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Research Article

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Intracellular pH Modulates the Generation of Superoxide Radicals by Human Neutrophils

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Abstract

The relationship of intracellular pH (pH_i) to superoxide radical (O_2^-) generation was investigated in chemotactic factor-stimulated human neutrophils. Exposure of cells to 100 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) caused activation of Na/H exchange which, in 140 mM Na medium (pH_0 7.40), led to a rise in pH_i from 7.22 to 7.80. This pH_i change was sensitive to amiloride (apparent K_i 78 μM), an inhibitor of Na/H countertransport. The time course of the alkalinization was similar to that of FMLP-stimulated O_2^- production, which was complete by 5 min. In the presence of 1 mM amiloride, which nearly blocked the pH_i transient elicited by FMLP, or in the absence of external Na, where intracellular acidification was observed in FMLP-stimulated cells, O_2^- release was still roughly 25–45% of normal. Thus, an alkalinization cannot be an obligatory requirement for O_2^- generation. By independently varying either pH_0, pH_i, or the internal or external concentrations of Na, both the direction and magnitude of the FMLP-induced pH_i transients could be altered. In each instance, the amount of O_2^- release correlated directly with pH_i and was enhanced by intracellular alkalinization.

In the absence of FMLP, a rise in pH_i to 7.7–7.8 by exposure of cells to 30 mM NH_4Cl, 10 μM monensin (a Na/H exchanging ionophore), or after a prepulse with 18% CO_2 did not result in O_2^- generation. Thus, these results imply that an alkalinization per se is not a sufficient trigger. Neutrophils exposed to 4 nM FMLP exhibited a threefold slower rate of alkalinization (reaching $pH_i \sim 7.80$ by 20–30 min) as compared to that obtained with 100 nM FMLP and did not release significant amounts of O_2^- under normal incubation conditions. However, these cells could be induced to generate O_2^- when the degree of alkalinization was enhanced by internal Na depletion or by pretreatment with 18% CO_2. Together, these results indicate a modulating effect of pH_i on O_2^- production and suggest that other functional responses of neutrophils may be regulated by their pH_i.

Introduction

In recent years, the effects of the ionic environment on neutrophil function have received much attention. To varying degrees, random motility, chemotaxis, aggregation, phagocytosis, O_2^- generation, and granule enzyme release have all been shown to be dependent on the extracellular concentrations of K, Na, and/or Ca (1–3). Although an inhibitory effect of high external K has often been attributed to a depolarization of the membrane potential (4, 5), the nature of the ionic requirements for Na and Ca remain largely unexplained. Our group and others (6, 7) have noted a marked reduction in the amount of O_2^- generation induced by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)[1] in human neutrophils as the extracellular concentration of Na, $[Na]_0$, was reduced from 140 mM to 0 by replacement with choline. It has also been observed that exposure of cells to FMLP leads to a striking depolarization of the transmembrane potential as measured by fluorescent cyanine dyes or by radiolabeled triphenylmethylphosphonium ion (5, 8–10). In view of this, alterations in responses consequent to variations in $[Na]_0$ have been ascribed to changes in the Na current (6, 7, 11), analogous to the role of external Na in propagating the action potential in excitable tissues such as muscle and nerve (12).

Recently, Naccache, Sha'afi, and co-workers reported that FMLP stimulates unidirectional in- and effluxes of ^{22}Na in rabbit peritoneal neutrophils (11, 13). The enhanced ^{22}Na fluxes occurred in association with a pH_i transient (i.e., prolonged alkalinization [14]), both of which were blocked by amiloride (15, 16), an inhibitor of Na/H exchange (17). These findings have been confirmed and extended in human neutrophils by our group (6, 18, 19). In these studies (18, 19), we observed that, after exposure of human neutrophils to 100 nM FMLP in 140 mM Na medium, pH_0 7.40, there was an increase in the steady-state intracellular Na concentration, $[Na]_i$, from ~30 to 60 meq/liter cell water and an increase in steady-state pH_i from a resting value of ~7.25 to 7.80. These steady-state values in FMLP-activated neutrophils are those predicted for an equilibrium distribution of Na and H across the cell membrane as mediated by a Na/H exchanger of 1:1 stoichiometry. The distribution, at chemical equilibrium, is governed by the equation:

$$\frac{[Na]_0}{[Na]_i} = \frac{[H]_0}{[H]_i}. \quad (1)$$

It thus appears that the chemotactic factor activates an amiloride-sensitive Na/H countertransport which mediates an alkalinization of pH_i via the net uptake of external Na in exchange for internal H.

The nature of the dependence of several different functional responses on $[Na]_0$ may therefore be due to the ion's serving as a substrate in the Na/H exchange reaction. This led us to investigate whether a pH_i transient, specifically an alkalinization, might be a requirement for or regulate O_2^- production. Considering the interrelationships between Na and H as dictated by Eq. 1, we were able to vary each of the four parameters independently in order to alter both the direction and magnitude of

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1. *Abbreviations used in this paper:* DMO, 5,5-dimethyloxazolidine-2,4-dione; DMSO, dimethylsulfoxide; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

the pH_i changes induced by FMLP. By measuring the release of O_2^- under these conditions, we were able to correlate pH_i responses with the degree of O_2^- generation and therefore, to determine the relationship of pH_i to O_2^- production. We find that, in cells activated by FMLP, an alkalinization of pH_i is not an obligatory requirement for O_2^- generation because substantial O_2^- release (25–45% of total) occurs in the presence of 1 mM amiloride (which nearly blocks the alkalinization, pH_i remaining unchanged at ~7.25) or in the nominal absence of extracellular Na (where pH_i acidifies to ~6.8). In addition, in the absence of FMLP, an alkalinization of pH_i to levels (7.7–7.8) obtained under normal conditions with an optimal dose (100 nM) of FMLP did not result in detectable O_2^- generation. Thus, an alkalinization of pH_i of this degree is not, by itself, a sufficient trigger. However, in cells exposed to 100 nM FMLP, we find that the pH_i appears to modulate the amount of O_2^- production, an alkalinization tending to enhance or potentiate the release of this product of the respiratory burst.

Methods

Incubation media

The standard medium used throughout this study had the following composition: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 5 mM Hepes buffer, pH 7.40, and 1 mg/ml of crystalline bovine serum albumin. To test the effects of varying external Na, media of appropriate $[Na]_0$ were prepared by equimolar substitution of *N*-methyl-D-glucamine for Na. *N*-methyl-D-glucamine, rather than choline, was chosen as the replacement for Na for the following reason. In preliminary studies, we found that even a short (~5 min) exposure to high choline concentrations (≥50 mM) caused marked inhibition of O_2^- release when cells were subsequently resuspended in 140 mM Na medium and stimulated with FMLP. In contrast, cells pretreated in Na-free, 140 mM *N*-methyl-D-glucamine-containing medium for 5 min displayed a normal O_2^- generation response when the cells were then exposed to FMLP in 140 mM Na. This suggests that at least part of the inhibitory effect of choline medium noted above is due, not to the reduction in external Na, but rather to the presence of choline or, perhaps, to a contaminant.

In some instances, the experimental media were buffered with 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pK'_a 6.0) or *N*-tris(hydroxymethyl)methylglycine (Tricine) (pK'_a 7.8) instead of Hepes (pK'_a 7.3), depending upon the extracellular pH (pH_0) of the media (5.9–8.0). Solutions containing 30 mM NH_4Cl were obtained by equimolar substitution of NH_4Cl for NaCl.

CO_2-containing solutions were prepared as follows: a stock solution containing 5 mM Hepes and normal amounts of other ions was gassed with a 20% CO_2/80% O_2 mixture. Sufficient $NaHCO_3$ (109 mM, replacing NaCl) was added to bring the pH_0 to 7.40. This stock was anaerobically diluted with different volumes of standard medium (pH_0 7.40) also containing 5 mM Hepes, in order to obtain solutions of different CO_2 concentrations (5 and 18%), but identical pH_0. The tubes containing these solutions, which were overlaid with mineral oil, were capped during the cell incubation.

