Intracellular Acidification-induced
Alkali Metal Cation/H⁺ Exchange
in Human Neutrophils

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ABSTRACT Pretreatment of isolated human neutrophils (resting pHᵢ ≈ 7.25 at pHₒ 7.40) with 30 mM NH₄Cl for 30 min leads to an intracellular acidification (pHᵢ ≈ 6.60) when the NH₄Cl prepulse is removed. Thereafter, in 140 mM Na⁺ medium, pHᵢ recovers exponentially with time (initial rate, ~0.12 pH/min) to reach the normal resting pHᵢ by ~20 min, a process that is accomplished mainly, if not exclusively, though an exchange of internal H⁺ for external Na⁺. This Na⁺/H⁺ countertransport is stimulated by external Na⁺ (Kₘ ≈ 21 mM) and by external Li⁺ (Kₘ ≈ 14 mM), though the maximal transport rate for Na⁺ is about twice that for Li⁺. Both Na⁺ and Li⁺ compete as substrates for the same translocation sites on the exchange carrier. Other alkali metal cations, such as K⁺, Rb⁺, or Cs⁺, do not promote pHᵢ recovery, owing to an apparent lack of affinity for the carrier. The exchange system is unaffected by ouabain or furosemide, but can be competitively inhibited by the diuretic amiloride (Kᵢ ≈ 8 μM). The influx of Na⁺ or Li⁺ is accompanied by an equivalent counterefflux of H⁺, indicating a 1:1 stoichiometry for the exchange reaction, a finding consistent with the lack of voltage sensitivity (i.e., electroneutrality) of pHᵢ recovery. These studies indicate that the predominant mechanism in human neutrophils for pHᵢ regulation after intracellular acidification is an amiloride-sensitive alkali metal cation/H⁺ exchange that shares a number of important features with similar recovery processes in a variety of other mammalian cell types.

INTRODUCTION

Neutrophils and other phagocytic cells play a vital role in normal host defense against microorganisms. Through the secretion of free radicals, degradative enzymes, and other toxic mediators of inflammation, they contribute to the pathogenesis of tissue injury in a number of diseases as well. In our previous address reprint requests to Dr. Louis Simchowitz, Dept. of Cell Biology and Physiology, Washington University School of Medicine, Box 8101, 660 S. Euclid Ave., St. Louis, MO 63110.

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report on intracellular pH (pHi) regulation in isolated human neutrophils (Simchowitz and Roos, 1985), we undertook a systematic investigation of the factors that control steady state pHi in resting cells. By identifying the ion pathways responsible for maintaining pHi homeostasis, these studies serve as a basis for examining the proposed pHi-dependent functions of these cells, which include phagocytosis, chemotaxis, superoxide radical generation, and degranulation (Segal et al., 1981; Klempner and Styrt, 1983; Simchowitz, 1985a; Simchowitz and Cragoe, 1986a). In our initial report (Simchowitz and Roos, 1985), we showed that human neutrophils possess two separate regulatory mechanisms for pHi homeostasis: (a) an Na⁺/H⁺ exchange that restores pHi to its normal resting value (~7.25 at pH₀ 7.40) after imposed acidification, and (b) a Cl⁻/HCO₃⁻ exchange that comes into play after imposed alkalinization.

The Na⁺/H⁺ exchange activity of human neutrophils exhibits two functionally distinct modes of operation, both of which seem to be quiescent in resting cells. The first, alluded to above, is activated by intracellular acidification to bring about pHi recovery to the normal steady state level (Grinstein and Furuya, 1984, 1986; Simchowitz and Roos, 1985). The second is stimulated by a variety of inducing agents, among them chemotactic factors and phorbol diesters. However, activation by these means leads to an intracellular alkalinization above the resting value (Molski et al., 1980; Sha’afi et al., 1981, 1982; Grinstein and Furuya, 1984; Grinstein et al., 1985; Simchowitz, 1985a-c). As noted above, this elevation of pHi has important implications for the functional behavior of these cells.

An Na⁺/H⁺ exchange mechanism has been implicated in the recovery from acidification in a variety of mammalian cell types, including rat lymphocytes (Grinstein et al., 1984), mouse soleus muscle fibers (Aickin and Thomas, 1977), sheep cardiac Purkinje fibers (Deitmer and Ellis, 1980), hamster lung fibroblasts (L’Allemain et al., 1984b), human epidermoid carcinoma cells (Rothenberg et al., 1983), and human neutrophils (Grinstein and Furuya, 1984, 1986; Simchowitz and Roos, 1985). In our previous study (Simchowitz and Roos, 1985), we examined the ionic basis of pHi recovery after imposed acid loads. After the initial fall in pHi, recovery could be completely ascribed to an Na⁺/H⁺ counter-transport, i.e., an exchange of external Na⁺ for internal H⁺. This conclusion was based on the observations that either removal of extracellular Na⁺ or the addition of amiloride, a well-recognized inhibitor of Na⁺/H⁺ exchange (Benos, 1982; Aronson, 1983), reduced the pHi recovery rate by ≥90%. The same conclusion was arrived at independently by Grinstein and Furuya (1984, 1986) on the basis of similar experimental evidence.

The purpose of the present work is to examine the properties of this acidification-induced Na⁺/H⁺ exchange in more detail. Specifically, we sought to characterize the interactions of this transport protein with respect to its substrates within the alkali metal cation series and to document more fully the nature of its sensitivity to amiloride and several selected analogues. For technical convenience, we chose to study a system where neutrophils were acidified by the NH₄Cl prepulse technique because of the faster rate and larger extent of the total pHi transient as compared with that after exposure to CO₂.

We find that external Na⁺ stimulates the Na⁺/H⁺ exchanger of human neutro-
phils with a $K_m$ of $\approx 21$ mM and that $\text{Li}^+$ ($K_m \approx 14$ mM) can serve as an effective replacement ion for $\text{Na}^+$. In contrast, $\text{K}^+$, $\text{Rb}^+$, and $\text{Cs}^+$ are unable to promote pH, recovery owing to a lack of affinity for the external translocation site of the carrier. The interactions of $\text{Na}^+$ and $\text{Li}^+$ with the exchanger can be competitively inhibited by amiloride ($K_i \approx 8 \mu M$) and by several of its more potent analogues, a finding in common with $\text{Na}^+/\text{H}^+$ exchangers of other cells (Vigne et al., 1984; L’Allemain et al., 1984a; Zhuang et al., 1984).

MATERIALS AND 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 crystalline bovine serum albumin. For studies involving $\text{Li}^+$, LiCl replaced NaCl. To test the effects of varying the external $\text{Na}^+$ or $\text{Li}^+$ concentrations ($[\text{Na}^+]_o$ or $[\text{Li}^+]_o$), media of appropriate $[\text{Na}^+]_o$ or $[\text{Li}^+]_o$ were prepared by equimolar substitution of N-methyl-D-glucamine for Na$^+$ or Li$^+$. For the NH$_4$Cl prepulse, solutions, which were $\text{Na}^+$ free (see below), contained 30 mM NH$_4$Cl, 110 mM N-methyl-D-glucamine HCl, and normal amounts of other ions.

Neutrophils

Human peripheral neutrophils were isolated by sequential dextran sedimentation at 37°C followed by Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) gradient centrifugation at room temperature (Boyum, 1968). Contaminating erythrocytes were removed by hypotonic lysis in distilled water for 30 s. The neutrophils were washed three times and then counted. The purity of the neutrophil suspensions averaged 98%, as judged by Wright’s staining. Viability averaged 99% as assessed by eosin Y exclusion, and was not affected by any of the agents or incubation conditions tested. The cells were routinely isolated using unrefrigerated centrifuges and medium that was warmed to 37°C. In addition, the cells were kept at 37°C before and during all assays.

The intracellular $\text{Na}^+$ concentration ($[\text{Na}^+]_i$) of steady state human neutrophils bathed in a 5 mM K$^+$, 140 mM Na$^+$ medium is 25–30 meq/liter cell water (Simchowitz et al., 1982; Simchowitz, 1985a; Grinstein and Furuya, 1986). However, all studies in this article were routinely performed using internally $\text{Na}^+$-depleted cells ($[\text{Na}^+]_i \leq 2$ meq/liter cell water), prepared by incubating them for $\approx 1$ h in $\text{Na}^+$-free, 5 mM K$^+$, 140 mM N-methyl-D-glucamine medium. The cells were then treated with 30 mM NH$_4$Cl (5 mM K$^+$, 110 mM N-methyl-D-glucamine) for 30 min as previously described (Simchowitz and Roos, 1985). Thus, the cells had been kept in $\text{Na}^+$-free media for a total of $\approx 1.5$ h before experimentation. As will be discussed later (see below), these cells maintained a normal cell volume as monitored by sensitive electronic cell-sizing techniques.

