Cytoplasmic pH Regulation in Thymic Lymphocytes by an Amiloride-sensitive Na\(^+\)/H\(^+\) Antiport

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ABSTRACT The mechanisms underlying cytoplasmic pH (pH\(_i\)) regulation in rat thymic lymphocytes were studied using trapped fluorescein derivatives as pH indicators. Cells that were acid-loaded with nigericin in choline\(^+\) media recovered normal pH\(_i\) upon addition of extracellular Na\(^+\) (Na\(_o^+\)). The cytoplasmic alkalinization was accompanied by medium acidification and an increase in cellular Na\(^+\) content and was probably mediated by a Na\(_o^+\)/H\(^+\) antiport. At normal [Na\(^+\)]\(_i\), Na\(_o^+\)/H\(^+\) exchange was undetectable at pH\(_i\) \(\geq 6.9\) but was markedly stimulated by internal acidification. Absolute rates of H\(^+\) efflux could be calculated from the Na\(_o^+\)-induced dpH\(_i\), using a buffering capacity of 25 mmol-liter\(^{-1}\)·pH\(^{-1}\), measured by titration of intact cells with NH\(_4\). At pH\(_i\) = 6.3, pH\(_o\) = 7.2, and [Na\(^+\)]\(_o\) = 140 mM, H\(^+\) extrusion reached 10 mmol-liter\(^{-1}\)·min\(^{-1}\). Na\(_o^+\)/H\(^+\) exchange was stimulated by internal Na\(^+\) depletion and inhibited by lowering pH\(_o\) and by addition of amiloride (apparent K\(_i\) = 2.5 \(\mu\)M). Inhibition by amiloride was competitive with respect to Na\(_o^+\). H\(^+\) could also exchange for Li\(^+\), but not for K\(^+\), Rb\(^+\), Cs\(^+\), or choline\(^+\). Na\(_o^+\)/H\(^+\) countertransport has an apparent 1:1 stoichiometry and is electrically silent. However, a small secondary hyperpolarization follows recovery from acid-loading in Na\(^+\) media. This hyperpolarization is amiloride- and ouabain-sensitive and probably reflects activation of the electrogenic Na\(^+\)-K\(^+\) pump. At normal Na\(^+\) values, the Na\(_o^+\)/H\(^+\) antiport of thymocytes is ideally suited for the regulation of pH\(_i\). The system can also restore [Na\(^+\)]\(_i\) in Na\(^+\)-depleted cells. In this instance the exchanger, in combination with the considerable cytoplasmic buffering power, will operate as a [Na\(^+\)]-regulatory mechanism.

INTRODUCTION

In view of the marked pH sensitivity of virtually all cellular processes, the importance of cytoplasmic pH (pH\(_i\)) regulation is unquestionable. With the possible exception of red blood cells, all mammalian cells appear to have pH\(_i\) levels that are higher than expected if H\(^+\) and HCO\(_3^-\) were passively distributed across the plasma membrane (Roos and Boron, 1981). The tendency of the cells...
to become acidified by the passive fluxes of $H^+$ and $HCO_3^-$, and by metabolic acid production must therefore be counteracted by regulatory acid-extruding mechanisms. Three basic types of $pHi$ regulatory mechanisms have been described: in invertebrate cells a system that requires extracellular $Na^+$ ($Na^+_o$) and $HCO_3^-$, as well as internal $Cl^-$, has been reported (Boron, 1977; Boron and De Weer, 1976; Thomas, 1977). This system can be inhibited by anion transport inhibitors, such as disulfonic stilbene derivatives (Thomas, 1976; Boron, 1977), and in some instances requires cellular ATP (Boron and De Weer, 1976; Russell and Boron, 1976). In mammalian cells two types of systems prevail: a coupled countertransport of $Na^+$ for intracellular $H^+$ ($H^+_i$) (Aickin and Thomas, 1977; Kinsella and Aronson, 1980) and a similar countertransport of $Cl^-$ for $HCO_3^-$ (Aickin and Thomas, 1977; see Roos and Boron, 1981, for review).

The $pHi$ of lymphocytes from different sources and of cultured lymphoblasts has been determined by a variety of techniques, including weak acid and base partition (Deutsch et al., 1979; Gerson and Kiefer, 1982, 1983), fluorescent indicator trapping (Rink et al., 1982; Gerson and Kiefer, 1982), $^31P$ and $^{19}F$ nuclear magnetic resonance (NMR) (Rink et al., 1982; Deutsch et al., 1982), and direct titration of freeze-thawed cells (Rink et al., 1982). In all cases, $pHi$ was $\sim 7.0$–7.1. For an extracellular $pH$ ($pH_o$) of 7.2, the Nernst potential for $H^+$ and $HCO_3^-$ would therefore be $-6$ to $-12$ mV. Because the membrane potential of lymphocytes has been reported to be in the $-40$ to $-60$ mV range (Deutsch et al., 1979; Grinstein et al., 1982), $H^+$ and $HCO_3^-$ must be maintained away from electrochemical equilibrium by a regulatory mechanism. The existence of a $pHi$-regulatory system in lymphocytes is further suggested by the constancy of $pHi$ upon changing the external $pH$ ($pH_o$) between 6.9 and 7.3 (Deutsch et al., 1982). The nature of the purported $pHi$-controlling mechanisms in lymphocytes is unknown. The presence of an inorganic anion exchanger has been inferred, in the case of peripheral blood lymphocytes, from the comparison of net and isotopic $Cl^-$ permeability (Grinstein et al., 1982). However, it is not known to what extent this system is involved in the control of $pHi$.

During the course of studies on the volume-regulating properties of blood lymphocytes, we were able to detect the operation of a $Na^+/H^+$ countertransport system (Grinstein et al., 1983), which could be activated by osmotically induced volume changes and also by acidification of the cytoplasm. The purpose of the present study was to examine the properties of this $Na^+_i/H^+_i$ antiport in lymphocytes and its possible role in $pHi$ regulation. Cells isolated from the rat thymus, a plentiful source of relatively homogeneous T-type lymphocytes, were used for these experiments. Intracellularly trapped fluorescent indicators were used to monitor $pHi$ and a novel acid-loading technique employing nigericin was used to manipulate $pHi$. Nominally $HCO_3^-$-free media were used throughout. This allowed us to study the $Na^+/H^+$ exchanger without interference by the $Cl^-/HCO_3^-$ exchanger, which could potentially regulate $pHi$ independently.

**Materials and Methods**

*Reagents*

Bovine serum albumin, gramicidin D, monensin, 2-[N-morpholino]ethanesulfonic acid (MES), DHPS, ouabain, and 9-amino acridine were purchased from Sigma Chemical Co.,
St. Louis, MO; nigericin and valinomycin were obtained from Calbiochem-Behring Corp., San Diego, CA; 5,6-dicarboxyfluorescein (DCF), its acetoxyethyl ester, and quin-2 acetoxyethyl ester were the kind gift of Dr. T. J. Rink, University of Cambridge; 3,3′dipropylthiadicarbocyanine [diS-C3-(5)] was the gift of Dr. A. Waggoner, Amherst College, MA; furosemide was obtained from Hoechst, Montreal, Canada; ionomycin was obtained from Squibb, Princeton, NJ; bumetanide was a gift from Lovens Kemiske Fabrik, Denmark; amiloride was a gift from Merck, Sharp & Dohme, Montreal, Canada; RPMI 1640 medium without HCO3− and trypan blue were from Grand Island Biochemicals, Grand Island, NY.