Cell isolation

Neutrophils were isolated from heparinized blood of normal donors by the standard procedure of dextran sedimentation at 37°C followed by Ficoll-Hypaque gradient centrifugation at room temperature (20). The cellular pellet was resuspended for 30 s in distilled water to lyse any red cells present. Isotonicity was then restored by addition of NaCl solution, after which the cells were washed twice in standard medium (pH_0 7.40). The cells were kept in this medium for 1 h at 37°C before experimentation. About 97% of the cell suspension consisted of neutrophils. Vital dyes, either eosin Y or trypan blue, when added to the suspension, were excluded by >99% of the cells, a generally accepted criterion of viability.

As previously reported (21), the resting cytoplasmic pH_i of steady-state human neutrophils bathed in nominally CO_2-free standard medium (pH_0 7.40) is ~7.25. Cells of more acidic pH_i were obtained by means of the undershoot of pH_i after an NH_4Cl prepulse as described below (21, but see reference 22 for review). Neutrophils were exposed to 30 mM NH_4Cl at pH_0 7.40 for 30 min at 37°C. Under these conditions, pH_i rises immediately to ~7.65 and then declines exponentially, approaching control values by 30 min. When, after 30 minutes, the NH_4Cl is removed and the cells resuspended in NH_4Cl-free standard medium (constant pH_0), pH_i strikingly falls to ~6.5, a value ~0.75 below control. After the undershoot, pH_i promptly recovers to near resting values by 15 min. The rapid initial pH_i rise upon exposure to NH_4Cl, which has been described in a variety of cell types (22), is due to the entry and subsequent protonation of NH_3. The ensuing recovery during maintained NH_4Cl exposure can be interpreted as due to the entry of H^+ equivalents. The resulting intracellular accumulation of H^+ equivalents leads to a pH_i undershoot when NH_4Cl is removed, because most of the intracellular NH_4^+ leaves as NH_3 (22).

Neutrophils of more alkaline pH_i were prepared by means of the overshoot of pH_i after a CO_2 prepulse as described below (21, 22). Cells were exposed to 5% or 18% CO_2 for 30 min at 37°C. Upon application of 18% CO_2 at constant pH_0 (7.40), pH_i falls immediately to ~6.80. Over the next 20–30 min, pH_i recovers by 0.2 U to ~7.00, after which it remains unchanged. When, after 30 min of CO_2 exposure, the cells are resuspended in CO_2-free standard medium (pH_0 7.40), the pH_i rapidly rises to ~7.75, that is, ~0.5 U above control. The pH_i then returns towards the original value over the course of the following 15 min. The pH_i fall upon CO_2 application is due to the entrance and subsequent hydration and dissociation of molecular CO_2. The subsequent alkalinization during maintained CO_2 exposure can be interpreted as the result of removal of H^+ equivalents from the cells. The resulting intracellular H^+ deficit leads to a pH_i overshoot upon CO_2 removal. For this study, cells were exposed to 5 or 18% CO_2. In the former instance, the pH_i changes were qualitatively similar to the above though of lesser magnitude.

Reagents

The following chemicals were purchased from Sigma Chemical Company, St. Louis, MO: 5,5-dimethyloxazolidine-2,4-dione (DMO), *N*-methyl-D-glucamine, HEPES, MES, Tricine, mineral oil, ferricytochrome *c* (type III), superoxide dismutase, crystalline bovine serum albumin, FMLP, and cytochalasin B. FMLP and cytochalasin B were dissolved at 1 mM and 5 mg/ml, respectively, in dimethylsulfoxide (DMSO, Fisher Scientific Co., St. Louis, MO) and then diluted in medium. The resultant final concentrations of DMSO (≤0.1%) had no detectable effect on any of the assays. Isotopes: $[^3H]H_2O$, [^{14}C]DMO, and [^{14}C]inulin were purchased from New England Nuclear, Boston, MA. Amiloride was a gift of Dr. Edward J. Cragoe, Jr., of the Merck, Sharp, & Dohme Research Institute, West Point, PA. Cells were pretreated with amiloride for 2 min before experimentation; longer preincubations with the drug were without further effect.

pH_i measurements with DMO

All experiments were conducted at 37°C. We derived pH_i from the steady-state distribution of the [^{14}C]-labeled weak acid, DMO, pK'_a 6.13 (23). The DMO method has been recently reviewed (22). The charged form of this compound is assumed to be impermeant, so that, at steady state, the uncharged partner is equilibrated across the plasma membrane. At known pH_0, pH_i can then be derived from the equilibrium distribution of the indicator. At the shortest practical exposure time, 15 s, equilibrium of DMO has already been achieved, as evidenced by unchanged distribution for the next 60 min (21). Thus, the probe is suitable for kinetic analyses during which pH_i is changing rapidly.

Samples of the neutrophil suspension (8–12 × 10^6 cells/ml), containing [^{14}C]DMO (1.0 μCi/ml) were incubated in plastic tubes at 37°C under various experimental conditions. Unlabeled indicator was added to a total concentration of 0.1 mM which does not affect pH_i as assessed by the fluorescence of 6-carboxyfluorescein, another pH_i probe (21). At intervals, triplicate aliquots of the cell suspensions were layered over 0.7 ml of Versilube F50 oil (Harwick Chemical Corp., Akron, OH) in 1.5-

ml plastic tubes, which were then centrifuged for ~30 s at 8,000 *g* in a microcentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The cells penetrate the oil and accumulate as a pellet, while the suspending medium remains above the oil. This layering method, introduced by Naccache et al. (13), allows cell separation in <5 s. The pellets were isolated and counted in a liquid scintillation counter (Beckman Instruments, Inc., LS 7000) after addition of 10 ml Aquasol-2 (New England Nuclear). In preliminary studies, [^{14}C]inulin was added as a marker for the extracellular space. The indicator content of the cells could thus be corrected for the medium trapped in the pellet. The extracellular water was 9±2% (n = 7) of total pellet water (21). In these inulin studies as well as in all others, total water was measured with [^{3}H]H_2O.

We have found that, upon addition of FMLP to a suspension of human neutrophils, only ~60% of the total number of cells actually exhibit a stimulation of ^{22}Na fluxes (19). With respect to ^{22}Na fluxes, the remainder of the cells (~40%) behave as nonresponders. Because the enhanced ^{22}Na in- and effluxes observed after exposure to FMLP may be solely ascribed to transport via Na/H exchange, it follows that only 60% of cells will respond with a pH_i change. This conclusion, that ^{22}Na fluxes and pH_i transients mirror the same transport process, is based on the quantitative (1:1) relationship between the two, as well as on their parallel and complete sensitivity to amiloride (18, 19). In our laboratory, the fraction of FMLP-responders (58.7±2.6%, n = 6) is similar to the value of 65.0±5.0% (n = 7) reported by Seligmann et al. (24), who observed functional heterogeneity to FMLP in human neutrophils by means of flow cytometry using a voltage-sensitive cyanine dye. Taking into account the existence of discrete neutrophil subpopulations, the DMO-derived pH_i in the bulk cell suspension, $\overline{pH}_{DMO}$, was used to calculate the cytoplasmic pH_i in the FMLP-responding subpopulation, pH_i^{FMLP}, according to the expression (22):

$$\overline{pH}_{DMO} = \log \sum_{n=1}^{j} f_j \cdot 10^{pH_j},$$

where f_j is the fractional volume of the jth compartment. Assuming a two-compartment model (i.e., FMLP-responding cells and nonresponders), the equation simplifies to:

$$\overline{pH}_{DMO} = \log [f_1 \cdot 10^{pH_i^{FMLP}} + f_2 \cdot 10^{pH_i^{resting}}],$$

where the fractional volumes of the FMLP-responder and nonresponder subpopulations, f_1 and f_2, were taken as 0.6 and 0.4, respectively; the pH_i of the nonresponders was taken as the control or resting pH_i in the absence of FMLP. In the figures and tables to follow, all reported pH_i values obtained with FMLP have been corrected for the presence of subpopulations, by assuming 60% responding cells.