The rationale for the use of $\text{Na}^+$-depleted cells is as follows: for the studies involving Li$^+$ fluxes, we sought to constrain the exchanger to engage exclusively in net Li$^+/\text{H}^+$ countertransport and thus avoid the concurrent exchange of internal Na$^+$ for external Li$^+$, since the latter would serve to complicate the quantitative comparisons that arise from simultaneous measurements of Li$^+$ and H$^+$ fluxes. In preliminary experiments, we had ascertained that the $K_m$ for internal Na$^+$ activation of $^{22}$Na$^+$ influx and the apparent $K_i$ for external Li$^+$ inhibition of $^{22}$Na$^+$ influx from a 1 mM Na$^+$ medium were the same in normal Na$^+$ as compared with Na$^+$-depleted cells. Thus, it is unlikely that the binding characteristics of the external translocation sites of the Na$^+/\text{H}^+$ exchange carrier were materially affected by internal Na$^+$ depletion.)
Reagents

All inorganic salts were obtained from Fisher Scientific Co., St. Louis, MO. The following reagents were purchased from Sigma Chemical Co., St. Louis, MO: crystalline bovine serum albumin, N-methyl-D-glucamine, ouabain, HEPES, and 5,5-dimethylloxazolidine-2,4-dione (DMO). Isotopes $^{22}\text{NaCl}$, $[^{14}\text{C}]\text{DMO}$, $[^{3}\text{H}]\text{H}_{2}\text{O}$, and $[^{14}\text{C}]\text{inulin}$ were purchased from New England Nuclear, Boston, MA; $^{22}\text{Na}^+$ was carrier free. Amiloride and its analogues were supplied by the Merck, Sharp & Dohme Research Institute, West Point, PA; the compounds had been synthesized as previously described (Cragoe et al., 1967; Cragoe, 1983).

Intracellular Ion Concentrations

Intracellular $\text{Li}^+$ ($[\text{Li}^+]_i$) and $\text{Na}^+$ ($[\text{Na}^+]_i$) concentrations were measured by flame photometry, as follows. Neutrophils ($10^7$/ml) were suspended in the experimental media described above. Triplicate aliquots were layered on 0.5 ml silicone oil (Versilube F-50, Harwick Chemical Corp., Akron, OH) contained in 1.5 ml plastic tubes and centrifuged for 1 min at 8,000 g in a microcentrifuge (Beckman Instruments, Inc., Fullerton, CA). Cell separation occurred in <5 s. The aqueous and oil phases were aspirated and discarded. The neutrophil pellets were lysed in 1.0% Triton X-100 in water (vol/vol) and analyzed for $\text{Li}^+$ or $\text{Na}^+$ in a flame photometer (Gilford Instruments, Oberlin, OH) using an internal Cs standard. Results are expressed in milliequivalents per liter of cell water. In separate experiments, using $[^{3}\text{H}]\text{H}_{2}\text{O}$ and $[^{14}\text{C}]\text{inulin}$ as markers for total and extracellular water, respectively, we determined cell water content to be $274 \pm 15$ (SEM) fl/cell (Simchowitz et al., 1982; Simchowitz and De Weer, 1986).

Unidirectional $^{22}\text{Na}^+$ Fluxes

The technique described by Naccache et al. (1977) was used. The incubations were performed at $37^\circ\text{C}$ in capped, plastic tubes (Falcon Plastics, Oxnard, CA) under various experimental conditions (neutrophils $8-12 \times 10^9$/ml). Influx experiments were performed in the presence of $^{22}\text{NaCl}$ (1.5 $\mu$Ci/ml). At stated intervals, triplicate aliquots of the cell suspensions were layered on silicone oil and centrifuged as described above. The neutrophil pellets were excised and counted in a gamma counter. Each individual uptake experiment was normalized to zero counts per minute at zero time by subtracting, from each data point, the zero-time value obtained in the absence of stimuli. This was done since the zero-time value, which represents label present in the extracellular space, sometimes varied considerably from day to day. For the efflux studies, neutrophils were first suspended at $2-3 \times 10^7$/ml and incubated with $^{22}\text{NaCl}$ (5 $\mu$Ci/ml) for 1-2 h at $37^\circ\text{C}$, after which the cells were washed twice in unlabeled medium. Triplicate samples were then taken at stated intervals and handled as described above. The data were normalized to 1.0 at zero time for each experiment by dividing the counts per minute at any time by the number of counts per minute present in the cell pellet at zero time. All flux units are given as milliequivalents per liter of cell water per minute (meq/liter·min).

Intracellular pH Measurements

We derived $\text{pH}_i$ from the distribution of the $^{14}\text{C}$-labeled weak acid DMO ($pK_a$ 6.15 at $37^\circ\text{C}$; Boron and Roos, 1976). The DMO method is based on the assumption that only the uncharged form is permeant, so that, at equilibrium, its concentration in cell water is equal to that in the external solution (see Roos and Boron, 1981, for review). As previously reported by our group (Simchowitz and Roos, 1985), the time required for DMO to equilibrate between neutrophils and medium is $\geq 15$ s. Thus, the probe is suitable for kinetic analyses during which $\text{pH}_i$ is changing rapidly.
Samples of the neutrophil suspensions (8–12 × 10⁶ cells/ml), containing [¹⁴C]DMO (1.0 μCi/ml), were incubated in plastic tubes at 37°C under various experimental conditions. The indicator concentration was 0.1 mM, which does not affect pHi significantly. At intervals, triplicate aliquots were layered on silicone oil and centrifuged as described above. The neutrophil pellets were isolated, placed in scintillation vials, and counted in a liquid scintillation counter (LS 7000, Beckman Instruments, Inc.) after addition of 10 ml of Aquasol-2 (New England Nuclear).

The indicator content of the cells was obtained after correction for the medium that was trapped in the pellet. Using [³²H]H₂O to estimate the total water space and, in separate aliquots, [¹⁴C]inulin to correct for trapped extracellular water, the intracellular water volume was determined. Internal pH was then calculated from the reworking of the Henderson-Hasselbalch equation (Roos and Boron, 1981).

**Data Analysis**

In many cases, the influx of ²²Na⁺, corrected for zero-time "uptake," followed equations of the form:

\[ C_t = C_\infty \left[ 1 - \exp(-kt) \right], \]

where \( C_t \) is the cell label at time \( t \), \( C_\infty \) is the cell label at steady state, and \( k \) is the rate coefficient. Eq. 1 was fitted to the data by a nonlinear least-squares program, and the initial influx rate was computed from the product \( kC_\infty \). Similar fits to the data were applied in the analysis of the pHi transients and of the [Na⁺] and [Li⁺] determinations by flame photometry. (The time course of pHi recovery and the associated Na⁺ and Li⁺ influxes might be expected to represent a complex function where the rate coefficient is constantly changing [e.g., decreasing as pHi approaches the resting value] rather than one that could be described by a simple exponential equation. However, this does not seem to be the case. As reported by Grinstein and Furuya [1984, 1986] and by our group [Simchowitz and Roos, 1985], the rate of recovery is a direct function of the degree of intracellular acidification between 6.5 and 7.2. Recovery is therefore linearly proportional to the magnitude of the fall in pHi from ~7.25, the proportionality constant in this case being the rate coefficient.) As indicated in the figure legends, the change in some of the measured variables often appeared to be linear over the period of study; in those cases, the influx rate was computed from the slope of the linear regression line.

The ²²Na⁺ effluxes followed single exponentials of the form:

\[ C_t = C_0 \exp(-kt), \]

where \( C_0 \) is the cell label at zero time. Curves representing the equation were fitted to the data by the least-squares method.

**RESULTS**

**Intracellular pH Studies**

For all of the experiments described herein, Na⁺-depleted neutrophils were pretreated with 30 mM NH₄Cl in 5 mM K⁺, 110 mM N-methyl-D-glucamine medium, pH 7.40. Under these conditions, pHi rises to ~7.65 immediately after NH₄Cl application and then recovers to its normal resting value over the ensuing 30-min interval during maintained NH₄Cl exposure (Simchowitz and Roos, 1985). The recovery represents the addition of H⁺ equivalents to the cytoplasm. This H⁺ accumulation leads to a pHi undershoot when the NH₄Cl prepulse is
removed by pelleting the cells and then resuspending them in NH₄Cl-free solutions. This technique was used routinely in all subsequent studies to acidify the cell interior, which in turn activates Na⁺/H⁺ exchange to restore pHᵢ to its normal level. Since the identical procedure was followed throughout this article, the pHᵢ of cells under all treatment conditions that are being compared was the same.