Isotopes

\[^{[H]}\text{polyethylene glycol (4,000 mol wt)}, \[^{[H]}\text{H}_2\text{O}, \text{and } [^{14}\text{C}]	ext{5,5-dimethyloxazolidine-2,4-dione (DMO)}\] were from New England Nuclear, Boston, MA.

Solutions

Stock solutions of DCF-acetoxyethyl ester, diS-C3-(5), ionomycin, quin-2, bumetanide, and furosemide were made in dimethylsulfoxide (DMSO). Nigericin, valinomycin, and monensin stocks were in ethanol. Amiloride, DIDS, and ouabain were kept in aqueous stocks.

Choline solution contained (in mM): 140 choline chloride (recrystallized from methanol), 10 glucose, 1 KCl, 1 CaCl2, 1 MgCl2, and 20 Tris-MES, pH 7.2. This solution was millipore-filtered and kept frozen. Na+ solution and K+ solution were prepared by isosmotic replacement of choline+ by Na+ or K+, respectively, but were otherwise identical. Hepes-RPMI was prepared by addition of 20 mM Hepes to HCO3−-free RPMI and titration with NaOH to pH 7.3. With the exception of the pH, and membrane potential experiments, where slightly (20%) hypertonic conditions were used, all other media were isotonic.

Cell Isolation and Characterization

Thymocytes were isolated from male Wistar rats weighing 200–250 g as follows: the freshly dissected thymus (0.3–0.6 g) was transferred into Na+ solution at room temperature. The thymus was finely minced with scissors and the cells were further released by gentle disruption in a Dounce tissue grinder fitted with a loose pestle. Large tissue fragments were removed by filtration through gauze and isolated cells were washed once in Na+ solution and resuspended in Hepes-RPMI. The cells were maintained at room temperature and always used within 8 h of isolation. Cell viability, which was determined by trypan blue exclusion, was >95% throughout this period.

Cell counting and sizing was performed with a Coulter Counter-Channelyzer combination (Coulter Electronics, Hialeah, FL). The shape factor determined by Segel et al. (1981) for human blood lymphocytes was applied to the volume determinations.

Intracellular Cation Content Manipulation and Determination

Cellular Na+ depletion was accomplished by incubating 5 × 10⁷ cells/ml in Na+-free medium for 1–2 h at 37°C. Similar results were obtained whether K+ or choline was used as a substitute.

Intracellular Na+ and K+ contents were measured by flame photometry (Photometer model 443; Instrumentation Laboratory, Inc., Lexington, MA) using Li+ as an internal standard. The samples (5 × 10⁷ cells) were sedimented at 250 g for 5 min, resuspended, and washed twice in 2,000 vol of an ice-cold medium containing 140 mM choline chloride and 20 mM Tris-HCl, pH 7.2. The final pellet was directly resuspended in Li+ standard solution (Instrumentation Laboratory). After vigorous vortexing the insoluble material
was sedimented and the supernatant was used for measurement. Intracellular concentrations were calculated using an average cell volume of 114 μm³, measured electronically, and assuming activity coefficients of 1.0.

\[ pH_i, Determinations \ with \ DCF \]

The carboxylated fluorescein derivative DCF was chosen for \( pH_i \) measurements because of its negligible leakage rate at room temperature (see below) and particularly because of its \( pK_a \) of ~6.3, which makes it ideal for \( pH_i \) determinations in acid-loaded cells. Cells (10⁵/ml) were loaded with DCF by incubation with the DCF-acetoxymethyl ester (1 μM) in Hepes-RPMI at 37°C. The parent compound diffuses across the membrane and is hydrolyzed by internal esterases in the cytoplasm, releasing the poorly permeant DCF, which can serve as a \( pH_i \) indicator (Rink et al., 1982). After 30 min the cells were sedimented and resuspended in fresh Hepes-RPMI to a final concentration of 10⁶ cells/ml. Direct observations by phase contrast and fluorescence microscopy showed that >90% of the cells take up the dye, which is distributed homogeneously throughout the cytoplasmic and nuclear spaces (Fig. 1, A and B). DCF was not accumulated by trypan-permeable cells.

Fluorescence of labeled cell suspensions was measured in a Perkin-Elmer (Norwalk, CT) 650-40 fluorescence spectrophotometer, attached to a Perkin-Elmer R-100 recorder. Excitation and emission wavelengths were 500 and 520 nm, and 5- and 10-nm slits were used, respectively. Routinely, \( pH_i \) measurements were performed in a 3-ml cuvette containing 1 ml of a thymocyte suspension (3 × 10⁶ cells/ml) in the indicated medium. Calibration of \( pH_i \) vs. fluorescence was carried out at the end of each experiment by disrupting the cells with 0.05% Triton X-100, followed by titration of the medium with concentrated Tris or MES. It has been reported (Rink et al., 1982) that the fluorescence emitted by intracellularly trapped fluorescein derivatives is somewhat decreased compared with the free dye because of a red shift. Therefore, a correction must be introduced if calibration is made after cell lysis. The necessary correction factor was determined for each batch of cells by the method of Thomas et al. (1979), using nigericin. The ionophore sets \( H^+/H^+ = K^+/K^+ \), so that if the cells are suspended in \( K^+ \) solution (which contains approximately the same \( K^+ \) concentration as the cytoplasm), \( H^+ \) will follow \( H^+ \). Under these conditions, as illustrated by Fig. 1C, disruption of the cells nevertheless increases DCF fluorescence, which confirms the occurrence of intracellular quenching. The fractional fluorescence increase was constant in the \( pH \) 5.9-7.0 range, so that a single correction factor was applied throughout. In 24 similar determinations the correction factor was 0.103 ± 0.011 (mean ± SE).

Dye leakage from the cells at room temperature was estimated by sedimentation of loaded cells at increasing time intervals and by analysis of fluorescence in both pellet and supernatant. In four experiments the fractional leakage measured over a period of 2.5–3 h averaged 0.04 ± 0.01 h⁻¹. Since the cells were always washed <3 h before the measurements, a maximum of 12% of the fluorescence was extracellular.

\[ Manipulation \ of \ pH_i \]

Preliminary experiments indicated that modifying \( pH_i \) by incubation in media of varying \( pH \), was a slow, poorly reproducible process. Much more rapid and reproducible results were obtained using nigericin. Cells could be acid-loaded by suspension in choline⁺ medium, \( pH \) 7.2, and addition of 0.3–0.7 μg/ml of nigericin. These concentrations had no effect on viability (in two experiments, concentrations of nigericin below 6 μg/ml did not affect viability within 20 min). A typical acid-loading experiment is illustrated in Fig. 2A. As shown in this figure, the effect of the ionophore could be readily stopped by
addition of concentrated albumin (5 mg/ml final). This provided a convenient method for the control of pH. It is presumed that albumin acts by binding nigericin, thereby removing it from the cell membranes and stopping its further action. Two types of experiments were performed: in some, like that in Fig. 2A, nigericin and albumin were added to the cuvette while recording proceeded. In other cases, however, the acid-loading and stopping procedures were performed in a test tube and were followed by centrifugation for removal of the albumin and residual nigericin. A parallel sample was treated in the fluorimeter cuvette to monitor the course and termination of acid-loading. The centrifuged sample was then resuspended in the medium indicated in the text and used.