Assay of superoxide radical generation

Continuous assay technique. Superoxide generation was measured by the reduction of ferricytochrome *c* as previously described (25). The production of O_2^- was assayed continuously by means of a recorder attached to a spectrophotometer (model 250, Gilford Instrument Laboratories, Oberlin, OH) in which the cuvette chamber was maintained at 37°C by means of circulating water. Experiments were performed in a total volume of 3.0 ml containing 2 × 10^6 neutrophils and 25 μM ferricytochrome *c*, to which 100 nM FMLP was added as a concentrated stock solution.

Discontinuous assay technique. All experiments were performed in duplicate in a volume of 0.4 ml in plastic tubes containing neutrophils (3–5 × 10^6/ml), ferricytochrome *c* (75 μM), and FMLP at final concentrations of 4 or 100 nM. The tubes were placed at 37°C and periodically agitated. After 5 min, the cells were pelleted by centrifugation at 8,000 *g* for 30 s in a microcentrifuge. The supernatants were decanted and the amount of reduced cytochrome *c* assayed as follows: 0.2 ml of supernatant was added to 2.8 ml of 0.1 M potassium phosphate buffer, pH 7.40, and the absorbance spectrum was measured at 549 nm in a spectrophotometer (Gilford Instrument Laboratories). Utilizing potassium ferricyanide (Fisher Scientific Co.) and sodium dithionite (J. T. Baker Chemical Co., Phillipsburgh, NJ), the amount of reduced cytochrome *c* and the total amount of cytochrome *c* present were calculated using an extinction coefficient of 21.1 mM^{-1} cm^{-1} at 550 nm (reduced-oxidized) (26). Appropriate control tubes demonstrated no spontaneous reduction of ferricytochrome *c* by FMLP, amiloride, or any of the experimental media in the absence of neutrophils.

The addition of superoxide dismutase (10 μg/ml, Sigma Chemical Co.) to the complete reaction mixtures inhibited FMLP-induced cytochrome *c* reduction by >90%, indicating that the reaction was specific for O_2^-. Superoxide generation is expressed as nanomoles of cytochrome *c* reduced per 10^6 neutrophils. It should be mentioned that none of the experimental manipulations (i.e., conditions of varying $[Na]_o$, $[Na]_i$, pH_o, or pH_i [see Results]) had any discernible effect on the time course of FMLP-stimulated O_2^- generation which was complete by 5 min (see Fig. 1).

Data analysis

In several instances, the time course of pH_i after exposure of cells to FMLP could be described by a single exponential equation of the form:

$$pH_t = pH_\infty - [pH_\infty - pH_{initial}] \exp(-kt) \quad (2)$$

where $pH_{initial}$, pH_t, and pH_∞ are the pH_i values at, respectively, zero time, time t, and after steady-state had been reached, and k is the rate coefficient. Curves representing the equation were fitted to the various groups of data by the least-squares method. The initial rate of pH_i change was derived from the expression $k[pH_\infty - pH_{initial}]$. In other cases, the pH_i course was nearly linear over the period of study, and the slope of the linear regression represented the rate of pH_i change.

In the statistical analyses, two-tailed comparisons were performed using multiple independent t tests on difference scores (27).

Results

Effect of amiloride. Exposure of human neutrophils to an optimal dose of FMLP (100 nM) led to the generation of O_2^- as measured by the reduction of cytochrome *c* (Fig. 1). Also shown is the time course of FMLP-induced O_2^- production in the presence of 1 mM amiloride, a drug which blocks Na/H countertransport (17). In both cases, the release of O_2^- was complete by 5 min, i.e., the duration was unaffected by amiloride although the extent of release was reduced by about half. [It should be emphasized that none of the drugs or experimental conditions (see below) had any effect on the time course of FMLP-stimulated O_2^- gen-

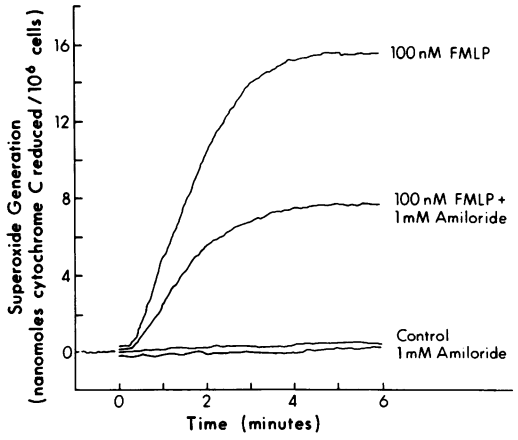

Figure 1. Time course of FMLP-induced O_2^- generation. Neutrophils were suspended in standard medium (140 mM Na, pH_o 7.40) in the presence or absence of 1 mM amiloride. At 0 time, FMLP was added to a final concentration of 100 nM. The generation of O_2^- was measured by continuous assay using cytochrome *c* (see Methods). The curves are tracings obtained with cells from a single donor.

eration. Thus, the differences to be described reflect changes in the total amounts of O_2^- release.]

The stimulation of respiratory burst activity was associated with an alkalinization of pH_i from 7.22 to 7.80 (Fig. 2), which followed a time course similar, but not identical, to that of FMLP-induced O_2^- release (Fig. 1). The rise in pH_i is mediated by a chemotactic factor-activated exchange of external Na for internal H. Fig. 2 also shows that 1 mM amiloride reduced the initial rate of alkalinization elicited by FMLP from 0.288±0.036 to 0.0061±0.0006 pH/min, an inhibition of 98%. However, at this concentration of amiloride, FMLP-stimulated O_2^- production was only partially reduced, i.e., to a level ~40% of the total in the absence of the drug (Fig. 1). From the data of Figs. 1 and 2, it is immediately apparent that an alkalinization of pH_i cannot be an absolute requirement for O_2^- generation because a considerable amount of O_2^- release was still observed in the presence of amiloride, which nearly abolished the pH_i transient.

The dose dependencies of amiloride inhibition of the pH_i and O_2^- responses induced by 100 nM FMLP are shown in Fig. 3. A Michaelis–Menten curve (apparent K_i 77.6±6.2 μM), fit to the pH_i data by a nonlinear least-squares program, extrapolated to a point that could not be experimentally or statistically distinguished from 0. This indicates that the pH_i response was completely sensitive to amiloride. On the other hand, the O_2^- release data could be nicely fit by an empirical curve for which two components were assumed: (*a*) an amiloride-sensitive fraction demonstrating Michaelis–Menten inhibition kinetics that was superimposed on (*b*) an amiloride-insensitive background. Using a least-squares program, the data are evidently well fit by a curve composed of an amiloride-sensitive (apparent K_i 92.8±20.5 μM) fraction (63.1±5.0% of total) and an amiloride-insensitive fraction (36.9±2.5% of total). It is of interest that, after taking this putative amiloride-resistant component of O_2^- generation into account, the apparent K_i's for amiloride inhibition of the pH_i response (78 μM) and of the amiloride-sensitive component of O_2^- production (93 μM) were similar.