The ionic basis of pHᵢ recovery after an imposed acidification, induced by removal of an NH₄Cl prepulse ("NH₄Cl undershoot"), was studied by examining the effects of a number of transport inhibitors and by different ion substitutions in the bathing medium. The time course of pHᵢ recovery after the undershoot is shown in Fig. 1. The pHᵢ immediately after NH₄Cl removal (estimated by back-extrapolation of the time course of recovery in the presence of amiloride) was ~6.62. Thereafter, in 140 mM Na⁺ medium, the pHᵢ recovered rapidly along an exponential time course (rate coefficient: 0.182 ± 0.015 min⁻¹) to reach a

**Figure 1.** Time course of DMO-derived pHᵢ upon removal of 30 mM NH₄Cl (solid lines). Results represent the means ± SEM of three to four separate experiments, each performed in triplicate. The pHᵢ was 7.40 throughout. The cells were first exposed to 30 mM NH₄Cl for 30 min and then resuspended at zero time in NH₄Cl-free solutions of the following compositions. (A) Medium containing 5 mM K⁺ and 140 mM Na⁺. The curve also uses results obtained in the presence of 0.1 mM ouabain or 0.4 mM furosemide. (B) 1 mM amiloride in 5 mM K⁺ media containing either 140 mM Na⁺ or 140 mM Li⁺. The line was fitted to both sets of data points combined. (C) 5 mM K⁺, 140 mM Li⁺ medium. (D) 25 mM Na⁺ and either 5 mM K⁺ (balance N-methyl-D-glucamine) or 120 mM K⁺. The curve was fitted to the combined data. The dashed line (E) represents the time course of pHᵢ of cells not exposed to NH₄Cl, but kept in 5 mM K⁺, 140 mM N-methyl-D-glucamine medium for 30 min. They were then resuspended in solutions identical to those described above. Since the pHᵢ's under all these conditions were indistinguishable, each of the points is the average of the various pHᵢ values. A horizontal line (curve E) was drawn at 7.29, the average of all of the control data points. Curves A, C, and D are single-exponential fits to the data, where the initial (derived from back-extrapolation of recovery in the presence of amiloride, curve B) and final pHᵢ values were 6.62 and 7.29, respectively. The initial rates (in pH units per minute) were: 0.1193 ± 0.0082, 0.0784 ± 0.0087, and 0.0634 ± 0.0062, respectively. For curve B, the data were fitted to a straight line with slope = 0.0112 ± 0.0012 pH/min.
normal resting value by 20 min of incubation: the initial recovery rate was 0.122 ± 0.010 pH/min (n = 3). This course was not significantly affected by 0.1 mM ouabain, which blocks the Na⁺/K⁺ pump, or by 0.4 mM furosemide, a loop diuretic; the initial recovery rate for the combined data (curve A) was 0.119 ± 0.008 pH/min (n = 9). In contrast, as previously reported (Simchowitz and Roos, 1985), the addition of 1 mM amiloride greatly slowed recovery (curve B): the initial rate was reduced to 0.0107 ± 0.0018 pH/min (n = 3), an inhibition of 91%. In our prior study, we also noted that recovery was dependent on the presence of Na⁺ in the external solutions: in its absence (equimolar replacement by N-methyl-D-glucamine), only a negligibly slow recovery was observed. The lack of appreciable recovery in media containing 140 mM N-methyl-D-glucamine strongly suggests that this ion is inert and cannot be transported by the carrier.

Apparently, Li⁺ can serve as an effective substitute for Na⁺ since, after the initial fall, the pH in 140 mM Li⁺ medium rose with time to reach a near-normal resting value by ~20 min (curve C). However, the initial recovery rate in Li⁺ medium was 0.0784 ± 0.0087 pH/min (n = 3), roughly two-thirds that in 140 mM Na⁺ medium (0.119 ± 0.008 pH/min, n = 9). Taking the average intrinsic intracellular buffering power (β) over the entire course of recovery as 55 mmol/liter cell water/unit pH (mM/pH; Simchowitz and Roos, 1985), these initial pH recovery rates signify H⁺ efflux rates of 6.55 ± 0.45 and 4.31 ± 0.47 meq/liter cell water/min in 140 mM Na⁺ and 140 mM Li⁺ medium, respectively. As with Na⁺, pH recovery in 140 mM Li⁺ was markedly suppressed by the addition of 1 mM amiloride (curve B): the recovery rate was reduced to 0.0117 ± 0.0017 pH/min (n = 3).

In other cell types, alkali metal cation/H⁺ exchange is electroneutral, a finding compatible with the observed 1:1 stoichiometry of the countertransport reaction (Rindler et al., 1979; Kinsella and Aronson, 1980, 1982; Moolenaar et al., 1981; Burnham et al., 1982; Grinstein et al., 1984). This also appears to be the case in neutrophils since pH recovery is independent of large changes in membrane voltage: at [Na⁺]₀ = 25 mM, the kinetics of recovery were indistinguishable at 5 mM K⁺ as compared with 120 mM K⁺ (curve D), where the membrane potential is approximately −60 and 0 mV (Simchowitz et al., 1982), respectively. As will be discussed immediately below, the slower recovery rate at 25 vs. 140 mM Na⁺ (curve D vs. curve A) results from decreased saturation of the carrier at the lower [Na⁺]₀.

This is shown more clearly in the next series of experiments (Fig. 2), where the alkali metal cation selectivity of the exchange reaction was investigated. For these studies, after pretreatment with 30 mM NH₄Cl, the cells were resuspended in NH₄Cl-free media, where the external concentrations of Na⁺, Li⁺, K⁺, Rb⁺, or Cs⁺ were varied between 0 and 140 mM (equivalent replacement by N-methyl-D-glucamine). As [Na⁺]₀ was raised, the initial rate of pH recovery increased along a Michaelis-Menten activation curve with an apparent $K_m(\text{Na}^+)$ of 30.8 ± 9.8 mM. Similarly, external Li⁺ stimulated the rate of recovery with an apparent $K_m(\text{Li}^+)$ of 11.2 ± 4.0 mM. However, the maximal transport rate for Li⁺, 0.0942 ± 0.0095 pH/min (H⁺ efflux rate: 5.18 ± 0.52 meq/liter·min), was substantially slower than that for Na⁺, 0.158 ± 0.018 pH/min (H⁺ efflux rate: 8.69 ± 0.99
meq/liter·min). Considering the apparently higher affinity exhibited for Li$^+$ as opposed to Na$^+$ binding to the external translocation site of the exchanger, the difference in $V_{\text{max}}$ values satisfactorily accounts for the twofold-slower rate of pH$_i$ recovery in 140 mM Li$^+$ as compared with 140 mM Na$^+$ (Fig. 1). Also shown in Fig. 2 is the absence of significant pH$_i$ recovery in 140 mM K$^+$, Rb$^+$, or Cs$^+$ media. The inefficacy of these three ions probably relates to a lack of affinity for the carrier (see below).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effect of different alkali metal cations on the rate of amiloride-sensitive pH$_i$ recovery after removal of an NH$_4$Cl prepulse. After a 30-min pretreatment with NH$_4$Cl, the cells were resuspended in 5 mM K$^+$ media in which the concentration of each of the ions (Na$^+$, Li$^+$, K$^+$, Rb$^+$, and Cs$^+$) was varied between 0 and 140 mM by equimolar replacement with N-methyl-D-glucamine. The DMO-derived pH$_i$ was measured at two or more times (between 1 and 10 min, as appropriate) during the course of pH$_i$ recovery. The initial recovery rates were calculated by fitting the pH$_i$ data points to single-exponential equations (Eq. 1) as in Fig. 1. Results represent three experiments in which the initial pH$_i$ recovery rates from intracellular acidification are plotted as a function of the external concentration of each ion. For convenience, the corresponding H$^+$ efflux rates (in meq/liter·min), calculated by multiplying the pH$_i$ recovery rates by the average intracellular buffering power, 55 mM/pH (Simchowitz and Roos, 1985), are also given as the right-hand ordinate. The activation curves for Na$^+$ and Li$^+$ follow Michaelis-Menten equations: for Na$^+$, the least-squares parameters are $K_m = 30.8 \pm 9.8$ mM and $V_{\text{max}} = 0.158 \pm 0.018$ pH/min (H$^+$ efflux = 8.69 ± 0.99 meq/liter·min); for Li$^+$, $K_m = 11.2 \pm 4.0$ mM and $V_{\text{max}} = 0.0942 \pm 0.0095$ pH/min (H$^+$ efflux = 5.18 ± 0.52 meq/liter·min). The recovery rates in K$^+$, Rb$^+$, and Cs$^+$ were similar and indistinguishable from zero: the combined set of data was fitted to a straight line with a least-squares slope of 0.000021 ± 0.000017 pH/min.