Figure 1. Fluorescence of DCF-stained thymic lymphocytes. (A) Phase-contrast micrograph of a representative thymocyte population. The cells were loaded with DCF, washed, and resuspended in Hepes-RPMI. Viability was >90%. (B) Fluorescence micrograph of the same field depicted in A. Microscopy was performed with an epi-illumination system (Carl Zeiss, New York) in a microscope (Universal Scientific, Inc., Atlanta, GA) using an HBO 50-W high-pressure mercury source. Emission was recorded on Ilford (Basildon, Essex, UK) HP5 film with an FX-35A dark box (Nikon, Inc., Garden City, NY) and the Microflex UFX (Nikon, Inc.) photomicrographic attachment. Notice that most of the cells are stained and that the fluorescence is homogeneously distributed throughout the cytoplasmic and nuclear spaces (X 450). (C) pH dependence of DCF fluorescence in solution (○) and inside thymocytes (O). Cells were loaded with DCF as described in Materials and Methods and suspended in K⁺ solution at the indicated pH. The cells were then treated with 0.7 μg/ml nigericin and fluorescence was recorded once the signal stabilized (open circles). This was followed by cell disruption by addition of concentrated Triton X-100 (final concentration 0.05%). The fluorescence of the released DCF was then recorded (solid circles). Light scattering was negligible both before and after detergent treatment and was not corrected for. The experiment illustrated is representative of 3 similar determinations in the pH 5.9–7.0 range and of 24 determinations at pH 7.0.
Determinations of Buffering Power and Calculation of $H^+$ Fluxes

Three methods were used for determining the cytoplasmic buffering power. (a) Direct measurement: 0.5–1.0 × 10⁹ cells were sedimented, washed in 150 mM NaCl, 0.5 mM Tris-MES, pH 7.0, and resuspended in 5 ml of the same medium. The cells were then disrupted by freeze-thawing twice and then sonicating for 20 s (Cell Disruptor model W140; Heat Systems-Ultrasonics, Inc., Plainview, NY). The cell lysate was used for fluorescence determination. Each experiment was followed by addition of Triton X-100 and calibration.¹

FIGURE 2. (A) Determination of the buffering power of thymocytes by NH₄⁺ titration. Cells were loaded with DCF as described and suspended in choline⁺ solution at 3 × 10⁶ cells/ml. A composite of typical fluorescence recordings is illustrated. Where indicated, 0.3 µg/ml nigericin was added. Acid-loading was stopped at different levels by addition of albumin (5 mg/ml final). The protein scavenges the ionophore and terminates acid-loading. Then, NH₄Cl (2 mM final) was added and recording was rapidly resumed. Discontinuities in the trace indicate the intervals when the sample compartment was opened for additions and stirring. The experiment was completed by cell disruption with detergent and calibration of fluorescence vs. pHᵢ (not illustrated). The calibration and the correction factor determined by the nigericin-K⁺ method were used for the calculation of pHᵢ (vertical scale). The traces are representative of the number of experiments reported in Fig. 3. (B) Evidence for the existence of a Na⁺/H⁺ exchanger in thymocytes. A composite of typical fluorescence recordings of DCF-labeled cells is illustrated. Thymocytes were DCF-stained, washed, and acid-loaded with nigericin in choline⁺ solution as described. Acidification was terminated by addition of albumin and washing. The cells were resuspended in a small volume of choline⁺ solution and recording was started upon dilution into 30 vol of either choline⁺ solution, Na⁺ solution, or Na⁺ solution containing 0.1 mM amiloride. The pHᵢ values were determined by calibration and correction as above. The time scale is the same as in A. The traces are representative of >10 experiments.

¹Because calibration was necessarily a posteriori, the desired value of pHᵢ reached during acid-loading could only be approximated. Therefore, the same type of experiment was carried out in different preparations at slightly varying pHᵢ levels. For this reason, individual values of pHᵢ are reported whenever this parameter could importantly affect the results (e.g., Fig. 5).
titration with KOH or HCl, taking into account the buffering capacity of the medium.

(b) NH₄Cl titration: The pH of DCF-loaded cells was set to a desired value by the nigericin-albumin technique. Then, 1–5 mM NH₄Cl was added and pH was recorded immediately (see Fig. 2A). The buffering capacity was calculated as Δ[NH₄⁺]/ΔpH, (see Roos and Boron, 1981). The concentration of [NH₄⁺] was calculated using a pK of 9.21 and assuming that NH₃ is in equilibrium across the membrane. Penetration of protonated NH₄⁺ was corrected by back-extrapolation (see Results). (c) K⁺/H⁺ exchange method: The buffering power was also measured as the amount of K⁺ that needs to be lost in order to change pH (i.e., Δ[K⁺]/ΔpH) when cells are suspended in choline⁺ medium with nigericin. DCF-labeled cells were exposed to the ionophore and ΔpH was recorded as described. Parallel aliquots were used for the determination of cellular K⁺ content before and after equilibration with nigericin.

The value of buffering power was used for the estimation of acid extrusion rates. The latter were calculated as the product of the rate of pH recovery (in pH·min⁻¹) times the buffering power (in mmol·liter⁻¹·pH⁻¹). The rate of pH recovery was measured directly from the fluorescence recordings (e.g., insets of Figs. 4–6).

Membrane Potential (E_m) Measurements

Membrane potential was estimated fluorimetrically as described (Grinstein et al., 1982) using 3 × 10⁶ cells/ml and 0.5 μM diS-C₅(5). The resting E_m was estimated by the “null-point” method (Waggoner, 1979), using valinomycin at various concentrations of external K⁺. Similar results were obtained with 1–3 μM valinomycin. E_K⁺ was calculated by the Nernst equation using an intracellular K⁺ concentration of 146.8 ± 10.5 mM (mean ± SE, n = 23), determined by flame photometry as described.

Determinations of Free Cytoplasmic Ca²⁺ Concentration ([Ca²⁺]₀)

[Ca²⁺]₀ was determined essentially as described by Tsien et al. (1982). Thymocyte suspensions (5 × 10⁷ cells/ml) in either Na⁺ or choline solutions were loaded for 30 min at 37°C with 10 μM quin-2 acetoxymethyl ester. The cells were then washed and resuspended in the required medium at 5–8 × 10⁶ cells/ml. Fluorescence was measured with excitation at 339 nm, using a 2-nm slit to minimize photolysis, and emission at 495 nm with a 15-nm slit. Calibration was made with ionomycin and Mn²⁺ as previously described (Rink et al., 1983).

Measurements of Acid Extrusion as ΔpH₀

5 × 10⁷ cells were washed in a choline⁺ solution buffered only with 0.5 mM Tris-MES, pH 7.3, and resuspended in 5 ml of the same medium. The pH of this suspension was measured under constant magnetic stirring with an Orion Research Inc. (Cambridge, MA) 601A digital Ionometer attached to an X vs. time flatbed recorder (model 7044; Hewlett-Packard Co., Palo Alto, CA). The buffering power of the medium was determined by titration with KOH or HCl.