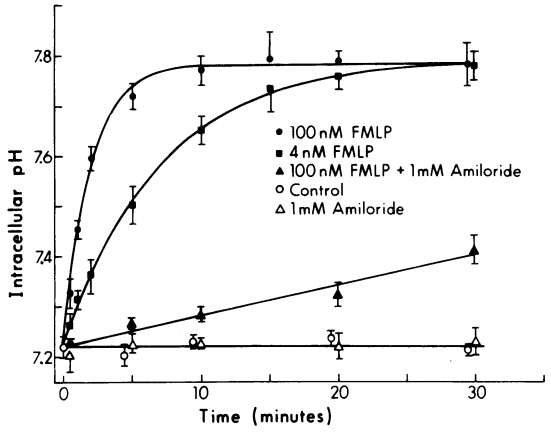

Figure 2. Time course of FMLP-induced pH_i changes. Neutrophils were suspended in standard medium (140 mM Na, pH_0 7.40) in the presence (Δ, ▲) or absence (o, • or ▪) of 1 mM amiloride. At zero time, 4 nM (▪) or 100 nM FMLP (•, ▲) was added. Controls, in the absence of FMLP, are also shown (o, Δ). At stated times, aliquots of the cell suspensions were taken for pH_i measurements using [^{14}C]DMO. The DMO-derived pH_i values have been adjusted for the presence of neutrophil subpopulations, assuming 60% FMLP-responding cells. Results represent the mean±standard error of the mean of three separate experiments, each performed in triplicate. For 4 nM and 100 nM FMLP, the curves are single exponential fits (Eq. 2) to the data. The initial rates were 0.0820±0.0124 and 0.288±0.036 pH/min, respectively; the final pH_i values were 7.80±0.03 and 7.79±0.02, respectively. The 100 nM FMLP plus 1 mM amiloride data were fit to a straight line (slope 0.0061±0.0006 pH/min). A horizontal line was drawn at 7.22, the average of the control and amiloride data points.

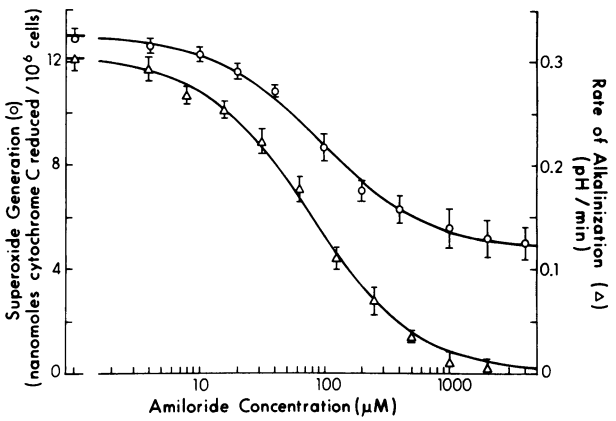

Figure 3. Effect of amiloride on FMLP-induced pH_i and O_2^- responses. All experiments were performed in standard medium (140 mM Na, pH_0 7.40). At zero time, 100 nM FMLP was added to the neutrophil suspensions in the presence of varying concentrations of amiloride (0–4,000 μM). Intracellular pH data (Δ): For each dose of amiloride, DMO-derived pH_i was measured at two different time points (0.5, 1, 2, 5, or 10 min as appropriate) during the course of the FMLP-stimulated alkalinization. The control (i.e., initial) pH_i and the two measured pH_i values were fitted to a single exponential equation (Eq. 2), from which the initial rate of pH_i change was calculated. The data were adjusted for neutrophil subpopulations by assuming 60% FMLP-responding cells. Results represent the mean±standard error of the mean of three separate experiments, each performed in triplicate. The data points, signifying the initial rates of FMLP-induced alkalinization, were fit by a least-squares program to a Michaelis–Menten inhibition equation which yielded an apparent K_i for amiloride of 77.6±6.2 μM. Superoxide radical generation (o): The amount of O_2^- release at 5 min was quantitated by cytochrome *c* reduction using a discontinuous assay technique. Results represent the mean±standard error of the mean of three separate experiments, each performed in duplicate. The data were fit by a least-squares program which assumed two separate components: (*a*) an amiloride-sensitive fraction, which demonstrated Michaelis–Menten inhibition (apparent K_i for amiloride 92.8±20.5 μM and which accounted for 63.1±5.0% of the total), that was superimposed on (*b*) an amiloride-resistant fraction (4.8±0.3 nmol cytochrome *c* reduced/10^6 cells, which comprised 36.9±2.5% of the total).

Effect of extracellular Na. In order to test further whether pH_i might at least partially regulate the O_2^- response, a number of other modifications in the experimental design were employed. In this series of experiments, both the direction and degree of the pH_i response induced by FMLP were varied by altering the Na gradient across the cell membrane, i.e., by changing the extracellular concentration of Na. Fig. 4 shows the results of varying $[Na]_0$ on FMLP-stimulated pH_i and O_2^- responses. For these studies, $[Na]_0$ was varied from 140 to nominally 0 mM by equimolar replacement with *N*-methyl-D-glucamine. As shown in Fig. 4, resting pH_i remained constant (at 7.20±0.01) over this range of $[Na]_0$. In contrast, the pH_i (measured at 5 min) in cells exposed to FMLP was strongly dependent on $[Na]_0$. The pH_i values in FMLP-responding cells rose as $[Na]_0$ was increased

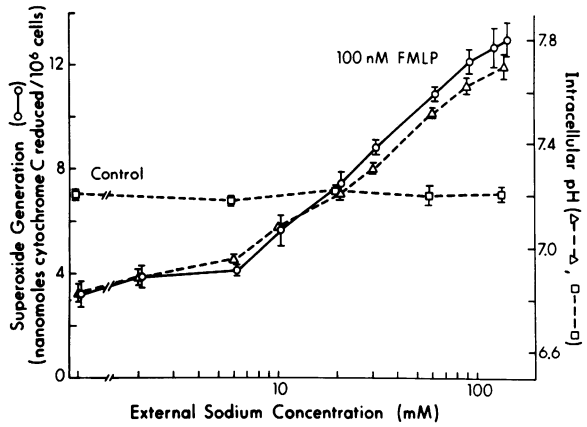

Figure 4. Effect of extracellular Na on FMLP-activated pH_i and O_2^- responses. Neutrophils were suspended at pH_0 7.40 in media in which the concentration of external Na was varied between 0 and 140 mM by equimolar replacement with *N*-methyl-D-glucamine. Experiments were performed in the presence (○, △) or absence (□) of 100 nM FMLP. After 5 min of incubation, samples were taken for measurements of DMO-derived pH_i (– – –) and O_2^- generation (——). Control O_2^- release under all conditions averaged ≤0.4 nmol cytochrome *c* reduced/10^6 cells (not shown). Results represent the mean±standard error of the mean of three (pH_i) or four (O_2^-) experiments.

between 0 and 140 mM. There was a crossover point (i.e., where FMLP caused no change in pH_i) at $[Na]_0 \sim 20$ mM. This value corresponds to the point ($[Na]_0 = 19.5$ mM) at which Na and H are at chemical equilibrium across the membrane according to Eq. 1. It is of note that below $[Na]_0$ 20 mM, where the Na gradient is reversed (now outward and greater than the outwardly directed H gradient), the addition of FMLP leads to a relative acidification.

Fig. 4 also shows the amount of O_2^- generated during a 5-min exposure to FMLP under comparable conditions. The assays were terminated at 5 min, by which time FMLP-induced O_2^- release under all conditions was complete. Although the production of O_2^- elicited by FMLP was dependent on $[Na]_0$, the relationship was not absolute in that appreciable (24–30% of total) O_2^- release occurred at very low $[Na]_0$ (2 mM) or in the nominal absence of extracellular Na. Chemical determinations of Na (by flame photometry) in the bathing medium indicated that the Na content did not exceed 0.5 mM in nominally Na-free medium. From the data, it is clear once again that an alkalinization of pH_i cannot be an absolute requirement for O_2^- production because at an $[Na]_0$ of 0–2 mM, where roughly 25% of total O_2^- release occurs, the pH_i of cells exposed to FMLP actually acidifies relative to control values.

