive increments in media Na^+ on H^+ secretion by stimulated neutrophils (Fig. 1). These studies indicate that V_{max} for H^+ secretion increased to 2.99 ± 0.42 fmol/cell per min and the K_m for Na^+ increased to 15.65 ± 0.62 mM ($n = 6$, $P < 0.05$). Con A-stimulated cells also showed an increase in Na^+-dependent H^+ secretion (data not shown).

These initial studies demonstrate that there is a moiety of H^+ secretion in both resting and stimulated neutrophils that is Na^+ dependent. Based on these observations we would suggest that there is a Na^+/H^+ antiporter in the plasma membrane of these cells. If this proposal is correct, amiloride, a known competitive inhibitor of this antiporter (21), should reduce the Na^+-dependent rate of acidification. Amiloride at 5 μM reduced the Na^+ (10 mM) augmented rate of acidification of resting and zymosan-stimulated neutrophils by 59 ± 1 and 54 ± 2%, respectively ($n = 11$, $P < 0.05$), and at 50 μM amiloride, H^+ secretion was reduced by >90%. In Fig. 2, the effect of amiloride (0–15 μM) on the Na^+ (4 or 8 mM) augmented rate of H^+ secretion is shown. The K_i for amiloride derived from Fig. 2 is 1.02 ± 0.04 μM in resting cells and similar values were obtained in zymosan-stimulated cells. The characteristics of the inhibitory effects of amiloride were also consistent with competitive antagonism. At 10 μM amiloride, the K_m for Na^+ in resting cells was increased from 3.22 ± 0.32 to 11.2 ± 0.61 mM ($n = 5$, $P < 0.05$), but V_{max} was not significantly altered.

The effect of amiloride and its more potent analogue, dimethylamiloride, were studied at physiological Na^+ concentrations, since the above studies were all done in low Na^+ media. Given the inhibition characteristics previously determined, one would not expect amiloride, in the same concentrations, to affect Na^+/H^+ exchange to a significant degree; however, dimethylamiloride appears in other cell types (22) to be approximately tenfold more potent than the parent compound; hence, some inhibition would be anticipated. The percent inhibition of the Na^+-dependent H^+ secretion by neutrophils in physiological Na^+ buffer (145 mM) was determined at 100- and 400-μm concentrations of the inhibitors. In resting cells, 100 μm dimethylamiloride achieved a 68.6 ± 10.3% inhibition, as opposed to 25.7 ± 2.3% for amiloride; in OZ-stimulated cells this concentration achieved 85.8 ± 8.9 and 36.0 ± 11.9% inhibition, respectively ($n = 4$). At 400 μm, inhibition seen in resting cells with dimethylamiloride was 78.4 ± 6.7%, with amiloride 45.4 ± 13.2%; in OZ-stimulated cells it was 119.4 ± 11.5 and 73.8 ± 18.8%, respectively ($n = 5$). Under all conditions, the differences between amiloride and the analogue were significant at the $P < 0.05$ level. This supports the finding of increased potency of the dimethylamiloride analogue seen in other cell types, and also demonstrates the importance of the Na^+/H^+ antiporter at physiological Na^+ concentrations. At these higher concentrations, neither amiloride nor dimethylamiloride altered cell viability significantly as assessed by trypan blue exclusion. Neither ouabain (100 μM), an Na^+-K^+ ATPase inhibitor, nor SITS (500 μM), an anion channel blocker, reduced H^+ secretion by resting or stimulated cells.

Cytoplast alkalinization. An alternative method to assess the presence of the Na^+/H^+ antiporter in neutrophils is to demonstrate that there is Na^+-dependent alkalinization of the cell. In these studies we used cytoplasts which are, in essence, large plasma membrane vesicles devoid of the nucleus and granules (14). These were used to avoid alteration of the fluorescent pH probe by HOCl formed by intact neutrophils in a myeloperoxidase-catalyzed reaction (17). The change in cytoplast pH was determined by the quench of acridine orange fluorescence (16). A representative experiment is presented in Fig. 3. Cytoplasts equilibrated to pH 6.0 were added to a cuvette containing Na^+-free media (pH 7.5) and acridine orange, and the fluorescence was continually monitored in a spectrofluorometer. With the addition of cytoplasts there was a rapid fall in fluorescence, achieving a stable value within 2 min. The decrement in fluorescence indicates that the interior of the cytoplasts are at a lower pH than the external media. When Na^+ was added there was an increase in fluorescence (alkalinization of the cytoplast). The initial rate of change in fluorescence after Na^+ addition is a function of the amount of Na^+ added to the system (Fig. 3).