Other Methods

Internal pH was also determined by partition of [¹⁴C]DMO essentially as described (Deutsch et al., 1979), using [¹⁴C]polyethylene glycol as an extracellular space maker. Unless otherwise indicated, all the experiments were carried out at room temperature (20–22°C). The results are presented either as representative traces or as the mean ± SE of the number of experiments indicated.
RESULTS

\(pH_i\) of Thymocytes

In six experiments using DCF, the \(pH_i\) of freshly isolated rat thymocytes suspended in Na\(^+\) medium at room temperature was 7.01 ± 0.06. Under these conditions \(pH_i\) remained constant (within 0.05 unit) for at least 15 min. The value of \(pH_i\) of cells suspended in K\(^+\) medium was not significantly different (7.05 ± 0.05, \(n = 4\)) and also remained relatively constant for up to 15 min. Immediately after resuspension in choline\(^+\) medium, \(pH_i\) was 7.06 ± 0.02 (\(n = 18\)). In most experiments \(pH_i\) declined slowly (0.013 ± 0.003 pH unit⋅min\(^{-1}\)) upon continuous incubation in choline\(^+\) medium.

An isotope distribution method was used to ascertain the accuracy of the fluorescent \(pH_i\) determinations. In four different preparations the steady state \(pH_i\), measured by \(^{14}C\)DMO equilibration in cells suspended in Na\(^+\) medium, averaged 7.1 ± 0.06. This value is not significantly different from that obtained with DCF.

Buffering Capacity of Thymocytes

Three different methods were used to estimate the intracellular buffering power of thymocytes. The first and simplest method involved direct titration of cells previously disrupted by repeated freeze-thawing cycles. As shown in Fig. 3 (shaded area), the buffering power estimated by this procedure was relatively constant in the pH 6.0–7.2 interval, ranging from 31 to 43 mmol-liter\(^{-1}\)⋅pH\(^{-1}\) in four preparations. A mean cellular volume of 114 μm\(^3\) was used for these calculations.

Fig. 2A illustrates the second method for determining the buffering power. DCF-loaded cells were suspended in choline\(^+\) medium and acidified by addition of nigericin. Acid-loading was stopped at different points with albumin, followed by addition of NH\(_4\)Cl to the medium. The ensuing cytoplasmic alkalinization was measured and the buffering power was calculated as described (see Roos and Boron, 1981, and Materials and Methods). As reported for other tissues, the rapid alkalinization was followed by a gradual acidification, which probably represents influx of the protonated base. A precise estimate of the alkalinization induced by NH\(_3\) uptake was obtained by back-extrapolation of this secondary acidification to the time of addition of extracellular NH\(_4\)^{+}. The data from 60 similar determinations are summarized in Fig. 3, in which the buffering power is plotted as a function of \(pH_i\). The midpoint of the NH\(_4\)^{+}-induced \(\Delta pH\) was considered for the assignment of \(pH_i\) (abscissa). The values obtained by this method were consistently lower than those calculated from direct titration (see Discussion), but they were similarly constant in the pH 6.4–7.2 interval and ~20% higher in the 6.2–6.4 range.

A third estimate of the buffering capacity was obtained by simultaneously measuring the \(\Delta pH\) and the loss of K\(^+\) induced by nigericin in choline\(^+\) medium over a 10-min period. The change in \(pH_i\) was estimated with DCF, while a parallel cell population was used for K\(^+\) content determinations by flame photom-
etry. The buffering power was then calculated as $\Delta[K^+] / \Delta \text{pH}$, as described for $\text{NH}_4^+$. In three similar experiments, values in excess of 50 mmol·liter$^{-1}$·pH$^{-1}$ were obtained.

As discussed below (see Discussion), the $\text{NH}_4^+$ titration method probably provides the most accurate estimate of the buffering capacity. For this reason, an average value of 25 mmol·liter$^{-1}$·pH$^{-1}$ was used for the calculation of $\text{H}^+$ (or equivalent) fluxes throughout the pH 6.0–7.2 range.

**Evidence for a Na$^+/H^+$ Exchange System in Thymocytes**

As shown in Fig. 2B, acid-loaded cells maintain a constant pH for long periods of time if suspended in choline$^+$ medium. In contrast, resuspension of acid-loaded cells in Na$^+$-containing solutions results in a rapid cytoplasmic alkalinization. This Na$^+$-induced alkalinization follows an exponential course and ceases when pH returns to 6.9–7.0. The rate of alkalinization depends on the starting pH, on the external and internal Na$^+$ concentrations, and on pHo. These properties are further analyzed below. In experiments to be discussed in detail below, it was found that the Na$^+$ content of the cells increases after recovery from acid-loading. Because a concomitant net uptake of Na$^+$ occurs, the alkalinization is probably mediated by Na$^+_\text{aq}/H^+\text{aq}$ exchange.
Dependence on pH$_i$

As illustrated in the inset of Fig. 4, the Na$^+$-induced rate of alkalinization depends critically on the cytoplasmic pH. At constant Na$^+$ and pH$_e$, the alkalinization rate increases with decreasing pH$_i$. Using the values determined above for the buffering capacity, the rate of alkalinization can be converted to a net efflux of H$^+$. The graph in Fig. 4 shows individual measurements of H$^+$ efflux obtained in seven preparations as a function of pH$_i$. Na$^+$/H$^+$ exchange was maximal at pH$_i$~6.2 (the lowest pH attainable with the nigericin technique under the conditions used), and decreased progressively as pH$_i$ was increased. No net H$^+$ efflux was induced by Na$^+$ at pH$_i$ > 6.9. Similar experiments were performed at various Na$^+$ concentrations. Although the absolute H$^+$ efflux rates varied with

\textsuperscript{2} An equivalent uptake of OH$^-$ or HCO$_3^-$ could equally account for the observed pH$_i$ changes. For simplicity, and in view of the effects of cytoplasmic pH on the fluxes, the Na$^+$-induced alkalinization will be interpreted throughout the manuscript as H$^+$ extrusion.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Effect of pH$_i$ on Na$^+$-induced H$^+$ efflux from thymocytes. Inset: typical fluorescence traces of Na$^+$-induced alkalinization. Cells stained with DCF were acid-loaded to the levels indicated on the ordinate. After removal of nigericin, alkalinization was triggered by resuspension in a medium containing 70 mM Na$^+$ and osmotically balanced with choline$^+$. pH$_e$ was 7.2 and the temperature was 20–22°C. Main graph: relation between pH$_i$ and H$^+$ extrusion rates. The latter were calculated from the initial rate of ΔpH$_i$, measured over the first minute in experiments like those in the inset, and given a uniform buffering power of 25 mmol·liter$^{-1}$·pH$^{-1}$ over the pH 6.0–7.2 range (see Fig. 3). Each type of symbol represents an individual experiment. pH$_i$ was determined by DCF fluorescence as described. [Na$^+$] = 70 mM; pH$_e$ = 7.2. Temperature 20–22°C.}
\end{figure}
[Na\(^+\)]\(_o\), the pH\(_i\)-dependence profiles were in all cases similar to that in Fig. 4 (not illustrated).

Dependence on [Na\(^+\)]\(_o\)

In acid-loaded cells (pH\(_i\), 6.35–6.45), the rate of alkalinization was proportional to the extracellular concentration of Na\(^+\), as illustrated in the inset to Fig. 5A.