Table I shows the relationship of $[Na]_0$ to O_2^- generation in cytochalasin B-treated cells. In the presence of 5 μg/ml cytochalasin B, the amount of O_2^- production induced by FMLP was constant and independent of external Na. Thus, cytochalasin B apparently releases the system from its dependence on $[Na]_0$. If the effect of $[Na]_0$, normally seen in the absence of cytochalasin B (Fig. 4), were related to a pH_i change, then one would predict that amiloride would have little or no effect on O_2^- release by cytochalasin B-treated cells, an expectation verified by the data of Table I. However, amiloride (1 mM) did block the FMLP-stimulated alkalinization in cytochalasin B-treated cells (data not shown: the pH_i responses were indistinguishable in the absence or presence of cytochalasin B, where all of the cells respond

Table I. Lack of Effect of External Sodium on FMLP-induced O_2^- Generation by Cytochalasin B-treated Cells

Conditions ($[Na]_0$)	FMLP-induced O_2^- generation
mM	*(nmol cytochrome c reduced/10^6 cells)*
0	24.7±3.6
1	22.3±2.3
5	23.8±3.1
10	22.5±1.4
30	21.8±3.1
60	24.8±1.8
90	22.5±2.9
140	23.8±2.9
140 + 1 mM amiloride	24.2±3.4

Experiments were performed as in Fig. 4 except that neutrophils were pretreated with 5 μg/ml cytochalasin B for 5 min before the addition of 100 nM FMLP. Results represent the mean±standard error of mean of three experiments.

to FMLP), similar to its efficacy in the absence of cytochalasin B (Figs. 2 and 3). In addition, the FMLP-activated pH_i values as a function of $[Na]_0$ were indistinguishable in normal (Fig. 4) vs. cytochalasin B-treated cells. At low external Na (≤2 mM), when pH_i acidifies to ~6.8, cytochalasin B-treated neutrophils still produced normal quantities of O_2^- (Table I) as compared with their counterparts in 140 mM Na medium where pH_i alkalinized to ~7.8. This point further dramatizes the fact that O_2^- production in the presence of cytochalasin B is independent of pH_i.

While clearly not an absolute requirement for O_2^- release, the data thus far presented do not rule out a modulating or regulatory role for pH_i on the O_2^- response. Because optimal O_2^- generation induced by FMLP is associated with an alkalinization of pH_i, a plausible hypothesis is that conditions which tend to increase pH_i may facilitate O_2^- production. The following series of experiments were undertaken to investigate this possibility.

Effect of extracellular pH. The effect of altering extracellular pH (pH_0) on the pH_i and O_2^- responses elicited by FMLP was tested. As shown in Fig. 5, varying pH_0 from 5.9 to 8.0, while causing slight changes in the pH_i of unstimulated cells, had a dramatic effect on the pH_i of FMLP-activated cells. As previously reported (21), extracellular acidification or alkalinization resulted in small pH_i changes in the same direction in resting cells. These pH_i transients may reflect H or OH ion permeability. The pH_i (measured at 5 min) in FMLP-responders, however, was strongly dependent on pH_0 (Fig. 5): raising pH_0 from 5.9 to 8.0 increased the pH_i of cells exposed to FMLP. There was a crossover point (i.e., where FMLP caused no pH_i change) at $pH_0 \sim 6.5$, at which point Na and H are distributed in chemical equilibrium across the membrane.

The results of O_2^- assays performed under similar conditions are also shown in Fig. 5. Relative to the amount of O_2^- generated under normal conditions (pH_0 7.4), lowering pH_0 towards 5.9 caused a reduction in the quantity of O_2^- released during the 5-min exposure to FMLP. In contrast, raising pH_0 above 7.4 resulted in enhanced O_2^- release. The quantities of O_2^- produced at pH_0 7.7 and 8.0 (15.7±0.9 and 16.5±0.6 nmol cytochrome *c* reduced/10^6 cells, respectively) were significantly greater (*P*

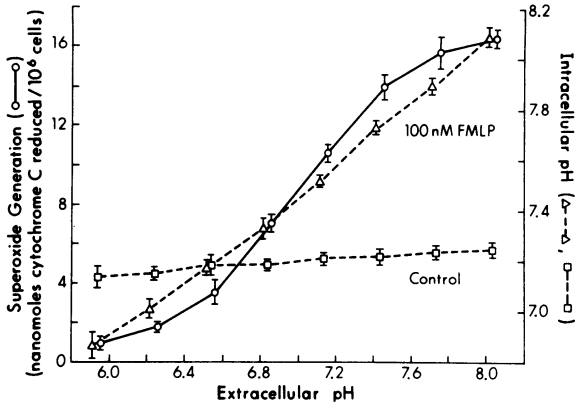

Figure 5. Effect of extracellular pH on FMLP-activated pH_i and O_2^- responses. Neutrophils were suspended in 140 mM Na media of varying pH_0 (5.9–8.0) in the presence (○, △) or absence (□) of 100 nM FMLP. After 5 min of incubation, samples were taken for measurements of DMO-derived pH_i (- - -) and O_2^- generation (——). Control O_2^- release under all conditions averaged ≤0.4 nmol cytochrome *c* reduced/10^6 cells (not shown). Results represent the mean±standard error of the mean of three (pH_i) or four (O_2^-) experiments.

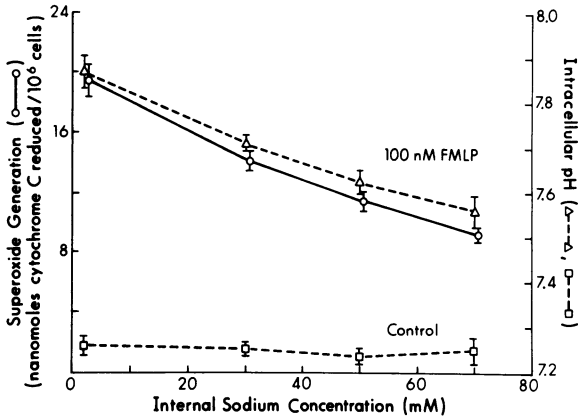

Figure 6. Effect of intracellular Na on FMLP-activated pH_i and O_2^- responses. Batches of neutrophils of varying internal Na concentrations were prepared as described in the text. As measured by flame photometry, the $[Na]_i$'s of the different cell preparations were as follows: Na-depleted (≤2 mM), normal Na (~30 mM), and Na-loaded (~50 and ~70 mM) (31). At 0 time, all cells were resuspended in standard medium (140 mM Na, pH_0 7.40) in the presence (○, △) or absence (□) of 100 nM FMLP. After 5 min of incubation, samples were taken for measurements of DMO-derived pH_i (- - -) and O_2^- production (——). Control O_2^- release under all conditions averaged ≤0.4 nmol cytochrome *c* reduced/10^6 cells (not shown). Results represent the mean±standard error of the mean of three (pH_i) or six (O_2^-) experiments.

< 0.05 and $P < 0.02$, respectively) than that at pH_0 7.4 (14.0±0.7 nmol reduced/10^6 cells). A comparison of the pH_i and O_2^- release data in the presence of FMLP indicates a positive correlation between the pH_i value and the amount of O_2^- release occurring in response to FMLP stimulation. In other experiments, O_2^- was generated in a cell-free system by the reaction of photo-reduced riboflavin and oxygen according to the method of Winterbourn et al. (28). The amount of O_2^- production detected by cytochrome *c* was constant and independent of pH_0 (5.9–8.0). These results suggest that the data of Fig. 5 were, in practice, little affected by any changes in the trapping efficiency of cytochrome *c* over this pH range or by possible prolongation of the half-life of O_2^- due to the presumed pH-dependence of the dismutation reaction: $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$.