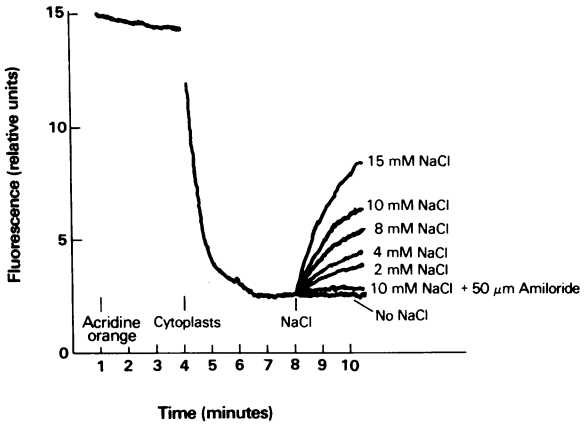

Figure 3. Representative experiment showing Na^+/H^+ exchange in cytoplasts. Cytoplasts were equilibrated in a pH 6.0 buffer and added (final concentration, 0.6 mg protein/ml) to a buffer containing acridine orange (0.6 μM), pH 7.5. A rapid quench in fluorescence was observed, but upon addition of NaCl a time-dependent rise in fluorescence (signifying cytosolic alkalinization) occurred. The Na^+-dependent response was ablated when the cytoplasts were also incubated with 50 μM amiloride.

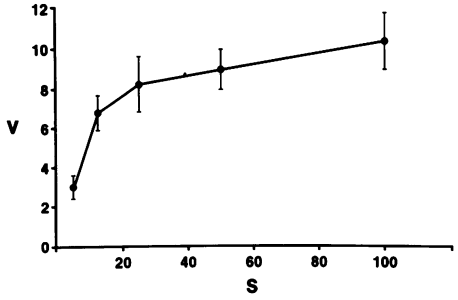

Figure 5. The rate of cytoplast alkalinization vs. media Na^+ concentration. V represents the rate of change of fluorescence in the first two minutes, and S the concentration of Na^+ in millimoles, $n = 5$. The rate of alkalinization did not significantly increase above 25 mM Na^+.

The addition of 50 μM amiloride to the cytoplasts markedly reduced the alkalinization induced by Na^+ addition. In Fig. 4 is presented a double-reciprocal plot of the rate of cytoplast alkalinization (the initial rate of increased fluorescence) vs. the concentration of Na^+. The estimated K_m for Na^+ by the cytoplasts, 6.57±0.57 mM, was not significantly different from that of the intact neutrophils. When these studies were performed in the presence of 10^{-4} M azide, an inhibitor of myeloperoxidase, similar results were obtained, indicating that the small contamination with myeloperoxidase had no effect on fluorescent measurements (17). In a separate group of cytoplast preparations, we also determined the rate of Na^+-dependent alkalinization over a wider range of Na^+ concentrations (Fig. 5) than was used for the kinetic studies (Fig. 3). At Na^+ concentrations > 25 mM, the rate of cytoplast alkalinization did not significantly increase, demonstrating saturation of the antiporter.

To rule out the possible contribution of Cl^--dependent H^+ transport pathways, Na^+-dependent alkalinization was also evaluated in the same Cl^--free buffer. The change in fluorescence with addition of 25 mM sodium cyclamate (30.7±1.6 fluorescence U/50 μg protein per min, $n = 5$) was not significantly different from that seen after addition of 25 mM NaCl (29.7±1.5 fluorescence U/50 μg protein per min, $n = 5$). To insure that the Na^+-dependent alkalinization noted above was an electroneutral process, the change in fluorescence upon addition of 25 mM NaCl was observed in a high K^+ buffer, with and without valinomycin (5 μg/ml). No significant difference was observed between valinomycin-free (20.3±4.9 fluorescence U/50 μg protein per min, $n = 5$), and valinomycin-containing media (18.8±1.2 fluorescence U/50 μg protein per min, $n = 5$).