The data of Fig. 1 indicate that 1 mM amiloride nearly abolishes pH$_i$ recovery in 140 mM Na$^+$ or Li$^+$. The dose dependences of amiloride inhibition in either 140 mM Na$^+$ or 140 mM Li$^+$ media were examined next (Fig. 3). In both cases, increasing the concentration of the drug between 0 and 4,000 μM led to a progressive reduction in the rates of pH$_i$ recovery. Each set of data could be fitted to a Michaelis-Menten inhibition equation, which yielded apparent $K_i$ values for amiloride of 68.2 ± 19.5 and 101.4 ± 28.4 μM in 140 mM Na$^+$ and
Li⁺ media, respectively. This inhibition is of the competitive type, as is demonstrated in the next set of experiments. Also displayed in Fig. 3 is the dose dependence of amiloride inhibition of pHᵢ recovery as a function of [Na⁺]₀. Reducing [Na⁺]₀ from 140 to 60 to 20 mM caused a shift in the apparent Kᵢ for amiloride to lower values (at [Na⁺]₀ = 140, 60, or 20 mM, the apparent Kᵢ = 68.2 ± 19.5, 37.9 ± 12.9, and 16.4 ± 6.8 μM, respectively), which indicates an increase in apparent affinity. This is shown in another way (inset to Fig. 3), where these apparent Kᵢ values for amiloride are graphed against [Na⁺]₀. This linear relationship is to be expected if Na ions were acting as competitive inhibitors of amiloride to its binding site. The true Kᵢ(amiloride) extrapolates to 9.72 ± 1.34 μM, and the inhibitory dissociation constant for Na⁺ is 22.9 ± 9.4 mM. The latter value compares favorably with the Kᵢ for Na⁺ derived from the [Na⁺]₀ dependence of pHᵢ recovery (30.8 mM, Fig. 2).

**Na⁺ and Li⁺ Movements**

The results presented thus far are consistent with the hypothesis that pHᵢ recovery after an NH₄Cl prepulse can be ascribed to an amiloride-sensitive exchange of external Na⁺ for internal H⁺ and that Li⁺ can serve as an effective substitute for Na⁺ in the countertransport reaction. In this section, we comple-
ment the studies on pH recovery with direct measurements of Na⁺ and Li⁺ fluxes.

*Internal ion contents and kinetics of influx.* The time courses of unidirectional ⁴²Na⁺ influx and net influxes of Na⁺ and Li⁺ (by flame photometry) from 140 mM media during recovery after the NH₄Cl undershoot are presented in Fig. 4. In each instance, the kinetics followed a single-exponential equation. As assessed by the uptake of ⁴²Na⁺ (Fig. 4A) and by chemical determinations of Na⁺ (Fig. 4B), the internal Na⁺ content rose at initial rates of 8.33 ± 1.17 and 7.87 ± 1.54
meq/liter·min (final extrapolated uptakes: 48.7 ± 2.7 and 55.0 ± 4.5 meq/liter cell water, respectively) to reach near-steady levels at 15–20 min.

The analysis of the Li+ influx data of Fig. 4C is slightly different from that involving Na+. In the case of Li+, leak influx into unstimulated cells is linear over the first hour (rate, ~0.45 meq/liter·min; unpublished observations), whereas passive Na+ leak influx rises exponentially toward a final value of ~30 meq/liter cell water, the normal resting [Na+]i (Simchowitz et al., 1982; Simchowitz, 1985b). Therefore, total Li+ influx was fitted as the sum of an exponenti-

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**Figure 4.** (opposite) Internal ion concentrations and time course of tracer and net Na+ and Li+ movements in neutrophils during pH, recovery from acidification. Aliquots of a neutrophil suspension were preincubated for 30 min in 30 mM NH4Cl, 5 mM K+, and 110 mM N-methyl-D-glucamine medium (solid lines). Controls (dashed line) were kept in 5 mM K+, 140 mM N-methyl-D-glucamine medium. After pelleting by centrifugation, the cells were resuspended at zero time either in 140 mM Na+, labeled with (panel A) or without (panel B) 1.5 μCi/ml 22Na+, or in 140 mM Li+ medium (panel C) in the presence (squares) or absence (circles) of 1 mM amiloride. The reaction mixtures were further incubated at 37°C and, at stated times, the cell pellets were assayed for radioactivity or for their internal Na+ or Li+ contents by flame photometry. Results represent the means ± SEM of four to five separate experiments, each performed in triplicate. (A) 22Na+ influx. The upper two curves (A and B, solid lines) were fitted by single exponentials. Curve A: initial influx rate = 8.33 ± 1.17 meq/liter·min and maximal uptake = 48.7 ± 2.7 meq/liter cell water. Curve B: initial influx rate = 1.28 ± 0.13 meq/liter·min. Curve C (dashed line) was fitted to the combined control and amiloride data by a single exponential where the final 22Na+ uptake was set equal to 30.0 meq/liter cell water, the normal resting intracellular Na+ concentration (Simchowitz et al., 1982; Simchowitz, 1985b). The initial influx rate was 0.987 ± 0.101 meq/liter·min. Thus, the initial 22Na+ influx rate due to Na+/H+ exchange following the NH4Cl undershoot was 8.33 ± 1.17 (curve A) minus 0.99 ± 0.10 (curve C) = 7.34 ± 1.27 meq/liter cell water.

(B) Na+ influx measured by flame photometry. See panel A above. Curve A: initial influx rate = 7.87 ± 1.54 meq/liter·min, maximal uptake = 55.0 ± 4.5 meq/liter cell water. For curves B and C, the initial influx rates are 1.29 ± 0.11 and 0.884 ± 0.076 meq/liter·min, respectively. The initial Na+ influx that may be ascribed to Na+/H+ exchange after the NH4Cl undershoot was 7.87 ± 1.54 (curve A) − 0.88 ± 0.08 (curve C) = 6.99 ± 1.62 meq/liter·min; that which can be attributed to Na+/H+ exchange in the presence of 1 mM amiloride was 1.29 ± 0.11 (curve B) − 0.88 ± 0.08 (curve C) = 0.41 ± 0.19 meq/liter·min. (C) Li+ influx measured by flame photometry. Curve A was fitted to the data as the sum of a single-exponential component, presumably reflecting Li+/H+ exchange, and a linear slope, equal to the control uptake, representing passive leak influx. The parameters of the exponential component were: initial influx rate = 4.01 ± 1.07 meq/liter·min and maximal uptake = 42.7 ± 11.2 meq/liter cell water; the slope was taken as 0.48 meq/liter·min (see below). Curves B and C were fitted to the individual sets of data as straight lines with slopes of 0.919 ± 0.141 and 0.479 ± 0.042 meq/liter·min, respectively. For curve C, the combined control and amiloride data were used. Thus, the initial Li+ influx rate that can be attributed to Li+/H+ exchange in the presence of 1 mM amiloride was 0.919 ± 0.141 (curve B) minus 0.479 ± 0.042 (curve C) = 0.440 ± 0.183 meq/liter·min.
tial component, presumably reflecting Li+/H+ exchange, and a linear slope, the leak flux. The acidification-induced rates given immediately below represent those for total influx. In all other instances, as in the figure legends and text to follow, the influx rates given for Na+ as well as for Li+ are those from which the leak fluxes have been subtracted. By contrast to Na+, the Li+ influx from a 140 mM Li+ medium was somewhat slower (total initial influx rate, 4.49 ± 1.07 meq/liter·min; final extrapolated uptake of the exponential component, 42.7 ± 11.2 meq/liter·min·cell water).

The near-steady internal Na+ contents at 20 min (46.8 ± 2.7 and 51.4 ± 4.8 meq/liter·cell water in A and B, respectively, of Fig. 4) represent maximal estimates for the uptake of Na+ through Na+/H+ exchange during that time period. The true extent of Na+ influx via Na+/H+ exchange is, however, overestimated as a result of Na+ accumulated by electrodiffusion. Taking net passive Na+ influx (Na+ efflux is negligibly small from these initially Na+-depleted cells) as ~0.9 meq/liter·min (control, Fig. 4, A and B) and subtracting the appropriate leak influx (i.e., 13.5 meq/liter·cell water at 20 min) from the total Na+ contents at 20 min, one derives values of 33.3 and 37.9 meq/liter·cell water for Na+ uptake through Na+/H+ exchange. Assuming an equivalent counterefflux of H+ and an average intrinsic intracellular buffering power of 55.0 mM/pH over the entire course of pH recovery (Simchowitz and Roos, 1985), an alkalinization of 0.61–0.69 pH units would be predicted at 20 min. These values are in good agreement with the 0.66 unit pH recovery actually observed (Fig. 1). A similar calculation for Li+ (taking leak influx as 0.48 meq/liter·min; Fig. 4C) gives an expected pH recovery of 0.66 units at 20 min, comparable to that actually observed (0.61 units; Fig. 1).