**Figure 5.** External Na\(^+\) ([Na\(^+\)]\(_o\)) dependence of Na\(^+\)/H\(^+\) exchange. (A) Inset: representative fluorescence traces of the alkalinization elicited by increasing Na\(^+\) concentrations. DCF-stained cells were acid-loaded, followed by removal of nigericin. Alkalinization was started by resuspension in isotonic media containing the indicated concentrations of Na\(^+\) (in millimolar). Choline\(^+\) was used as an osmotic balance. pH\(_i\) = 7.2. (A) Main graph: dependence of H\(^+\) extrusion on [Na\(^+\)]\(_o\). Data from four experiments like that illustrated in the inset are summarized. The H\(^+\) efflux rates were calculated as in Fig. 4. Each symbol is representative of one experiment for which pH\(_i\) at the time of addition of Na\(^+\) is indicated. \(^1\) pH\(_o\) = 7.2. Temperature 20–22°C. (B) Lineweaver-Burk linearization of the data of A. Some of the points in A have been omitted from the drawing, because of overlap, but were used for the calculation of the line. The latter was calculated by least squares and had a correlation coefficient of 0.967.
A summary of data obtained in four different preparations is presented in Fig. 5A (main graph). The activation of H⁺ efflux by Na⁺ follows a rectangular hyperbola compatible with Michaelis-Menten-type kinetics. This was confirmed by linearizing the data according to Lineweaver-Burk (Fig. 5B) or Eadie-Hofstee (not shown). In both cases straight lines with significant correlation coefficients were obtained. From these plots the maximum rate of Na⁺/H⁺ exchange at pHᵢ 6.35–6.45 and pHₒ 7.2 was found to be 13.6 mmol H⁺·liter⁻¹·min⁻¹, and half-maximal stimulation was attained with 59 mM Na⁺.
Dependence on pH₆

As shown in Fig. 6A, Na⁺/H⁺ exchange was markedly inhibited by lowering pH₆. Na⁺-induced alkalinization was completely abolished at pH₆ ≤ 6.0 and increased progressively at higher pH₆ values, reaching a maximum at ~7.6. At pH 6.3–6.4, using 70 mM Na⁺, maximal exchange rates of 10 mmol H⁺ liter⁻¹ min⁻¹ were obtained (Fig. 6A) and half-maximal exchange was reached at pH₆ ≈ 6.9.

Experiments like that in Fig. 6A were performed at various Na⁺ concentrations in order to define the mechanism of inhibition by H⁺. Graphical analysis of the data (Fig. 6B) indicated that the inhibitory effect of H⁺ is not purely competitive. In three similar experiments the apparent Kᵢ for H⁺ ranged from 1.6 to 3.8 × 10⁻⁸ M.

Dependence on [Na⁺]ᵢ

The effect of cytoplasmic [Na⁺] on Na⁺/H⁺ exchange was determined at different values of pH₆. The rate of H⁺ efflux induced by a constant amount of Na⁺ (70 mM) was compared in cells with normal Na⁺ content and in Na⁺-depleted cells. Depletion was achieved by incubation in Na⁺-free media (choline⁺ or K⁺ substitution) for 60–120 min at 37°C. The effectiveness of the depletion procedure was confirmed by flame photometry: whereas freshly prepared cells contained 1.69 ± 0.3 nmol Na⁺ 10⁶ cells⁻¹ (equivalent to 14.8 ± 2.6 mM), the content of depleted cells dropped to 0.38 ± 0.14 nmol Na⁺ 10⁶ cells⁻¹ (equivalent to 3.4 ± 1.2 mM).

Fig. 7 illustrates the effects of Na⁺ depletion on Na⁺-induced H⁺ efflux. As discussed in reference to Fig. 4, Na⁺/H⁺ exchange is greatly activated in normal cells by reducing pH₆ (see solid symbols in Fig. 7). A similar activation was noted in Na⁺-depleted cells (open symbols). However, for identical levels of pH₆ and Na⁺, H⁺ efflux was substantially higher in Na⁺-depleted cells. This was true at all the pH₆ values explored. It is noteworthy that whereas no net H⁺ efflux occurs in normal cells at pH₆ ≥ 6.9, a very substantial flux is observed in this range in Na⁺-depleted cells.
Attempts were also made to analyze the effects of elevated $\text{Na}^+$ on the exchange mechanism. However, in cells loaded with $\text{Na}^+$ by incubation with ouabain in $\text{K}^+$-free media, the $\text{pH}_i$ could not be adequately manipulated with nigericin. Because $\text{K}^+$ depletion had occurred, only reduced acidifications could be accomplished by addition of the ionophore, which carries $\text{K}^+$ much more efficiently than $\text{Na}^+$.

**Effect of Elevated $[\text{Ca}^{2+}]_o$.**

In cells that depend on the operation of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger for the regulation of $[\text{Ca}^{2+}]_i$, removal of $\text{Na}^+$ results in an increased $[\text{Ca}^{2+}]_i$. Because our $\text{Na}^+$-depletion procedure requires prolonged incubation in $\text{Na}^+$-free solutions, an increased $[\text{Ca}^{2+}]_i$ might be involved in the activation of the $\text{Na}^+/\text{H}^+$ exchanger, as suggested for fibroblasts (Villereal, 1981). Three types of experiments were performed to test this possibility.

First, the free cytoplasmic $\text{Ca}^{2+}$ levels were compared in $\text{Na}^+$-depleted and in untreated cells using quin-2, a novel fluorescent indicator (Tsien et al., 1982). In five determinations, $[\text{Ca}^{2+}]_i$ in control cells averaged $143 \pm 23$ nM. In $\text{Na}^+$-depleted cells, $[\text{Ca}^{2+}]_i$, measured in the same choline medium used for depletion, was not significantly different ($152 \pm 17$ nM, $n = 5$). These results are consistent with the observations of Tsien et al. (1982), who found no effect of $\text{Na}^+$ removal.

![Figure 7. Effects of $\text{Na}^+$ depletion on $\text{Na}^+$-induced $\text{H}^+$ efflux. $\text{H}^+$ efflux was measured as $\Delta \text{pH}_i$ in DCF-stained and acid-loaded cells as described for Figs. 4-6. Normal cells (solid symbols) and $\text{Na}^+$-depleted cells (open symbols) were compared. $\text{Na}^+$ depletion was achieved by preincubation in $\text{Na}^+$-free solutions as described in Materials and Methods. The levels of $[\text{Na}^+]_i$ for the individual experiments, determined by flame photometry at the onset of the fluorescence measurements, were (in mM): (O) 1.5; (□) 5.9; (△) 2.8; (●) 11.8; (■) 20.1; (▲) 13. As indicated by equivalent symbols, cells from the same preparation were used for paired control and $\text{Na}^+$ depletion determinations. Abscissa: $\text{pH}_i$ at the time of addition of $\text{Na}^+$. Ordinate: $\text{H}^+$ efflux $[\text{Na}^+]_o = 70$ mM. $\text{pH}_o = 7.2$. Temperature 20–22°C.](image-url)
on \([Ca^{2+}]_i\), in murine and porcine lymphocytes. Second, experiments like that in Fig. 7 were carried out with cells depleted of Na⁺ by incubation in a Ca²⁺-free choline solution containing 2 mM Mg²⁺ and 0.1 mM EGTA. The resulting stimulation of Na⁺/H⁺ exchange was identical to that observed in Ca²⁺-containing media. Third, the effects of elevated \([Ca^{2+}]_i\), on Na⁺/H⁺ exchange were tested directly using ionophores. Ionomycin, a nonfluorescent, highly Ca²⁺-selective ionophore, was used to increase \([Ca^{2+}]_i\), while pHᵢ was simultaneously recorded. In order to minimize potential pHᵢ changes through Ca²⁺/H⁺ exchange via ionomycin, the extracellular Ca²⁺ concentration was reduced to 0.1 mM, which is still three orders of magnitude higher than \([Ca^{2+}]_i\), (see below). Results of a typical experiment are illustrated in Fig. 8A. Addition of the ionophore had no significant effect on pHᵢ. Longer incubations with ionomycin (up to 10 min) were equally ineffectual. Similar negative results were obtained in the presence of