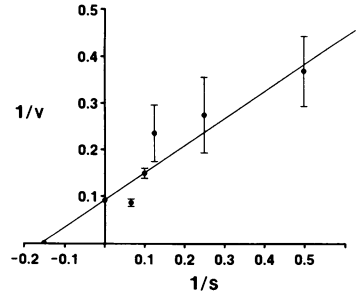

Figure 4. Double-reciprocal plot of the rate of cytoplast alkalinization vs. media Na^+ concentration. V is expressed as the initial rate of change in fluorescence and S as Na^+ in millimolars. The line was drawn by linear regression analysis. The equation is $Y = (0.09\pm0.02) + (0.60\pm0.11)(X)$; ($r = 0.79$, $n = 20$, $P < 0.05$).

Antiporter function in the stimulated neutrophil. The Na^+/H^+ antiporter may be an important regulator of the respiratory burst function of neutrophils stimulated by a variety of agonists. To evaluate the role of the Na^+/H^+ antiporter in this process we determined the effect of various Na^+ and amiloride concentrations on O_2^- production by stimulated neutrophils (Fig. 6). The rate of O_2^- generation by neutrophils incubated in physiologic control buffer (PBS) was compared with that of neutrophils incubated in (*a*) Na^+-free choline buffer; (*b*) 10 mM Na^+-containing choline buffer; (*c*) 10 mM Na^+-containing choline buffer with 50 μM amiloride; and (*d*) PBS with 50 μM amiloride. In the absence of Na^+, O_2^- production was significantly reduced (30% of control buffer) in neutrophils stimulated with zymosan or Con A. In 10 mM Na^+-containing media, the rate of O_2^- production was not different than control with both agonists. However, when 50 μM amiloride was added to this low Na^+ media, O_2^- production was reduced to the same level as that in Na^+-free media. This concentration of amiloride in low Na^+-media was shown in the above studies to completely inhibit Na^+-dependent H^+ secretion by neutrophils. The presence of 50 μM amiloride in PBS did not significantly affect O_2^- generation in cells stimulated by OZ or by Con A (Fig. 6).

The effect of dimethylamiloride in a physiologic buffer was again compared with amiloride at 100- and 400-μm concentrations. In OZ-stimulated cells, 100 μm dimethylamiloride decreased O_2^- production by 68.3±19.1% compared with 39.3±10.8% for amiloride; at 400 μm, 102.0±16.6% compared with 82.5±17.1% was observed ($n = 4$, $P < 0.05$). Thus, inhibition of Na^+-dependent H^+ secretion by removal of extracellular Na^+, addition of amiloride at low concentrations of extracellular Na^+, or addition of a potent amiloride analogue to physiological Na^+ buffers, markedly reduces O_2^- production by stimulated neutrophils. In parallel studies of burst activation, the measurement of oxygen consumption exhibited the same Na^+ dependence as seen in the O_2^- assays (data not shown). These findings cannot be explained by an inhibitory effect of amiloride on the NADPH-oxidase. The respiratory burst enzymatic activity is assessed by the SOD-inhibitable reduction of cytochrome *c* in a 27,000 *g* particulate preparation harvested from zymosan-stimulated