The 22Na+ influx rate, as well as the initial uptake rates of Na+ and Li+ measured chemically, were markedly inhibited (by ~90%) in the presence of 1 mM amiloride (Fig. 4, A–C). Again, this is as expected since these fluxes were mediated principally by transport through a Na+/H+ or Li+/H+ exchange mechanism that is activated by intracellular acidification.

From the data of Fig. 4, the mean total initial Na+ and Li+ influx rates from 140 mM media are 8.33 or 7.87 and 4.49 meq/liter·min, respectively. Subtracting the passive leak influx rates (0.99 or 0.88, and 0.48 meq/liter·min for Na+ and Li+, respectively), one calculates values of 7.34 or 6.99, and 4.01 meq/liter·min for Na+ and Li+ influx rates owing to Na+/H+ or Li+/H+ exchange. These rates compare favorably with the computed initial H+ efflux rates, 6.55, 7.24, and 6.82 (for Na+) and 4.31, 5.01, and 5.43 (for Li+) meq/liter·min·derived from the initial pH recovery rates in 140 mM Na+ and 140 mM Li+ media (Figs. 1–3).

Substrate Selectivity

Substrate saturation is shown in Fig. 5, where the external Na+ dependence of 22Na+ influx is depicted. The relationship obeyed saturation kinetics: as [Na+]o was raised from 1.25 to 140 mM (replacing N-methyl-D-glucamine), the initial rate of 22Na+ influx rose along a Michaelis-Menten activation curve, with an apparent Km(Na+) of 20.5 ± 5.5 mM, similar to the value of 30.8 ± 9.8 mM
derived from the $[\text{Na}^+]_o$ dependence of pH$_i$ recovery (Fig. 2). Similarly, external Li$^+$ stimulated its own rate of influx in a saturable fashion, with an apparent $K_m(\text{Li}^+)$ of $15.8 \pm 6.4$ mM. This can be compared with the value of $11.2 \pm 4.0$ mM obtained from the dependence of the pH$_i$ recovery rate on external Li$^+$ (Fig. 2). In harmony with several other determinations presented in this study, the maximal transport rate for Li$^+$ ($6.20 \pm 0.76$ meq/liter·min) was somewhat less than that for Na$^+$ ($9.47 \pm 0.83$ meq/liter·min).

Substrate interactions between Na$^+$ and Li$^+$ are documented in Fig. 6, where the ability of external Li$^+$ to compete with Na$^+$ at the external transport site of the exchanger was tested. In these experiments, $^{22}\text{Na}^+$ influx from a 1 mM Na$^+$

Figure 5. Stimulation of $^{22}\text{Na}^+$ and Li$^+$ influx as a function of the extracellular concentrations of Na$^+$ and Li$^+$. See legend to Fig. 4. Experiments were performed in 5 mM K$^+$ media in which either $[\text{Na}^+]_o$ or $[\text{Li}^+]_o$ was varied between 1.25 and 140 mM by equimolar replacement with N-methyl-d-glucamine. The uptakes of $^{22}\text{Na}^+$ or Li$^+$ (by flame photometry) were measured as described in Fig. 2, and the initial influx rates were calculated by fitting the internal Na$^+$ and Li$^+$ contents to single-exponential equations (Eq. 1). The data points were then fitted to Michaelis-Menten activation curves. The equations yielded the following kinetic parameters: for Na$^+$, apparent $K_m = 20.5 \pm 5.5$ mM and $V_{\text{max}} = 9.47 \pm 0.83$ meq/liter·min; for Li$^+$, apparent $K_m = 15.8 \pm 6.4$ mM and $V_{\text{max}} = 6.20 \pm 0.76$ meq/liter·min. Results are from three experiments.

medium was measured in the presence of 0–140 mM Li$^+$. Increasing $[\text{Li}^+]_o$ between 0 and 140 mM resulted in a progressive reduction in the rate of $^{22}\text{Na}^+$ entry into the cells with an apparent $K_m(\text{Li}^+)$ of $9.55 \pm 1.92$ mM. This is similar to the values of $11.2 \pm 4.0$ and $15.8 \pm 6.4$ mM for the apparent $K_m$ values for external Li$^+$ activation of pH$_i$ recovery and Li$^+$ influx, respectively (Figs. 2 and 5). This equality is as expected for a system characterized by competition kinetics, where the relationship between the true and apparent $K_m$ values for a substrate $S_1$ in the presence of a competing substrate $S_2$ is given by:

$$\text{apparent } K_m(S_1) = \text{true } K_m(S_1) \left[ 1 + \frac{[S_2]}{K_m(S_2)} \right]$$

(3)
Entering the appropriate values for Na\(^+\) (i.e., \([S_2] = 1\) mM and \(K_m(S_2) \approx 25\) mM), the apparent \(K_m\) for Li\(^+\) (i.e., \([S_1]\) should be nearly the same as the true \(K_m\) for Li\(^+\) measured in the absence of Na\(^+\), since \([Na^+]_o\) is \(\ll K_m(Na^+)\). Also shown in Fig. 6 is the complete absence of effect on \(^{22}\)Na\(^+\) influx of K\(^+\), Rb\(^+\), and Cs\(^+\) at concentrations ranging from 10 to 140 mM in the external solutions. In analogy to other cell types (Kinsella and Aronson, 1980; Burnham et al., 1982; Grinstein et al., 1984), the Na\(^+\)/H\(^+\) exchanger of human neutrophils appears to lack affinity for these cations.

The data of Fig. 6 show that external Li\(^+\) competitively inhibits \(^{22}\)Na\(^+\) influx. The converse also holds (Fig. 7): the initial rate of Li\(^+\) influx from a 40 mM Li\(^+\) medium is inhibited by external Na\(^+\), with an apparent \(K_m(Na^+)\) of 72.6 ± 19.0 mM. Again, this is as expected from Eq. 3 since the true \(K_m(Na^+)\) is \(\sim 25\) mM and the true \(K_m(Li^+)\) is \(\sim 15\) mM.

**Inhibition by Amiloride and Its Analogues**

In Fig. 4, we observed that 1 mM amiloride reduced the influx rates of Na\(^+\) and Li\(^+\) by \(-90\%\). The inhibitory effect of amiloride is further shown in Fig. 8, where the initial rates of \(^{22}\)Na\(^+\) or Li\(^+\) influx from 140 mM Na\(^+\) or Li\(^+\) media are plotted against the external concentration of amiloride (0–2,000 \(\mu\)M). In both instances, as the amount of added drug was gradually raised, the rates of
$^{22}\text{Na}^+$ and $\text{Li}^+$ influx were progressively reduced. The relationship could be fitted by Michaelis-Menten kinetics, with apparent $K_i$ values for amiloride of 51.2 ± 12.9 and 87.3 ± 15.9 μM in 140 mM $\text{Na}^+$ and 140 mM $\text{Li}^+$, respectively. These determinations are in good agreement with the apparent $K_i$ values of 68.2 ± 19.5 and 101.4 ± 28.4 μM measured for amiloride inhibition of pH$_i$ recovery in 140 mM $\text{Na}^+$ and $\text{Li}^+$ medium, respectively (Fig. 3).

**Figure 7.** Inhibition by external Na$^+$ of Li$^+$ influx from a 40 mM Li$^+$ medium. After the NH$_4$Cl prepulse, the cells were resuspended in 5 mM K$^+$, 40 mM Li$^+$ medium containing various concentrations of Na$^+$ (0–100 mM, replacing N-methyl-~glucamine). The influx of Li$^+$ was measured and the initial influx rates were calculated from Eq. 1. The curve is a least-squares fit of a Michaelis-Menten inhibition equation to the data. The apparent $K_i$(Na$^+$) is 72.6 ± 19.0 mM. Results are from three experiments.