It is important to bear in mind that our group has previously reported that the binding of tritiated-FMLP is not affected by the removal of extracellular Na and that binding appears to be independent of the nature of the monovalent cation in the bathing medium (6). Also, variations in pH_0 over the range employed in this study (6–8) have only a modest effect on the binding of FMLP (29, 30). A slight reduction in binding has been reported as pH_0 is raised from 7.4 to 8.0, where the amount of O_2^- generation is actually increasing (Fig. 5). Thus, the observed experimental results in Figs. 4 and 5 are unlikely to be due to variations in the binding of FMLP to its cell surface receptors.

Effect of intracellular Na. Fig. 6 shows the results of experiments designed to test the effects of varying the initial internal Na concentration, $[Na]_i$, on pH_i values and O_2^- generation after FMLP stimulation. For these studies, cells of different $[Na]_i$'s were prepared as follows: batches of Na-depleted cells ($[Na]_i$ ~ 2 mM) were prepared by incubation of cells (1.5 hours, 37°C) in Na-free medium, where Na was completely replaced by *N*-methyl-D-glucamine (31). Batches of Na-loaded cells ($[Na]_i$ ~ 50 or ~70 mM) were obtained by incubating cells for 1.5 or 3 h, respectively, in ice-cold, K-free saline as previously described (31). Normal Na cells ($[Na]_i$ ~ 30 mM) were kept in standard (140 mM Na) medium at 37°C throughout (31). After appropriate incubations, all cells were pelleted and resuspended in 140 mM Na medium (pH_0 7.40) in the presence or absence of FMLP. Aliquots of the cell suspensions were then taken for pH_i determinations and for measurements of O_2^- release after 5 min. Resting pH_i was the same (~7.25) for all $[Na]_i$ conditions. In each of the four sets, pH_i (at 5 min) rose after the addition of FMLP though the magnitudes of the pH_i increments were dependent on the initial (i.e., resting) values of $[Na]_i$. Relative to normal Na cells (initial $[Na]_i$ ~ 30 mM), the pH_i of Na-depleted cells (initial $[Na]_i$ ~ 2 mM) rose to a higher pH_i value (7.87±0.04 vs. 7.71±0.02). By contrast, the pH_i of FMLP-stimulated Na-loaded cells (initial $[Na]_i$ ~ 70 mM) increased to only 7.56±0.05. As also shown in Fig. 6, the O_2^- generation responses (in nanomoles of cytochrome *c* reduced/10^6 cells) to FMLP paralleled the pH_i changes: relative to normal Na cells (14.2±0.8), Na-depleted cells released more (19.5±1.2, *P* vs. normal Na cells < 0.001), while Na-loaded cells released less O_2^- (9.3±0.6, *P* vs. normal Na cells < 0.002).

Effect of intracellular pH. A series of experiments were performed to test the effects of variations in the initial pH_i on FMLP-stimulated pH_i and O_2^- production by 5 min. For these studies, cells of more acidic pH_i relative to normal (pH_i ~ 7.25) were obtained by the undershoot of pH_i after an NH_4Cl prepulse and cells of more alkaline pH_i were prepared by the overshoot of pH_i after pretreatment of cells with CO_2 (see Methods). For the experiments shown in Fig. 7, cells were preincubated with either 30 mM NH_4Cl, standard medium, 5% CO_2, or 18% CO_2, all at a constant pH_0 of 7.40. After 30 min at 37°C, the cells were spun down and resuspended in 140 mM Na medium, pH_0 7.40, in the presence or absence of FMLP. Samples were then taken for pH_i measurements at 5 min.

In the absence of FMLP, the pH_i of control cells after the NH_4Cl undershoot and CO_2 overshoot was rapidly recovering (i.e., returning to near normal values by 15 min [21]). For clarity,

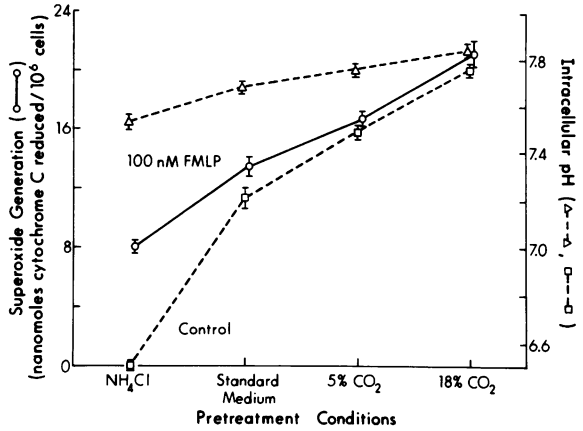

Figure 7. Effect of intracellular pH on FMLP-activated pH_i and O_2^- responses. Batches of neutrophils of different pH_i were prepared by pretreating cells with either 30 mM NH_4Cl, 5% CO_2, 18% CO_2, or standard medium, each at pH_0 7.40 (see Methods). At zero time, all cells were resuspended in standard medium (140 mM Na, pH_0 7.40) in the presence (o, Δ) or absence (□) of 100 nM FMLP. After 5 min of incubation, samples were taken for measurements of DMO-derived pH_i (- - -) and O_2^- production (——). In the absence of FMLP, the pH_i of control cells previously exposed to 30 mM NH_4Cl, 5% CO_2, or 18% CO_2 was rapidly recovering (i.e., returning towards the normal resting pH_i of ~7.2). For clarity, only the initial pH_i values (□) of control cells are shown for comparison. Control O_2^- release under all conditions averaged ≤0.4 nmol cytochrome *c* reduced/10^6 cells (not shown). Results represent the mean±standard error of the mean of three (pH_i) or four (O_2^-) experiments.

only the initial pH_i values are shown (Fig. 7). In each of the four batches of cells, the addition of FMLP led to an alkalinization of pH_i though both the relative pH_i increments (ΔpH_i's) as well as the absolute pH_i values induced by FMLP differed among the cell preparations and were related to the initial pH_i's. The ΔpH_i was least in cells preincubated with 18% CO_2, where initial pH_i was highest (7.76±0.03), though pH_i in response to FMLP alkalinized to the highest level (7.84±0.04). By comparison, ΔpH_i was greatest in cells preincubated with 30 mM NH_4Cl, where initial pH_i was lowest (6.50±0.02), but pH_i in response to FMLP rose to only 7.53±0.04.

For convenience, the O_2^- responses of separate aliquots of these cells are also shown in Fig. 7. There was a good correlation between the pH_i induced by FMLP at 5 min and the quantity of O_2^- produced. Cells pretreated with 18% CO_2, where pH_i rose to the highest value (7.84) generated the most O_2^- (21.3±1.0 nmol cytochrome *c* reduced/10^6 cells), while cells pretreated with 30 mM NH_4Cl, where pH_i alkalinized to only 7.53, generated the least O_2^- (8.1±0.5 nmol reduced/10^6 cells). In both instances, the amounts of O_2^- released were significantly different ($P < 0.01$) from that produced by cells kept in standard medium (13.5±0.8 nmol reduced/10^6 cells) where pH_i rose to an intermediate value of 7.68±0.03 after the addition of FMLP. Thus, it appears that the critical effect in regulating the O_2^- response relates to the absolute level to which pH_i rises rather than to the relative increment in pH_i induced by FMLP.