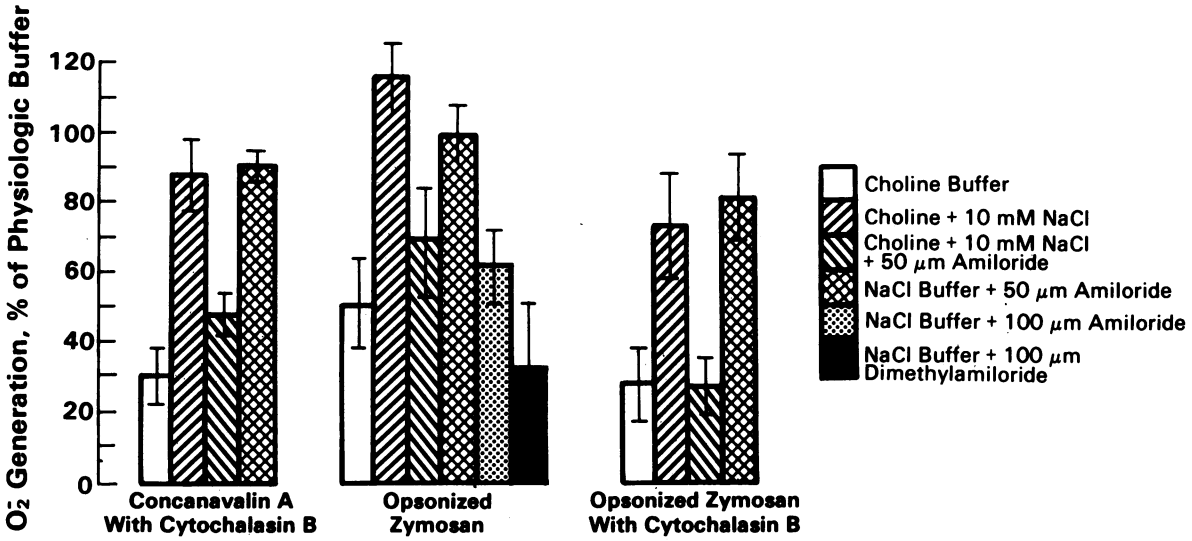

Figure 6. Effect of amiloride and various extracellular Na^+ concentration on O_2^- generation by Con-A and zymosan-stimulated neutrophils. The results obtained in different buffers are expressed as a percentage of that obtained in PBS (nanomoles cytochrome *c* reduced per minute per 10^6 cells, mean±SEM, $n = 5$; Con A = 10.05±0.71; OZ = 16.38±3.12, OZ with cytochalasin b = 5.95±1.87). A difference at the $P < 0.05$ level is observed between (*a*) O_2^- generated in choline alone and choline with 10-mM NaCl buffers, and (*b*) O_2^- generated in choline with 10 mM NaCl, and that same buffer with the addition of 50 μM amiloride. Amiloride at 50 μM had no significant effect in physiologic buffers, but 100 μm amiloride and 100 μm dimethylamiloride resulted in inhibition as shown.

neutrophils (2). In our standard Na^+-free choline buffer, the addition of 100 μM amiloride did not significantly alter the NADPH-oxidase activity (control: 8.68±2.20 vs. choline buffer: 7.52±2.20 nmol O_2^-/mg per min, mean±SD, $n = 5$); thus, an effect on the assay of O_2^- or the oxidase directly cannot account for the inhibitory effect of amiloride. Con A binding was not found to be significantly altered by changes in extracellular Na^+ (data not shown).

The role of the antiporter in degranulation was investigated by measuring release of lysozyme from Con A-stimulated cells. The marked inhibition of release in Na^+-free media (41.9±7.5% of physiologic media, $n = 6$) could be corrected to near normal levels (80.9±6.7%, $n = 6$) with addition of 10 mM NaCl, but again inhibited (49.7±5.6%, $n = 6$) in the presence of 50 μM amiloride. No effect of 50 μM amiloride was seen in the physiologic buffer, again supporting a competitive inhibiting role for amiloride. This degranulation response was similar to O_2^- generation in various concentrations of Na^+, and in the presence of amiloride as shown in Fig. 6.

Effect of monensin on the respiratory burst. We interpret our data as demonstrating that the respiratory burst of stimulated neutrophils requires an active Na^+/H^+ antiporter. However, since a cascade of events is involved upon stimulation of the cell, we sought to determine the effect of Na^+/H^+ exchange alone on the respiratory burst. Monensin mediates Na^+/H^+ exchange, and hence, upon its addition, cell alkalinization necessarily occurs with Na^+ influx. Neutrophils that had been incubated with the pH probe carboxyfluorescein were suspended in PBS, and a consistent rise in fluorescence was noted with addition of monensin (10 μM), signifying cell alkalinization. However, under the same conditions, no O_2^- generation could be detected, in accord with a previous report (6). This suggests that while extracellular Na^+ is necessary for a normal respiratory burst, its influx into the cell under these conditions is inadequate by itself to initiate NADPH-oxidase activation.