![Figure 8](image-url)

**Figure 8.** (A) Effect of \([Ca^{2+}]_i\), on pHᵢ. Thymocytes were DCF-labeled and used for fluorescence determination as described in Materials and Methods. The cells were suspended in Na⁺ solution containing 0.1 mM Ca⁺ and, where indicated, 1 µM ionomycin was added. Calibration after disruption with Triton X-100 and the correction factor determined by the nigericin-K⁺ method were used for the estimation of pHᵢ (vertical scale). The pHᵢ remained constant for at least 10 min after addition of the ionophore. pHᵢ = 7.2. Temperature 20-22°C. The trace is representative of five similar determinations. (B) Effect of ionomycin on \([Ca^{2+}]_i\). Cells were loaded with quin-2 and suspended in Na⁺ solution containing 0.1 mM Ca⁺. A typical fluorescence trace is illustrated (excitation: 339 nm; emission: 495 nm). Where indicated, 1 µM ionomycin was added and recording was resumed. Finally, an additional 2 mM Ca⁺ was mixed into the suspension. The \([Ca^{2+}]_i\), calibration was obtained by saturation of the quin-2 signal, followed by quenching with Mn²⁺ (see Rink et al., 1983).
amiloride, which indicates that \(\text{Na}^+/\text{H}^+\) exchange was not active but balanced by an opposing flux.

That addition of ionomycin indeed elevated \([\text{Ca}^{2+}]_i\), was measured directly with quin-2 (Fig. 8B). Under conditions identical to those of Fig. 8A, addition of the ionophore rapidly increased \([\text{Ca}^{2+}]_i\), more than 10-fold. Increasing \([\text{Ca}^{2+}]_o\), from 0.1 to 2.1 mM enhanced quin-2 fluorescence only slightly, which indicates that saturation of the dye (which occurs at >10 \(\mu\text{M} \text{Ca}^{2+}\)) was nearly complete at the lower \(\text{Ca}^{2+}\) concentration. Taken together, the above results strongly suggest that \(\text{Na}^+/\text{H}^+\) exchange in thymocytes is not regulated by \([\text{Ca}^{2+}]_i\).

Selectivity of the \(\text{Na}^+/\text{H}^+\) Exchanger

The cation selectivity of the external site of the exchanger was investigated by measuring the ability of a variety of monovalent cations to generate \(\text{H}^+\) efflux in acid-loaded cells. The rate of change of \(\text{pH}_i\) upon addition of 70 mM of the chloride salts of the test cation was measured in cells previously acidified to \(\text{pH}_i\) 6.2–6.45 by the nigericin technique. Choline, which was previously shown to be inert with respect to the exchanger (see Fig. 2B), was used for osmotic balance. The specificity of the effects produced by the cations was established using amiloride, a selective inhibitor of the exchanger (see below). The results of these experiments are summarized in Table I. Maximal effects were obtained with \(\text{Na}^+\) in all the experiments. Of all the other cations tested, only \(\text{Li}^+\) promoted amiloride-sensitive \(\text{H}^+\) efflux. At equimolar concentrations, \(\text{Li}^+\) was 42\% as effective as \(\text{Na}^+\). \(\text{K}^+, \text{Rb}^+, \text{and Cs}^+\) were totally ineffective. In experiments where nigericin was quenched with albumin but not washed, addition of \(\text{K}^+\) (but not \(\text{Rb}^+\) or \(\text{Cs}^+\)) produced a small but significant alkalinization. The latter, however, was not inhibited by amiloride and was not observed in washed cells, which suggests that it was mediated by a small amount of residual nigericin.

| Cation | \(\text{H}^+\) efflux | % |
|--------|----------------------|---|
| \(\text{Na}^+\) | 8.9±2.9 | 100 |
| \(\text{Li}^+\) | 3.7±1.8 | 42 |
| \(\text{K}^+\) | 0 | 0 |
| \(\text{Rb}^+\) | 0.06±0.06 | 0.7 |
| \(\text{Cs}^+\) | 0 | 0 |

Thymocytes labeled with DCF were acid-loaded in choline\(^+\) medium with nigericin as described in Materials and Methods. The data are from three experiments in which the \(\text{pH}_i\)'s were 6.25, 6.3, and 6.45, respectively. Acid-loading was terminated by addition of albumin with or without washing. \(\text{H}^+\) efflux was calculated from the initial rate of \(\Delta\text{pH}\) recorded upon resuspension of the cells in media containing 70 mM of the chloride salt of the test cation and osmotically balanced with choline chloride. Paired measurements were made in media with 100 \(\mu\text{M} \text{amiloride and the diuretic-sensitive fluxes are reported as absolute rates or as the percent of the effect obtained with Na}^+\). \(\text{pH}_i\) was 7.2 in all cases; the temperature was 20–22°C. Data are the means ± SE of three to four determinations.
External Anion Dependence

In some cell types, Na⁺-mediated changes in intracellular pH are tightly coupled to the movement of inorganic anions (Thomas, 1976; Russell and Boron, 1976). In squid axons and in barnacle muscle fibers, external HCO₃⁻ is essential (Boron, 1977; Boron and Russell, 1983). However, this anion is unlikely to play a significant role in our experiments, which were carried out in nominally HCO₃⁻-free solutions. The possible participation of Cl⁻, the major anion in the media used, was assessed by ion-substitution experiments. Cells were acid-loaded with nigericin in media containing the gluconate⁻ or SO₄²⁻ salts of either choline⁺ or Rb⁺, which are transported poorly, if at all, by the ionophore. The different anions or the use of Rb⁺ instead of choline⁺ did not affect the acidifying properties of nigericin. Exchange was initiated after removal of the ionophore by addition of the Na⁺ salts of the substituent anions or, for comparison, NaCl.

**TABLE II**

**Effect of Inhibitors on Na⁺⁺-induced Alkalization**

| Inhibitor   | Concentration | H⁺ efflux | %     |
|-------------|---------------|-----------|-------|
| None        | —             | 100       |       |
| Bumetanide  | 0.1           | 98.7±3.5  | (8)   |
| Furosemide  | 1.0           | 89.4±4.4  | (10)  |
| Ouabain     | 1.0           | 108.0±7.6 | (7)   |
| DIDS        | 0.1           | 101.5±4.2 | (6)   |
| Amiloride   | 0.05          | 0±0       | (4)   |

Thymocytes were labeled with DCF and acid-loaded as described in Materials and Methods. pHI ranged between 6.25 and 6.40 and pHₖ was always 7.2. H⁺ efflux was calculated from the initial rate of ΔpH, recorded upon addition of 70 mM NaCl. The results are expressed as percent of the control value, which in 15 determinations averaged 7.6±0.9 mmol-l⁻¹.min⁻¹, calculated using a buffering power of 25 mmol-l⁻¹.pH⁻¹. The temperature was 20-22°C. The data are means ± SE of the number of determinations shown in parenthesis.

Na⁺⁺/H⁺ exchange was not affected by SO₄²⁻ substitution: in six determinations the response was 93 ± 6% of that observed in Cl⁻ medium. Complete substitution of Cl⁻ by gluconate resulted in a drop in Na⁺⁺/H⁺ exchange rate to 64 ± 3% (n = 6) of the control. However, it is presently not clear whether the effect is due to removal of Cl⁻ or to inhibition by gluconate, since partial (50%) substitution with gluconate produced a similar effect. In all cases the Na⁺⁺-induced fluxes could be fully inhibited by amiloride.