The preceding sections all provide evidence in support of the argument that an alkalinization of pH_i facilitates O_2^- generation in the presence of FMLP. However, this relationship does not appear to hold true in the absence of FMLP. For cells pretreated with 18% CO_2, pH_i rose initially to ~7.75 (21), a value similar to that achieved by normal Na cells exposed to 100 nM FMLP in 140 mM Na (19), yet no O_2^- release could be detected during a 30-min assay. A further dissociation between an alkalinization of pH_i and function in the absence of FMLP could be achieved by other means: namely, exposure of cells to NH_4Cl or to monensin, an ionophore that promotes the exchange of alkali metal cations, especially Na, for H (32). When cells are exposed to 30 mM NH_4Cl at pH_0 7.40, pH_i rises dramatically to ~7.65 and then gradually recovers to near control levels (7.25) within 30 min (21). The pH_i of cells exposed to 10 μM monensin in 140 mM Na medium at pH 7.40 undergoes an alkalinization whose time course and final extent (~7.80) are indistinguishable from that obtained with 100 nM FMLP (19). However, no O_2^- production could be detected during 30 min of incubation in either instance, i.e., with NH_4Cl or monensin, even though the alkalinization in the latter case is sustained. These results indicate that an alkalinization of pH_i, per se, is not a sufficient trigger for O_2^- production. Thus, FMLP must be generating some additional excitatory signal that is critical to the O_2^- response.

Subthreshold FMLP concentrations. In a previous publication (6), our group reported that the dose-response range for stimulation of ^{22}Na fluxes by FMLP extended over 2.5–3 logs, from ~0.1 nM to a maximum at ~100 nM. In contrast, the dose-response range for the induction of O_2^- release by FMLP is narrow, comprising only 1 log, from 10 to 100 nM. We have been unable to show a statistically significant or reproducible increase in O_2^- production above control values in the presence of 4 nM FMLP. This is true even though ^{22}Na uptake into cells exposed to 4 nM FMLP for 5 min is roughly half the amount achieved in response to 100 nM FMLP, an optimal dose (6). In that Na influx via Na/H exchange occurs by a 1:1 countertransport of Na for H (18, 19), the uptake of ^{22}Na should mirror the pH_i change. Therefore, one would predict that in cells exposed to 4 nM FMLP, pH_i would rise at a slower rate than in cells treated with 100 nM FMLP, that the degree of alkalinization at 5 min in the former case should be about one-half of the latter, but that steady-state pH_i would be the same in the two instances (i.e., chemical equilibrium distribution for Na and H as dictated by Eq. 1). These predictions are verified by the data of Fig. 2. As can be seen, the pH_i of cells exposed to 4 nM FMLP approached ~7.80 by ~30 min, the same value reached at ~10 min in cells exposed to 100 nM FMLP. However, with 4 nM FMLP, no O_2^- release above control could be detected during a 30-min incubation (data not shown).

These data imply that even an alkalinization of this magnitude (of ~0.6 to pH_i ~ 7.80) induced by FMLP is not a sufficient trigger if the pH_i rise is sufficiently slow or delayed. Apparently, if the induced pH_i change is to modulate the functional activity of these cells, it must do so early, presumably within the first few seconds when the critical triggering signal is generated by FMLP. Whatever the exact nature of this event, we hypothesized that it might be potentiated by a rise in pH_i. We predicted, therefore, that if the rate and/or amplitude of the alkalinization could be increased by modifying the experimental conditions, then O_2^- release in response to 4 nM FMLP might become measurable in that some threshold effect would then be exceeded. To test this hypothesis, we used Na-depleted cells and cells pretreated with 18% CO_2, two maneuvers associated with enhanced O_2^- release in cells stimulated by 100 nM FMLP (Figs. 6 and 7). The results of these experiments are shown in Table II. In the case of initially Na-depleted cells and for cells after a CO_2 prepulse, pH_i in response to 4 nM FMLP was higher than

Table II. Potentiation of a Subthreshold FMLP Dose by Intracellular Alkalinization

Cells*	pH_i‡ Control	pH_i‡ 4 nM FMLP	O_2^- generation‡ Control	O_2^- generation‡ 4 nM FMLP
Normal Na	7.28±0.01	7.51±0.03	0.4±0.2	0.4±0.1
Na-depleted	7.27±0.03	7.61±0.03	0.3±0.1	3.2±0.3‖
18% CO_2-pretreated	7.46±0.02§	7.63±0.02	0.4±0.1	5.2±0.5‖

* Batches of normal Na ($[Na]_i$ ~ 30 mM) and Na-depleted ($[Na]_i$ ~ 2 mM) cells were prepared by preincubating neutrophils in standard medium (140 mM Na, pH_0 7.40) or in Na-free medium (*N*-methyl-D-glucamine replacement), respectively, for 1.5 h at 37°C (see text). Another aliquot of the cell suspension was exposed to 18% CO_2 (pH_0 7.40) for 30 min. At zero time, all cells were resuspended in standard medium (140 mM Na, pH_0 7.40) in the presence or absence of 4 nM FMLP.

‡ At 5 min, DMO-derived pH_i and O_2^- release (from cytochrome *c* reduction) were measured. The amount of O_2^- generation is expressed as nanomoles of cytochrome *c* reduced per 10^6 neutrophils. The results are the mean±standard error of the mean of three separate experiments.

§ The initial pH_i value was 7.79±0.03.

‖ P vs. control < 0.005.

for normal Na cells at 2 min (not shown) and at 5 min. In both instances, significant amounts of O_2^- release were now observed (P vs. control < 0.005). Under these conditions, there were no differences in either the amounts or kinetics of specific [^{3}H]FMLP binding to the neutrophils. Thus, the finding of increased O_2^- generation by Na-depleted cells and by those pretreated with 18% CO_2 are unlikely to be due to altered receptor–ligand interactions.

Discussion

The regulation of pH_i is currently receiving considerable attention as the importance of pH_i to a number of cell functions becomes increasingly apparent. For example, pH_i has been shown to play a role in egg fertilization, mechanical properties of muscle, cell growth and division, and epithelial transport and secretion (for reviews, see References 22 and 33). Recently, proposals have been set forth that several functions of neutrophils may be regulated by their pH_i (16, 34). The present study was undertaken to define the potential role of chemotactic factor-induced pH_i transients in human neutrophils on a specific functional response: that of O_2^- production. The results demonstrate a direct relationship between an intracellular alkalinization and the amount of O_2^- generated by FMLP-activated cells. In an analogous way, a correlation between an alkalinization and the functional responses of other stimulated cells has been reported in rat splenic lymphocytes (35) and in hamster pulmonary fibroblasts (36), where the proliferative responses to mitogens or growth factors, as well as the pH_i changes, are inhibited by amiloride. Comparable results are also seen for serotonin secretion by thrombin-stimulated human platelets (37), for insulin release by rat and mouse pancreatic islet cells (38), and for the early developmental behavior following sea urchin egg fertilization (39). All of these observations strongly implicate pH_i as an important element in stimulus-response coupling.

Our group (19) and others (14) have previously reported that exposure of human or rabbit neutrophils to FMLP leads to an alkalinization of pH_i. In contrast to the single phase of alkalinization in FMLP-activated human neutrophils noted in the present study, Molski et al. (14) observed a biphasic pH_i response in rabbit neutrophils: a small (~0.05 U) acidification within the first minute followed by a sustained alkalinization of ~0.2 U. These magnitudes are understandably smaller than those reported in this paper which were corrected for the presence of the 60% FMLP-responding subpopulation. However, the reason for the disparity in the data regarding the initial relative acidification is not at all apparent. The nature of these pH_i transients has been examined in some detail. The alkalinization results from activation of an amiloride-sensitive Na/H countertransport mechanism that, under normally prevailing conditions, mediates a 1:1 exchange of external Na for internal H leading to a rise in pH_i from ~7.25 to ~7.80 (19). Several lines of evidence indicate that this alkalinization cannot be an obligatory requirement or sine qua non for O_2^- generation. First, roughly 40% of O_2^- release induced by 100 nM FMLP occurs in the presence of 1 mM amiloride (Figs. 1 and 3), conditions under which the alkalinization is almost completely abolished (Figs. 2 and 3). Second, compared to the response at $[Na]_0$ 140 mM, ~25% of maximal O_2^- production occurs in the nominal absence of extracellular Na, where, because the Na gradient has been reversed, pH_i actually acidifies after FMLP stimulation (Fig. 4). Third, exposure of cells to 4 nM FMLP leads to an alkalinization of the same degree (to ~7.8) at 30 min as that observed with 100 nM FMLP at 10 min (Fig. 2), yet causes no O_2^- release. Fourth, in the absence of FMLP, raising pH_i to 7.7–7.8 by means of NH_4Cl, monensin, or after a CO_2 prepulse resulted in no demonstrable O_2^- generation above control. The foregoing arguments leave little doubt that some other, as yet, unidentified early excitatory signal induced by FMLP is critical to activation of the enzyme system responsible for the generation of O_2^-.