Discussion

The protons secreted by the human neutrophil are derived from at least two sources: in the resting cell, lactic acid, and in the stimulated cell, primarily from the HMP shunt, by the dissociation of hydrated CO_2 ($H_2CO_3 \rightarrow H^+ + HCO_3^-$) (3, 4). In this study, we have characterized an important mechanism of H^+ secretion in the neutrophil as the amiloride-sensitive Na^+/H^+ antiporter. In the absence of Na^+, minimal H^+ secretion does occur, but this rate of secretion increases markedly with addition of millimolar concentrations of Na^+. The K_m for Na^+, the K_i for amiloride, the electroneutrality and the insensitivity to ouabain, and SITS strongly support our hypothesis that this antiporter is the same as described in the renal microvillus and in several other tissues (13, 23). The studies with cytoplasts used an entirely different method of measuring H^+ flux—the pH-dependent change in fluorescence of acridine orange. By this method, similar Na^+ dependence and sensitivity to amiloride was observed, thus corroborating the finding of the Na^+/H^+ antiporter in the intact cell.

In the stimulated neutrophil, the Na^+/H^+ antiporter responds with an apparent increased V_{max} and a rise in the K_m for Na^+. With cell stimulation, a host of metabolic changes rapidly occur (24) including pH change, Na^+ influx, protein phosphorylation, and fusion of granules with the plasma membrane. All of these events, with others, may change the ionic gradients and the number and efficiency of the antiporter units in the membrane, contributing to the change in the kinetics of the system (13, 25). Which entities are critical are yet to be defined.

With the complex metabolic events of the stimulated neutrophil, it is not surprising that a considerable rise in Na^+-independent H^+ secretion is also seen. This may reflect increased lactate production, as well as activation of a magnesium-dependent electrogenic proton-translocating H^+-ATPase (26). Despite these other mechanisms, a Na^+-dependent and amiloride-sen-

sitive pathway for H^+ secretion is important in the stimulated neutrophil.

The functional role of the Na^+/H^+ antiporter has been initially characterized in these studies with the antiporter inhibitor, amiloride. When extracellular Na^+ is varied, receptor function may be altered (as recently demonstrated with neutrophils stimulated with FMLP [8]), and discernment of antiporter function from effects on receptor-ligand binding may not be made. In our studies of Con A-stimulated cells, decreased binding could not be involved as explaining the inhibitory effects seen with amiloride or in low Na^+-containing buffers. Amiloride at 50-μM concentrations effectively inhibited O_2^- generation in low Na^+-containing buffers, when cells were stimulated with Con A or OZ. At physiologic Na^+ concentrations with higher amiloride concentrations, and with dimethylamiloride, this inhibition was again observed, supporting a competitive inhibitory role for amiloride in this process. Degranulation also appears to be dependent upon a functional Na^+/H^+ antiporter. In Con A-stimulated cells, both absence of Na^+ or presence of amiloride in low Na^+ media markedly inhibited lysozyme release, whereas amiloride had no effect in physiologic Na^+ concentrations. Hence, the Na^+/H^+ antiporter appears to be a crucial entity for normal degranulation and respiratory burst activation in the human neutrophil.

Given the sensitivity of the respiratory burst to extracellular Na^+ concentration and to amiloride at low Na^+ concentrations, it might be expected that Na^+/H^+ exchange initiated by an ionophore such as monensin would stimulate the burst. However, while mediating Na^+/H^+ exchange to a similar extent as observed with OZ or Con A, monensin was unable by itself to activate O_2^- production. Extracellular Na^+ then is crucial for normal cell activation, but its influx, with resultant intracellular alkalinization, is insufficient alone. This supports a secondary role for the Na^+/H^+ antiporter (27), a modulator but not an initiator of cell activation. How the antiporter modulates the respiratory burst is unclear, however its contribution to the acid base homeostasis in the cell is significant; hence, it may affect cellular function by its role in maintaining intracellular pH, which in many systems modifies cell function (28, 29).

Amiloride has been shown to block Na^+/Ca^{++} exchange in erythroleukemia cells (30) and in microsomes of rat cerebral cortex (31, 32); hence, theoretically amiloride could mediate its effect on the respiratory burst through its direct effects on calcium flux. However, these previous studies show that only at high amiloride concentrations (1–2 mM) is the Na^+/Ca^{++} exchange affected. Hence, the concentrations of amiloride found in our study to inhibit O_2^- generation are consonant with blocking Na^+/H^+ exchange rather than directly effecting Na^+/Ca^{++} exchange.