**Figure 8.** Inhibition by amiloride and three of its analogues of $^{22}\text{Na}^+$ and $\text{Li}^+$ influx into Na$^+$-depleted neutrophils from 140 mM Na$^+$ or Li$^+$ medium. Cells were exposed to 30 mM NH$_4$Cl for 30 min and then resuspended in 140 mM Na$^+$ medium (labeled with 1.5 μCi/ml $^{22}\text{Na}^+$) or in 140 mM Li$^+$ medium, each in the presence of various concentrations of amiloride (0–2,000 μM). Also shown are the effects of three amiloride derivatives: compounds I, O, and MM, the 5-N,N-dimethyl, 5-N,N-diethyl, and 5-N,N-hexamethylene analogues of amiloride, respectively. Controls, which had been kept in 140 mM N-methyl-d-glucamine medium throughout, were otherwise handled in the same manner. Influxes were measured as in Fig. 4 and the initial influx rates were computed from Eq. 1. The curves are least-squares fits of Michaelis-Menten inhibition equations to the data. The equations yielded apparent $K_i$ values for amiloride of 51.2 ± 12.9 and 87.3 ± 15.9 μM in 140 mM Na$^+$ or Li$^+$ medium, respectively; the apparent $K_i$ values for compounds I, O, and MM in 140 mM Na$^+$ medium were 6.39 ± 1.39, 0.646 ± 0.145, and 0.105 ± 0.029 μM, respectively. Results are from three experiments.
In addition, the data of Fig. 8 document the effects of three more potent amiloride derivatives, compounds I, O, and MM (the 5-N,N-dimethyl, 5-N,N-diethyl, and 5-N,N-hexamethylene analogues of amiloride, respectively) on $^{22}\text{Na}^+$ influx from a 140 mM Na$^+$ medium. The apparent $K_i$ values were 6.39 $\pm$ 1.39, 0.646 $\pm$ 0.145, and 0.105 $\pm$ 0.029 $\mu$M for compounds I, O, and MM, respectively. Thus, these drugs were ~10, 90, and 570 times more potent than the parent molecule amiloride.

**Kinetics of $^{22}\text{Na}^+$ Efflux**

Data on the efflux of $^{22}\text{Na}^+$ from Na$^+$-depleted cells ([Na$^+$]$_i$ $\leq$ 2 meq/liter cell water) after NH$_4$Cl removal are presented in Fig. 9. The experiments were conducted in 5 mM K$^+$ media containing, at 140 mM, either Na$^+$, Li$^+$, or N-methyl-D-glucamine, each in the presence of 0.1 mM ouabain in order to block the active efflux of $^{22}\text{Na}^+$ mediated by the Na$^+$/K$^+$ pump. Assuming an [Na$^+$]$_i$ of 1 mM for these Na$^+$-depleted cells, the control (i.e., resting) efflux rates into the three different media were similar: the rate for the combined data was 0.00874 $\pm$ 0.00063 meq/liter-min. However, intracellular acidification, after NH$_4$Cl withdrawal, caused an increase in the rate of $^{22}\text{Na}^+$ efflux from the cells. In 140 mM N-methyl-D-glucamine, where pH recovery is negligibly slow (Simchowitz...
and Roos, 1985), the $^{22}\text{Na}^+$ efflux rate increased to $0.0174 \pm 0.0028$ meq/liter·min, twofold faster than control.

In contrast, resuspension of neutrophils in 140 mM Na$^+$ medium resulted in a transient stimulation of $^{22}\text{Na}^+$ efflux, whose time course roughly paralleled that of pH recovery under the same conditions (Fig. 1). The initial efflux rate into 140 mM Na$^+$ was $0.0765 \pm 0.0127$ meq/liter·min, ninefold greater than that of controls. The initial acidification-induced $^{22}\text{Na}^+$ efflux rate was thus $0.0765 - 0.0087$ (control) = $0.0678$ meq/liter·min. Similarly, the acidification-activated increase in $^{22}\text{Na}^+$ efflux rate in 140 mM Li$^+$ medium was also transient, with an initial efflux rate of $0.0280 \pm 0.0075$ meq/liter·min, threefold higher than the control rate and about half that of Na$^+$. The augmentation of $^{22}\text{Na}^+$ efflux that is associated with pH recovery from acidification was markedly sensitive to 1 mM amiloride (not shown).

Exchange Stoichiometry

In our first analysis of the results of Fig. 4 (see above), we commented that there was a close correspondence between the magnitudes of Na$^+$ or Li$^+$ influx on the one hand and that of H$^+$ efflux on the other. These findings were taken to imply a 1:1 stoichiometry (hence, electroneutrality), an observation in keeping with the lack of voltage sensitivity of the exchange (Fig. 1). This point is illustrated in a more definitive way in Fig. 10, where the amiloride-sensitive Na$^+$ or Li$^+$ influx is plotted against the corresponding carrier-mediated H$^+$ efflux at a given external concentration of Na$^+$, Li$^+$ (1.25-140 mM), or amiloride (0-2,000 μM) concentration. The $^{22}\text{Na}^+$ and Li$^+$ influx data were taken from Figs. 2, 3, 5, and 8. The rates of pH recovery in Na$^+$ and Li$^+$ media, taken from Figs. 2 and 3, were converted to H$^+$ efflux rates by multiplying them by the average intrinsic intracellular buffering power, 55 mM/pH (Simchowitz and Roos, 1985). The open symbols refer to data on the [Na$^+$], or [Li$^+$], dependence of $^{22}\text{Na}^+$ or Li$^+$ influx or of pH recovery (Figs. 2 and 5). The filled symbols represent data in the presence of various concentrations of amiloride (Figs. 3 and 8). The line of identity where the influx/efflux ratio = 1.0 is shown for comparison. The least-squares slopes of the lines (not shown) representing average $^{22}\text{Na}^+$ or Li$^+$ influx/H$^+$ efflux were $1.09 \pm 0.04$ and $0.90 \pm 0.04$, respectively.
DISCUSSION

Kinetic Properties

We have investigated the ability of Na\(^+\) and other ions of the alkali metal series to be handled by the Na\(^+\)/H\(^+\) exchange mechanism of human neutrophils. This carrier-mediated countertransport system functions physiologically in pH\(_i\) regulation to restore pH\(_i\) to its normal resting value (~7.25 at pH\(_o\), 7.40) after imposed acidification (Grinstein and Furuya, 1984, 1986; Simchowitz and Roos, 1985). By measuring the DMO-derived pH\(_i\) during the time course of pH\(_i\) recovery after removal of an NH\(_4\)Cl prepulse and correlating this with the uptake of \(^{22}\text{Na}^+\) and chemical determinations of Na\(^+\) or Li\(^+\) contents by flame photometry, we were able to characterize several properties of this cation exchange. These include substrate saturation, substrate competition, specific inhibition, and countertransport.

The substrate and inhibitor interactions described above can be quantitatively accounted for on the basis of a simple equilibrium carrier model that exhibits competition kinetics, similar to our analysis of anion exchange in these cells (Simchowitz et al., 1986). The theoretical curves (e.g., straight lines, single exponentials, Michaelis-Menten equations) for all of the figures in this article were obtained by conventional least-squares techniques. As mentioned throughout the text, the apparent $K_m(\text{Na}^+)$, apparent $K_m(\text{Li}^+)$, and apparent $K_i(\text{amiloride})$ values thus derived were in good general agreement with each other. Since the Michaelis-Menten constants for all of the kinetic curves should all be functions of the three true constants in various algebraic combinations, we used a program (designed by Dr. Paul De Weer) to least-squares fit a unique $K_m$ or $K_i$ value to all of the data simultaneously. This program, together with the least-squares criteria governing these formulations, has been described in one of our previous reports on anion exchange (Simchowitz et al., 1986).

Table I lists the refined values for the true $K_m(\text{Na}^+)$, $K_m(\text{Li}^+)$, and $K_i(\text{amiloride})$, as well as the appropriate derived (apparent) constants. From comparisons with other cell types, it is evident that the intracellular acidification-induced Na\(^+\)/H\(^+\) exchange activity of human neutrophils shares a number of important general features of Na\(^+\)/H\(^+\) countertransport systems with respect to alkali metal cation selectivity, inhibitor sensitivity, and stoichiometry (for reviews, see Benos, 1982; Aronson, 1983; Krulwich, 1983).