**Effects of Inhibitors**

The effects of a number of transport inhibitors on Na⁺⁺/H⁺ exchange were tested and the results are summarized in Table II. DMSO, the solvent used as a vehicle for delivery of hydrophobic reagents, had no effect. Similarly, the loop diuretics furosemide and bumetanide failed to inhibit the exchange significantly. Inhibition of the Na⁺⁺-K⁺ pump with ouabain (1 mM) had no effect on Na⁺⁺/H⁺ exchange, which indicates that different mechanisms are involved. Moreover,
the system is also different from the anion-dependent Na⁺-induced alkalinization observed in acid-loaded invertebrate cells, inasmuch as disulfonic stilbene derivatives (such as DIDS) are not inhibitory in thymocytes (Table II). Of the inhibitors tested, only amiloride had a substantial effect on Na⁺/H⁺ exchange. A typical experiment is illustrated in Fig. 2B, where the diuretic completely inhibited Na⁺-induced alkalinization of acid-loaded cells. As noted in Table II, full inhibition could be attained with 50 μM of this agent. The concentration dependence and inhibitory mechanism of amiloride were further investigated and the results are summarized in Fig. 9. Acid-loaded cells (pH, 6.25–6.35) were exposed to Na⁺-
containing media in the presence of increasing concentrations of amiloride. The type of inhibition was determined by repeating the experiments at three different concentrations of Na+. Because the exchanger displays Michaelis-Menten-type kinetics with respect to Na+, the data were calculated by the method of Dixon and are summarized in Fig. 9. At all Na+ concentrations, a linear relationship was found between the inverse of the H+ efflux and amiloride concentration. Within error, the three lines meet at a single point. The Vmax with respect to Na+, calculated from the value of the ordinate at the point of intersection, is very similar to that calculated from the Lineweaver-Burk linearization of Fig. 5B (15.4 vs. 13.6 mmol H+·liter⁻¹·min⁻¹). These data provide strong evidence that amiloride inhibits by competing with Na+ for binding to the transport site. The Ks calculated from the negative of the value of the abscissa at the point of intersection is 2.5 μM.

Inhibition by amiloride was fully reversible. In three experiments cells preincubated with 50 μM of the inhibitor and then briefly washed displayed normal rates of Na+/H+ exchange.

Measurement of Na+/H+ Exchange as an Extracellular Acidification

Changes in cytoplasmic pH are not necessarily the result of the translocation of H+ (or equivalent) across the plasma membrane. Thus, changes in buffering capacity, metabolic acid production, or acid-base exchanges with intracellular organelles would also alter pHi. To ensure that the Na+-induced alkalinization is a consequence of transmembrane flux of acid (or equivalent), the predicted changes in pHo were measured. Concentrated cell suspensions were made in lightly buffered media and the changes in pHo were monitored as described in Materials and Methods. Fig. 10 shows the results of a series of typical experiments. Under the conditions used, thymocytes suspended in choline medium acidify the medium at a barely detectable rate, presumably as a result of metabolic acid production. Addition of nigericin to these cells produced a marked alkalinization of the medium, with a time course that mirrors the intracellular acidification. As shown in Fig. 10A, addition of Na+ rapidly reversed the alkalinization; the time course of the Na+-induced effect also closely parallels the internal alkalinization recorded with DCF. If monensin, an ionophore capable of Na+/H+ exchange, is then added, only a small further acidification is measured. Fig. 10B shows the effects of amiloride. The diuretic did not affect the alkalinization induced by nigericin but completely blocked the effect of Na+. That the inward Na+ gradient persisted in these conditions was demonstrated using monensin, which rapidly acidified the solution. Addition of monensin to nigericin-treated cells in the absence of Na+ had no effect on pHo (not illustrated). The trace in Fig. 10C illustrates the specificity of the Na+/H+ exchanger, as well as that of nigericin. When Rb+ is added to acid-loaded cells, no change in the medium pH is observed, which is consistent with the constancy of pHo under identical conditions. Subsequent addition of Na+ produced the expected acidification, which could be drastically terminated by amiloride. In the presence of the inhibitor, a second alkalinization, probably effected by nigericin, was observed.
Stoichiometry of Na⁺/H⁺ Exchange

The Na⁺ dependence of the cytoplasmic alkalinization, together with preliminary determinations of Na⁺ gain during recovery from acid-loading, suggested a Na⁺/H⁺ exchange mechanism. Experiments were carried out to quantitate the Na⁺ uptake and to determine the stoichiometry of the exchanger. The stoichiometry was estimated by comparing the efflux of H⁺, measured either as an internal alkalinization or external acidification, with the net Na⁺ uptake. In all cases, the cells were acid-loaded with nigericin and pHᵢ was monitored with DCF in a parallel sample.

**Figure 10.** Na⁺-induced extracellular acidification in thymocyte suspensions. Isolated thymocytes were suspended in lightly buffered choline⁺ medium at 10⁷ cells/ml. This suspension was magnetically stirred and used for pHᵢ determinations as described in Materials and Methods. (A) Where indicated, 0.7 µg/ml nigericin was added. After 7 min, 30 mM NaCl was added as a small volume of concentrated solution, making the medium slightly (~20%) hypertonic. Finally, 10 µM monensin was added. The buffering capacity of the medium was determined at the end of each experiment by titration with KOH or HCl. The time scale is applicable to A–C. (B) Amiloride (50 µM final) was added at the onset of the recording. After 2 min, nigericin (0.7 µg/ml) was added, followed by 30 mM NaCl, where indicated. Finally, 10 µM monensin was added. (C) Cells were treated with nigericin as in A, followed by addition of 30 mM RbCl. Where indicated, 30 mM NaCl was also added and finally amiloride (50 µM final) was included. Note that nigericin was present throughout the recording in A–C, partially counteracting the Na⁺-induced acidification.
Thymocytes have been reported to take up \(^{22}\text{Na}^+\) at extraordinarily rapid rates \((t_{1/2} < 3\text{ min}; \text{Lichtman et al., 1972; Averdunk, 1976})\). Because these measurements were performed under conditions where both the cation content and the pH are in a steady state, isotope uptake probably reflects \(\text{Na}^+/\text{Na}^+\) exchange. These large basal transport rates would surely prevent an accurate estimation of the \(\text{Na}^+\) taken up in exchange for \(\text{H}^+\). For this reason, the net change of \(\text{Na}^+\) content, rather than isotopic fluxes, was used to determine the stoichiometry. Ouabain (1 mM) was present to prevent backflux of the \(\text{Na}^+\) taken up, and the experiments were performed both in the absence and presence of fully inhibitory concentrations of amiloride, to eliminate the possible contribution of alternative \(\text{Na}^+\) leakage pathways. Resuspending acid-loaded cells (pH \(6.25-6.35\)) in \(\text{Na}^+\) medium containing ouabain resulted in a clear increase in the \(\text{Na}^+\) content. In 11 experiments, the amiloride- (100 \(\mu\text{M}\)) sensitive fraction of the uptake was equivalent to \(15.6 \pm 2.3\) mmol·liter\(^{-1}\). The latter value was obtained 7 min after resuspension in the \(\text{Na}^+\) medium. For technical reasons, no efforts were made to determine the initial rate of \(\text{H}^+\)-induced \(\text{Na}^+\) uptake. No significant changes in the \(\text{K}^+\) content of the cells occurred during \(\text{Na}^+/\text{H}^+\) exchange (in the same 11 experiments, a net \(\text{K}^+\) gain of \(1.8 \pm 2.9\) mmol·liter\(^{-1}\) was recorded).