By the K_m for Na^+ and inhibition characteristics of amiloride, a Na^+/H^+ antiporter similar to that in other cells has been defined in the neutrophil. What specific role the Na^+/H^+ antiporter has in the activation sequence of the respiratory burst oxidase and degranulation response is yet to be defined, however, this Na^+/H^+ antiporter appears linked to these functions. Whether the antiporter passively accommodates to the increased H^+ load of the stimulated cell, is directly modified by phosphorylation, as recently shown in HL-60 cells (11), or is modified by phospholipase activation as in fibroblasts (27), are important relationships to define for its functional characterization. While each of these activities are activated in the stimulated neutrophil (24), their respective relations to each other are yet to be elucidated.

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References

1. Badwey, J., and M. L. Karnovsky. 1980. Active oxygen species and the functions of phagocytic leukocytes. *Annu. Rev. Biochem.* 49: 695–726.

2. Light, D. R., C. Walsh, A. M. O'Callaghan, E. J. Goetzl, and A. I. Tauber. 1981. Characteristics of the cofactor requirements for the O_2^--generating NADPH-oxidase of human polymorphonuclear leukocytes. *Biochemistry.* 20:1468–1476.

3. Borregaard, N., J. H. Schwartz, and A. I. Tauber. 1984. Proton secretion by stimulated neutrophils: significance of hexose monophosphate shunt activity as source of electrons and protons for the respiratory burst. *J. Clin. Invest.* 74:455–459.

4. van Zweiten, R., R. Wever, M. N. Hamers, R. S. Weening, and D. Roos. 1981. Extracellular proton release by stimulated neutrophils. *J. Clin. Invest.* 68:310–313.

5. Simchowitz, L., and I. Spilberg. 1979. Chemotactic factor-induced generation of superoxide radicals by human neutrophils: evidence for the role of sodium. *J. Immunol.* 123:2428–2435.

6. Korchak, H. M., and G. Weissmann. 1980. Stimulus-response coupling in the human neutrophil. Transmembrane potential and the role of extracellular Na^+. *Biochim. Biophys. Acta.* 601:180–194.

7. Bianca, V. D., P. Bellavite, P. DeTogni, R. Fumarulo, and F. Rossi. 1983. Studies on stimulus-response coupling in human neutrophils. I. Role of monovalent cations in the respiratory and secretory response to N-formylmethionylleucylphenylalanine. *Biochim. Biophys. Acta.* 755: 497–505.

8. DeTogni, P., V. D. Bianca, P. Bellavite, M. Grzeskowiak, and F. Rossi. 1983. Studies on stimulus-response coupling in human neutrophils. II. Relationships between the effects of changes of external ionic composition on the properties of N-formylmethionylleucylphenylalanine receptors and on the respiratory and secretory responses. *Biochim. Biophys. Acta.* 755:506–513.

9. Simchowitz, L., I. Spilberg, and P. deWeer. 1982. Sodium and potassium fluxes and membrane potential of human neutrophils. Evidence for an electrogenic sodium pump. *J. Gen. Physiol.* 79:453–479.

10. Sha'afi, R. I., T. F. P. Molski, and P. H. Naccache. 1981. Chemotactic factors activate differentiable permeation pathways for sodium and calcium in rabbit neutrophils. Effect of amiloride. *Biochem. Biophys. Res. Commun.* 99:1271–1275.

11. Besterman, J. M., and P. Cuatrecasas. 1984. Phorbol esters rapidly stimulate amiloride-sensitive Na^+/H^+ exchange in a human leukemic cell line. *J. Cell Biol.* 99:340–343.

12. Grinstein, S., and W. Furuya. 1984. Amiloride-sensitive Na^+/H^+ exchange in human neutrophils: mechanism of activation by chemotactic factors. *Biochem. Biophys. Res. Commun.* 122:755–762.

13. Aronson, P. S. 1983. Mechanisms of active H^+ secretion in the proximal tubule. *Am. J. Physiol.* 14:F647–F659.

14. Roos, D., A. A. Voetman, and L. J. Meerrhof. 1983. Functional activity of enucleated human polymorphonuclear leukocytes. *J. Cell Biol.* 97:368–377.