Ion selectivity. The $K_m$ for external Na\(^+\), one of the physiologically relevant substrates, is ~21 mM, a value that can be compared with the range of 5–59 mM reported for rabbit renal proximal tubule vesicles (Kinsella and Aronson, 1981b; Warnock et al., 1982; Burnham et al., 1982; Aronson et al., 1983), cultured dog kidney cells (Rindler et al., 1979), rat thymocytes (Grinstein et al., 1984), human A431 epidermoid carcinoma cells (Rothenberg et al., 1983), hamster pulmonary fibroblasts (Paris and Pouysségur, 1983; L'Allemain et al., 1984b), mouse neuroblastoma cells (Moolenaar et al., 1981), and chick myoblasts (Vigne et al., 1982). Apparently, like a variety of other cell types (Rindler et al., 1979; Moolenaar et al., 1981; Burnham et al., 1982; Aronson, 1983; Paris and Pouysségur, 1983), Li\(^+\) may serve as an effective substitute for Na\(^+\), albeit with...
an even slightly higher affinity (\(K_m \approx 14 \text{ mM}\)), whereas \(K^+, Rb^+, \text{ and } Cs^+\) cannot replace \(Na^+\) (Kinsella and Aronson, 1980; Burnham et al., 1982; Aronson, 1983; Grinstein et al., 1984). In other cells, including rabbit brush border vesicles (Kinsella and Aronson, 1981a; Burnham et al., 1982; Aronson, 1983; Aronson et al., 1983), dog kidney cells (Rindler et al., 1979), and hamster lung fibroblasts (Paris and Pouyssegur, 1983), apparent \(K_m\) values of 1–12 mM for \(Li^+\) have been reported. However, the transport rate for \(Li^+\) across the neutrophil cell membrane is twofold slower than for \(Na^+\), similar to the findings in rabbit renal tubules (Kinsella and Aronson, 1981a), rat thymocytes (Grinstein et al., 1984), and hamster lung fibroblasts (L’Allemain et al., 1984b). The lack of efficacy of \(K^+, Rb^+, \text{ and } Cs^+\) appears to relate to the carrier’s being devoid of affinity, or nearly so, for these ions. Thus, the order of relative affinities is \(Li^+ > Na^+ > K^+ > Rb^+ > Cs^+\), a relationship that is also obtained in rabbit renal proximal tubule cells (Kinsella and Aronson, 1980, 1981a; Burnham et al., 1982; Aronson, 1983; Aronson et al., 1983), mouse neuroblastoma cells (Moolenaar et al., 1981), hamster lung fibroblasts (Paris and Pouyssegur, 1983; L’Allemain et al., 1984b), and rat thymic lymphocytes (Grinstein et al., 1984).

**Inhibitor sensitivity.** Ouabain and furosemide have no appreciable effect on \(Na^+/H^+\) exchange, which can be blocked by the diuretic amiloride. Amiloride acts as a competitive inhibitor of substrate binding to the external translocation site of the carrier (for reviews, see Benos, 1982; Aronson, 1983). The drug’s \(K_i\) of \(\sim 8 \mu M\) in human neutrophils falls within the range of 1–30 \(\mu M\) reported in rabbit renal microvilli (Kinsella and Aronson, 1981b; Aronson et al., 1983).

### Table I

| Ligand | True Condition | Value |
|-------|---------------|-------|
| \(Na^+\) | 20.9±3.9 mM* | 40 mM \(Li^+\) | 79.8±14.9 mM* |
| \(Li^+\) | 14.2±3.4 mM* | 20 mM \(Na^+\) | 15.7±2.0 \(\mu M\) |
| Amiloride | 8.02±1.11 \(\mu M\) | 60 mM \(Na^+\) | 51.0±4.4 \(\mu M\) |
| | | 140 mM \(Na^+\) | 61.7±10.4 \(\mu M\) |
| | | 140 mM \(Li^+\) | 87.1±15.3 \(\mu M\) |

True and apparent kinetic constants were obtained by least-squares fitting the single-site competition kinetics carrier model described in the text to all of the data derived with \(Na^+, Li^+, \text{ and amiloride in Figs. 2, 3, and 5–8 simultaneously. The model assumes that N-methyl-D-glucamine has negligible affinity for the exchange carrier. The footnotes explain in which figures the true and apparent constants apply.}*

* \(^*\) Figs. 2, 3, and 5–8.
\(^{t}\) Fig. 7.
\(^{!}\) Figs. 3 and 8.
\(^{m}\) Fig. 5.
cultured dog kidney cells (Rindler et al., 1979), rat thymocytes (Grinstein et al., 1984), hamster lung fibroblasts (Paris and Pouysségur, 1983), chick myoblasts (Vigne et al., 1982), and mouse 3T3 fibroblasts (Frelin et al., 1983). In addition, three derivatives of amiloride, the 5-N,N-dimethyl, 5-N,N-diethyl, and 5-N,N-hexamethylene analogues of amiloride, proved to be more active than the parent compound with potencies 10, 90, and 570 times, respectively, that of amiloride. This rank order of effectiveness of the four compounds, as well as their apparent Kᵢ values, is similar to that reported by our group for inhibition of chemotactic factor-activated Na⁺/H⁺ exchange in human neutrophils (Simchowitz and Craigoe, 1986b). Comparable data for these and closely related derivatives of amiloride have been reported for hamster pulmonary fibroblasts (L'Allemain et al., 1984a), chick myocytes (Vigne et al., 1984), and human epidermoid carcinoma cells (Zhuang et al., 1984).

Stoichiometry. Alkali metal cation/H⁺ exchange is independent of membrane voltage and is therefore electroneutral, a finding consistent with a 1:1 stoichiometry observed in all other cell types to date (Rindler et al., 1979; Kinsella and Aronson, 1980, 1982; Moolenaar et al., 1981; Burnham et al., 1982; Aronson, 1983, Grinstein et al., 1984). This equality, that Na⁺ or Li⁺ influx is accompanied by an equivalent efflux of H⁺, was directly demonstrated in the present study (Fig. 10).

In a closely related series of studies, Grinstein and Furuya (1984, 1986) investigated the basic properties of the Na⁺/H⁺ exchanger of human neutrophils in pHi recovery from acid loads. They also found pHi recovery to be amiloride sensitive and Na⁺ dependent. In addition, recovery was associated with a net uptake of Na⁺ and with extrusion of acid equivalents into the external solution, both of which were inhibited by amiloride. The rate of pHi recovery was linearly related to the degree of intracellular acidification between 6.5 and 7.2, in agreement with our previous observations (Simchowitz and Roos, 1985). By contrast, however, Grinstein and Furuya (1986) found recovery to be stimulated by external Na⁺ with a Kᵢ of 74 mM, a value that differs substantially from our estimate of ~21 mM (Table I). Moreover, they derived a Kᵢ for amiloride of 24 μM, rather different from our value of ~8 μM (Table I). Note that in both instances, the estimates of Grinstein and Furuya (1986) for the true kinetic constants of Na⁺ and amiloride are threefold greater than ours, whereas the two laboratories find remarkably similar apparent Kᵢ values for amiloride in 140 mM Na⁺ medium (~75 μM).

Cell Volume

In this article, we have made few references to cell volume, even though changes in cell size would be expected to take place during: (a) incubation in Na⁺-free medium, (b) pretreatment with NH₄Cl, and (c) removal of NH₄Cl. Such secondary volume effects could, in theory and in practice, complicate the interpretation of our results.

(a) Depletion of internal Na⁺ from its normal value of 25–30 (Simchowitz et al., 1982; Simchowitz, 1985b; Grinstein and Furuya, 1986) to ~0 meq/liter of cell water by incubating cells in 140 mM N-methyl-D-glucamine could result in the loss of ~60 meq/liter of osmotically active particles (assuming an equivalent
loss of anions, e.g., Cl\(^-\)) from the intracellular water space, leading to a reduction of \(~60\%/300\) or \(~20\%\) of cell volume (where 300 represents the total cell osmolality in milliosmoles per liter of cell water). In fact, when measured by sensitive electronic cell-sizing techniques (particle size analyzer, model 180XY, Particle Data, Inc., Elmhurst, IL), we found cell volume to be indistinguishable from that of normal-Na\(^+\) cells. This indicates that the cells gained solute particles from the extracellular fluid, namely N-methyl-D-glucamine, a point for which we have other evidence (see Discussion).

(b) During the pretreatment with NH\(_4\)Cl, several different ionic events are taking place that should lead to an increase in cell volume. These processes include the rise in internal NH\(_3^+\) and the net influx of Cl\(^-\) through Cl\(^-\)/HCO\(_3^-\) exchange that is responsible for pH\(_i\) recovery from the NH\(_4\)Cl-induced alkalinization (Simchowitz and Roos, 1985). However, the slight enlargement in cell size seems to activate a volume-regulatory mechanism, which, by analogy to other cell types (Cala, 1980; Kregenow, 1981; Spring and Ericson, 1982; Grinstein et al., 1982), restores cell volume to near normal, since no substantial (>7%) or sustained cell volume transient could be identified.