Nonetheless, it appears that the pH_i level seems to play a modulating or regulatory role at one or more steps in the pathway leading to O_2^- release. For instance, when either $[Na]_0$, $[Na]_i$, pH_0, or pH_i was varied independently, there was a direct, positive correlation between the pH_i value in FMLP-stimulated cells and the amount of O_2^- released by 5 min. It is important to remember that the 5-min pH_i value cannot, in and of itself, be the direct modulating factor in that O_2^- production is complete by 4–5 min. Rather, the relationship of pH_i to this functional response may best be viewed in terms of this pH_i's reflecting the pH_i level prevailing at some critical juncture in time. Presumably, this period occurs early in the time course of events, possibly within the first few seconds after ligand–receptor interaction.

During the respiratory burst, there is a net generation of protons which can be detected as an acidification of the bathing medium (40, 41). At first sight, it might indeed seem that the associated pH_i changes observed here are a direct result of the proton efflux and that an intracellular alkalinization is not unexpected. Actually, there is no theoretical reason to suppose that an acid efflux must be coupled to or be mirrored by an intracellular alkalinization. In fact, no a priori conclusion can be drawn about either the magnitude or direction of pH_i from the quantity of H^+ equivalents entering the bathing medium. (*a*) Cytosolic pH_i may remain unaffected if the H^+ equivalents are generated at the membrane surface, a distinct possibility in this case considering the plasma membrane localization of the O_2^--forming NADPH oxidase. (*b*) Cytosolic pH_i may actually fall as H^+ equivalents are generated inside, some of them diffusing into the external medium. (*c*) Cytosolic pH_i may rise because of a separate mechanism such as an exchange of internal H for external Na.

It is important to emphasize that FMLP-activated Na/H exchange is a distinct, independent phenomenon, divorced from the release of protons associated with the formation of O_2^- or its reaction products. This point is graphically demonstrated by the data of Fig. 2 wherein the final pH_i in stimulated cells is the same (~7.80) after exposure to either 4 or 100 nM FMLP even though no O_2^- generation can be detected at the lower stimulus concentration, whereas the latter elicits a lot.

The mechanism by which pH_i exerts its regulatory role is unclear at present. In other cells, where this question has been addressed, numerous metabolic events have been shown to be pH-sensitive. These include intracellular calcium and cyclic nucleotide levels, protein and DNA synthesis, energy metabolism, contractile apparatus, and membrane permeability to ions (for review, see Reference 33). It is tempting to speculate that the potentiation of O_2^- production seen at relatively alkaline pH_i values represents the titration of a critical functional group(s) on a protein(s) in the pathway leading to O_2^- generation. From the data presented, this protein might be expected to display a pH optimum in the region of 8.0. A number of investigators working with cell-free preparations derived from zymosan- or phorbol myristate acetate-stimulated neutrophils have all reported a pH optimum for the O_2^--forming activity (NADPH oxidoreductase) in the range of 7.0–7.5 (42–44). A slight decrease in activity is generally seen as pH is raised to 8.0. Thus, it appears unlikely that the observed enhancement of O_2^--generating capacity at alkaline pH_i can be attributed to a direct effect on this enzyme. However, it should be remembered that the properties of any integral membrane protein may be different in intact cells as compared to those in partially purified subcellular preparations. It seems then that the mechanistic explanation underlying the effect of pH_i on O_2^- generation awaits further identification of the pH dependencies of the reaction sequence leading to the production of O_2^-.

In this context, it is intriguing that Bormann et al. (45) have recently reported activation by FMLP of a phospholipase activity in rabbit neutrophil membranes. The optimal pH was 8.0–8.5 with activity rapidly decreasing at pH values below 7.0. It is tempting to speculate that pH_i might be acting through this route. This is especially appealing because changes in the turnover of phospholipids, in particular the release and subsequent metabolism of arachidonic acid to prostaglandins, leukotrienes, and other mediators, are thought to be important biochemical events in chemotactic factor-induced activation of neutrophils.

In this study, relatively high (i.e., millimolar) concentrations of amiloride were required to block FMLP-stimulated Na/H exchange and O_2^- generation. In other cell types, these concentrations have also been shown to inhibit Na conductive pathways (46), Na/Ca exchange (47), Na/K ATPase activity (48), and bicarbonate transport (49), and to penetrate the cell interior to affect protein synthesis (17). Thus, a question arises as to the specificity of amiloride for the Na/H exchanger of human neutrophils: whether the effect of amiloride on O_2^- production is related solely to abrogation of Na/H exchange rather than to some other property. It is unlikely that the drug's suppression of O_2^- release is related to inhibition of Na channels in that we have found that amiloride is without effect on passive, electrodiffusive Na fluxes in human neutrophils (18). In addition, an effect on Na/Ca exchange is unlikely since amiloride does not inhibit ^{45}Ca fluxes (15) and since benzyl-amiloride, which blocks Na/Ca exchange in neutrophils by ~75% (unpublished observations), does not alter FMLP-induced O_2^- generation. Finally, an effect on the Na/K pump can be ruled out because ouabain has no effect on O_2^- release (7) and because amiloride does not inhibit the active pump-mediated efflux of ^{22}Na from neutrophils (unpublished observations).

In contrast to the observation of a reduction in the amount of O_2^- release with decreasing $[Na]_0$, Della Bianca and co-workers (50) reported enhancement of O_2^- generation at low $[Na]_0$ (equivalent replacement by K). The authors noted that the increase in functional responsiveness correlated with an increase in the affinity and number of FMLP receptors (51). The explanation for the difference between this and the present study is not immediately apparent. However, it should be pointed out that the studies of Rossi and colleagues were conducted in Ca-free media containing 1 mM KCN, 17 mM phosphate, and 5 μg/ml cytochalasin B, conditions very different from those reported here. Moreover, altered receptor function caused by the different experimental media cannot explain our results because a 1.5-h preincubation in Na-free medium (to cause internal Na depletion) or a 30-min pretreatment with 18% CO_2, two maneuvers associated with higher FMLP-induced pH_i and O_2^- generation, had no effect on either the time course or extent of [^{3}H]FMLP binding at 4 nM FMLP.

Pfefferkorn (52) has presented evidence that several functional responses of human monocytes, including O_2^- generation, occur independently of external Na, but that a dependence on $[Na]_0$ could be induced by pretreating cells with isotonic NH_4Cl. The author implied that the $[Na]_0$ dependence of a variety of neutrophil functions might be an artifact resulting from the use of NH_4Cl to lyse contaminating erythrocytes during the purification procedure. Although this observation could be invoked to explain our original data on O_2^- production (6), it cannot explain the present results because the neutrophils were rid of erythrocytes by hypotonic lysis. In addition, it should be noted that other investigators, who did not use the NH_4Cl lysis technique, have also observed a number of different neutrophil responses, such as O_2^- generation (7), chemotaxis (4), and degranulation (7, 53), to be dependent on external Na.

In summary, the data presented in this study indicate a modulating effect of intracellular pH on O_2^- generation by FMLP-activated human neutrophils. These results suggest that other functional responses of these cells may be regulated by their pH_i.

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