15. Borregaard, N., and A. I. Tauber. 1984. Subcellular localization of the human neutrophil NADPH-oxidase. b-cytochrome and associated flavoprotein. *J. Biol. Chem.* 259:47–52.

16. Lee, M. C., J. G. Forte, and D. Epel. 1982. The use of fluorescent amines for the measurement of pHi; applications in liposomes, gastric microsomes and sea urchin gametes. *In* Intracellular pH: Its Measure-

ment, Regulation and Utilization in Cellular Function. R. Nuccitelli and D. W. Deamer, editors. Alan R. Liss Inc., NY. 135–160.

17. Hurst, J. K., J. M. Albrich, T. R. Green, H. Rosen, and S. Klebanoff. 1984. Myeloperoxidase-dependent fluorescein chlorination by stimulated neutrophils. *J. Biol. Chem.* 259:4812–4821.

18. Klass, H. J., J. Hopkins, G. Neale, and T. J. Peters. 1977. The estimation of serum lysozyme: a comparison of four assay methods. *Biochem. Med.* 18:52–57.

19. DiVirgilio, F., and B. D. Gomperts. 1983. Ionophore monensin induces Na^+-dependent secretion from rabbit neutrophils. Requirement for intracellular Ca^{2+} stores. *Biochim. Biophys. Acta.* 763:292–298.

20. Zerbe, G. O., P. G. Archer, P. Banchero, and A. J. Lechner. 1982. On comparing regression lines with unequal slopes. *Am. J. Physiol.* 242: R178–R180.

21. Benos, D. J. 1982. Amiloride: a molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* 242:C131–C145.

22. L'Allemain, G., A. Franchi, E. Cragoe, Jr., and J. Pouysseger. 1984. Blockade of the Na^+/H^+ antiport abolishes growth factor-induced DNA synthesis in fibroblasts. *J. Biol. Chem.* 259:4313–4319.

23. Ives, H. E., and F. C. Rector. 1984. Proton transport and cell function. *J. Clin. Invest.* 73:285–290.

24. Tauber, A. I., N. Borregaard, E. R. Simons, and J. Wright. 1983. Chronic granulomatous disease: a syndrome of phagocyte oxidase deficiencies. *Medicine.* 62:286–309.

25. Grinstein, S., J. D. Goetz, and A. Rothstein. 1984. Na^+ fluxes in thymic lymphocytes. *J. Gen. Physiol.* 84:585–600.

26. Mollinedo, F., and D. L. Schneider. 1984. Subcellular localization of cytochrome b and ubiquinone in a tertiary granule of resting human neutrophils and evidence for a proton pump ATPase. *J. Biol. Chem.* 259:7143–7150.

27. Vincentini, L. M., R. J. Miller, and M. L. Villereal. 1984. Evidence for a role of phospholipase activity in the serum stimulation of Na^+ flux in human fibroblasts. *J. Biol. Chem.* 259:6912–6919.

28. Busa, W. B., and R. Nuccitelli. 1984. Metabolic regulation via intracellular pH. *Am. J. Physiol.* 246:R409–R438.

29. Tauber, A. I., and E. J. Goetzl. 1979. Structural and catalytic properties of the solubilized superoxide-generating activity of human polymorphonuclear leukocytes. *Biochemistry.* 18:5576–5584.

30. Smith, R. L., I. G. Macara, R. Levenson, D. Housman, and L. Cantly. 1982. Evidence that a Na^+-Ca^{2+} antiport system regulates murine erythroleukemia cell differentiation. *J. Biol. Chem.* 257:773–780.

31. Schellenberg, G. D., L. Anderson, and P. D. Swanson. 1983. Inhibition of Na^+-Ca^{2+} exchange in rat brain by amiloride. *Mol. Pharmacol.* 24:251–258.

32. Swanson, P. D., G. D. Schellenberg, and L. Anderson. 1983. Effects of amiloride and analogue on Na^+-Ca^{2+} exchange in synaptic plasmalemma vesicles and on metabolism in cerebral slices. *J. Neurochem.* 41:S160C.