(c) After removal of the NH\(_4\)Cl prepulse, there is an immediate fall in pHi as the accumulated intracellular NH\(_3^+\) (~30 mM) exits the neutrophil as NH\(_3\), leaving behind a proton. This would be expected to cause a small volume loss of ~10%. These considerations are especially pertinent since it is well known that cell shrinkage per se activates Na\(^+\)/H\(^+\) exchange in other biological systems (Kregenow, 1981; Spring and Ericson, 1982; Cala, 1980, 1983). However, this reduction in neutrophil volume should be very transient, since, during the ensuing pH\(_i\) recovery owing to Na\(^+\)/H\(^+\) countertransport, the influx of Na\(^+\) in exchange for H\(^+\) leads to the net uptake of osmotically active particles.

When measured electronically using a particle-size analyzer system, the predicted cell volume transients could be easily detected during all stages of the experimental protocol as noted above. The cell volume changes were all relatively minor in degree, never exceeding 7%. Thus, during all phases of the experiment, the neutrophil volume is for the most part maintained at or very near normal values. For this reason, cell volume transients were largely ignored in our calculations and cell volume was assumed to be effectively constant throughout this study.

Resting pH\(_i\) of Na\(^+\)-depleted Cells

The set of points that constitute line E in Fig. 1 represents the pH\(_i\) (7.29) of neutrophils that had been kept in Na\(^+\)-free medium for 1.5 h. One might have expected the pH\(_i\) of these Na\(^+\)-depleted cells to be lower than that of their counterparts (normal-Na\(^+\) cells) that are continuously bathed in 140 mM Na\(^+\) medium since metabolic acid production, for example, can no longer be controlled by Na\(^+\)/H\(^+\) exchange. However, the two batches of cells exhibit remarkably similar pH\(_i\) values (~7.25; Simchowitz and Roos, 1985). Conceivably, the pH\(_i\) could also have drifted downward through reversal of the Na\(^+\)/H\(^+\) exchanger (i.e., net internal Na\(^+\)/external H\(^+\) exchange since the Na\(^+\) gradient is now directed outward in Na\(^+\)-free medium) or through passive H\(^+\) and/or OH\(^-\) leaks.

The failure to observe depression of pH\(_i\) via reversed Na\(^+\)/H\(^+\) exchange can
easily be accounted for by the lack of appreciable Na+/H+ exchange activity in resting neutrophils (Sha'afi et al., 1981; Simchowitz, 1985b; Grinstein and Furuya, 1986). This point is also corroborated by the lack of effect of amiloride on the resting pH of cells bathed in 140 mM Na+ medium (Simchowitz and Roos, 1985).

The resting potential of cells bathed in 5 mM K+ medium is approximately −60 mV (Seligmann and Gallin, 1980; Simchowitz et al., 1982). Since thermodynamic considerations dictate a pH of ~6.40 (one unit lower than pHo) for passive H+ distribution, this large negative membrane voltage constitutes a strong driving force for net passive H+ influx and OH− efflux by current-carrying pathways. However, as the total membrane conductance is well matched by the sum of the individual specific conductances of K+, Na+, and Cl− (Simchowitz et al., 1982; Simchowitz and De Weer, 1986), it would appear that, in all likelihood, the permeabilities of H+ and OH− are very small.

The explanation for the relatively high resting pH of Na+-depleted cells is probably related to permeation of N-methyl-D-glucamine as the free base (pKₐ 9.6). In our prior work (Simchowitz and Roos, 1985), we alluded to the point that some entry of the compound occurs by nonionic diffusion. This is evident in the small degree of intracellular alkalization (~0.1 unit) that is seen within the first 30 min upon resuspension of normal cells in 140 mM N-methyl-D-glucamine. This slow alkalinizing effect probably counterbalances the cumulative rate of acidifying influences owing to a combination of as yet poorly defined mechanisms so that pH remains relatively unchanged. Using a buffering power of 55 mM/pH, the gradual internal alkalinization signifies an influx rate of the free base form of N-methyl-D-glucamine of ~0.2 meq/liter·min, which by 1.5 h results in the intracellular accumulation of ~20 meq/liter of cell water of N-methyl-D-glucamine. This degree of uptake corresponds closely in amount to the net loss of Na+ (25–30 meq/liter of cell water) from the cells. These calculations explain why Na+-depleted neutrophils retain a normal cell volume.

A related issue stems from the fact that the extent of pH recovery in 25 mM Na+ medium appears to be similar to that in 140 mM Na+ (Fig. 1). This finding requires some explanation. At an intracellular buffering power of 55 mM/pH, the amount of Na+ that must enter the cell via Na+/H+ exchange to raise pH from 6.62 to 7.29 (Fig. 1) is 37 meq/liter of cell water. If all of this Na+ were to accumulate inside the cells, the intracellular Na+ concentration would actually exceed the prevailing external Na+ concentration (25 mM), which might seem difficult to reconcile with a downhill one-for-one exchange of Na+ and H+. Such an explanation is indeed readily compatible with the energetics of the system. From thermodynamic considerations, given [Na+]o = 25 mM, pHe = 7.40, and pH = ~7.25 at complete recovery, Na+ exchange will be in chemical equilibrium when [Na+]i/[Na+]o = [H+]i/[H+]o = 1.4, or when [Na+]i = ~35 mM. This value is not appreciably different from that calculated above (37 mM). From the standpoint of thermodynamics, the data are therefore consistent with a 1:1 stoichiometry for Na+/H+ exchange and one need invoke no other factors to explain the observation that cells bathed in low-Na+ (25 mM) medium recover to the same extent as those in physiological (140 mM Na+) medium. Another possible explanation (although not relevant here) is that the Na+/K+ pump.
extrudes Na⁺ entering via Na⁺/H⁺ exchange, thereby preserving the driving force for H⁺ extrusion. However, this mechanism cannot explain the data of Fig. 1, since recovery was still complete when the pump was inhibited by 0.1 mM ouabain.

Comparison with Chemotactic Factor-stimulated Na⁺/H⁺ Exchange

As mentioned in the Introduction, there are at least two functionally distinct, physiologically relevant modes of operation of Na⁺/H⁺ exchange activity in human neutrophils: one, activated by intracellular acidification, restores pHᵢ to its normal resting value (Grinstein and Furuya, 1984, 1986; Simchowitz and Roos, 1985); the second, induced by chemotactic factors, phorbol diesters, and other stimuli, brings about an intracellular alkalinization (Molski et al., 1980; Sha'afi et al., 1981, 1982; Grinstein and Furuya, 1984; Grinstein et al., 1985; Simchowitz, 1985a–c). In the latter case, the rise in pHᵢ has been shown to enhance several stimulated responses and pHᵢ transients in general have been noted to play an important regulatory role in the functional behavior of these cells (Segal et al., 1981; Klempner and Styrt, 1983; Simchowitz, 1985a; Simchowitz and Cragoe, 1986a). Thus, knowledge of the factors governing pHᵢ regulation is essential to our understanding of the intracellular machinery underlying stimulus-response coupling.

One of the immediate aims in obtaining kinetic parameters for the acidification-induced Na⁺/H⁺ exchanger is to compare these values with those of the chemotactic factor-stimulated Na⁺/H⁺ exchange mechanism whose action results in cytoplasmic alkalinization. From our previous work (Simchowitz, 1985b, c) in neutrophils activated by exposure to the chemotactic tripeptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), it appears that the binding constants of this stimulated exchange carrier for external Na⁺ (Kᵣ = 26–35 mM) and for amiloride (Kᵢ = ~11 µM) are quite similar to those described in the present studies for the acidification-induced Na⁺/H⁺ exchanger. Measurements of FMLP-stimulated Li⁺ fluxes and pHᵢ transients as a function of the external Li⁺ concentration (data not shown) indicate that Kᵣ(Li⁺) is also comparable for the two exchange systems (~15 mM). These findings would seem to support the hypothesis that one is dealing with the same or at least two very closely related transport proteins that may be subject to dual control: by intracellular acidification and by chemotactic factors or phorbol esters. Similar studies on the binding affinities for H⁺, Na⁺, and Li⁺ at the internal translocation sites of the carrier should be useful in gaining insight into the biochemical mechanisms of activation of the two exchange systems and provide the basis for probing the nature of the internal regulatory sites of carrier activity.

In summary, in response to internal acidification, an otherwise quiescent Na⁺/H⁺ countertransport mechanism is activated to restore pHᵢ to its normal resting value. This recovery process is mainly, if not entirely, accomplished through an amiloride-sensitive exchange of external Na⁺ for internal H⁺, in which Li⁺ can substitute effectively for Na⁺.

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