In experiments like those of Fig. 10, the change in pH\(_{\text{ex}}\) induced by \(\text{Na}^+\) does not accurately reflect the extent of \(\text{Na}^+_2/\text{H}^+_2\) exchange since nigericin was also present. The ionophore will tend to exchange \(\text{K}^+\) for \(\text{H}^+\), particularly if \(\text{H}^+\)/\(\text{H}^0\) is driven away from \(\text{K}^+\)/\(\text{K}^0\) by the amiloride-sensitive \(\text{Na}^+_2/\text{H}^+_2\) exchange. Thus, accurate measurements of \(\text{H}^+\) efflux could be obtained only after removal of nigericin by albumin and washing. In five experiments, resuspension of acid-loaded cells (pH \(6.25-6.35\)) in 40 mM \(\text{Na}^+\) produced a total acid secretion of \(17.7 \pm 1.3\) mmol·liter\(^{-1}\), measured after 7 min. The initial rate of acid production was \(8.7 \pm 1.3\) mmol·liter\(^{-1}·\text{min}^{-1}\). This number somewhat exceeds the value calculated from the fluorescence measurements of pH\(_{\text{i}}\) under equivalent conditions (e.g., Fig. 5), which probably reflects an underestimation of the buffering power used for the flux calculations (see Discussion). The close correspondence of \(\text{H}^+\) extrusion and \(\text{Na}^+\) uptake suggests a 1:1 stoichiometry.

**Reversibility of the Activation of \(\text{Na}^+_2/\text{H}^+_2\) Exchange by pH\(_{\text{i}}\).**

In some epithelial systems, activation of \(\text{H}^+\) extrusion is mediated by fusion of intracellular vesicles containing transporters with the plasma membrane (Gluck et al., 1982). The possibility that \(\text{Na}^+_2/\text{H}^+_2\) exchange in thymocytes is activated by pH\(_{\text{i}}\) through a similar mechanism was explored. The assumption was made that if fusion of vesicles were the only mechanism involved, then activation by acidification would revert rather slowly even if pH\(_{\text{i}}\) was returned to normal levels. To test this hypothesis, cells were acid-loaded in a lightly buffered, high-\(\text{K}^+\) medium by lowering pH\(_{\text{o}}\) to 6.2 in the presence of nigericin. Under these conditions pH\(_{\text{i}}\) tends to equal pH\(_{\text{o}}\) (see Materials and Methods). After pH\(_{\text{i}}\) reached 6.2, concentrated buffer was added to bring pH\(_{\text{o}}\), and therefore pH\(_{\text{i}}\), to 7.0. Nigericin was then removed with albumin with or without washing and the effects of \(\text{Na}^+_2\) on pH\(_{\text{i}}\) were tested both in \(\text{K}^+\)- and choline\(^+\)-based media. No net \(\text{Na}^+_2/\text{H}^+_2\) exchange was detectable in these cells. Thus, restoration of pH\(_{\text{i}}\) causes
rapid cessation of H+ efflux. The activation and inactivation of the exchange system are rapid, and exposure of intracellular exchangers is unlikely.

**Effects of Na+/H+ Exchange on Membrane Potential**

Membrane potential determinations were undertaken to determine whether Na+/H+ exchange is electrogenic or electroneutral. The fluorescent cyanine dye diS-C$_3$-(5), which has been successfully applied to lymphocytes from various tissues and species (Rink et al., 1980; Grinstein et al., 1982), was used for these determinations. Determinations of the resting membrane potential ($E_m$) of cells suspended in choline$^+$ medium were performed by the null-point titration method, using 1 µM valinomycin. In four preparations $E_m$ averaged $-76 \pm 2$ mV. This potential is higher than those reported for other lymphocytes using the same and other detection methods (Deutsch et al., 1979; Grinstein et al., 1982). The difference can be attributed to the absence of Na$^+$ and the reduced concentration of K$^+$ in the medium.

Addition of 30 mM Na$^+$ to cells in choline$^+$ solution resulted in an increase in fluorescence equivalent to 4–5% of the initial dye fluorescence. This represents a depolarization of ~5 mV. The depolarization is associated with a limited Na$^+$ permeability, rather than with an osmotic artifact, since equiosmolar sucrose or tetraethylammonium chloride had no effect. The effect of Na$^+$ on acid-loaded cells was analyzed next and the results are summarized in Fig. 11. Addition of the electrically silent cation-exchanger nigericin to cells in choline$^+$ medium had practically no effect, as expected, on $E_m$ (Fig. 11A). A small gradual depolarization was occasionally observed, which probably results from accumulation of K$^+$ (which leaves the thymocytes via nigericin) in unstirred layers surrounding the cells. Addition of 30 mM Na$^+$ to these acid-loaded cells consistently produced a biphasic change in $E_m$: an initial small depolarization was followed by a more prolonged hyperpolarization (Fig. 11A). The former is probably analogous to the Na$^+$-induced depolarization observed in normal (not acid-loaded) cells. The latter is probably due to activation of an electrogenic Na$^+$ pump following intracellular Na$^+$ accumulation via the Na$^+$/H$^+$ antiport. The evidence supporting this hypothesis is presented in Fig. 11, B and C. In the presence of amiloride, addition of Na$^+$ to acid-loaded cells produced only the depolarizing phase, which in this case was identical to that observed in normal cells. This indicates that the hyperpolarization is secondary to Na$_2^+$/H$^+$ exchange. Fig. 11C provides evidence that the Na$^+$-K$^+$ pump is involved. Cells were first treated with nigericin in choline$^+$ solution in the presence of ouabain. The ionophore produced a gradual depolarization that was more pronounced than that noted in the absence of ouabain. This suggests that accumulation of K$^+$ around the cells is partly prevented, in control cells, by inward pumping through the ATPase. Upon addition of extracellular Na$^+$, a rapid depolarization, resembling that in Fig. 11B, was recorded, followed by a depolarizing phase indicative of continued K$^+$ loss. This secondary phase of K$^+$ loss is mediated by nigericin, as the Na$_2^+$-H$^+$ exchange drives H$^+$ out of the cell away from K$^+$/K$^+$.

Further evidence of the involvement of the Na$^+$-K$^+$ pump in the hyperpolarizing phase was obtained by manipulating K$^+$. In choline$^+$ medium containing
elevated K⁺ (5 mM), the secondary hyperpolarization was more pronounced. In contrast, complete removal of K⁺ from the medium substantially decreased, but did not entirely abolish, the hyperpolarizing phase. The latter can be explained by the availability of some K⁺, which leaks through nigericin, in the vicinity of the cells.

**Figure 11.** Effect of Na⁺/H⁺ exchange on membrane potential. Potential was measured fluorimetrically using 0.5 μM diS-C₂(5) as described in Materials and Methods. 3 × 10⁶ cells/ml in choline⁺ medium were pre-equilibrated with the dye until a stable signal was obtained (4–6 min). The traces in A–C start after equilibration has been reached. (A) Where indicated, nigericin (0.7 μg/ml) was added to the suspension. The 2-min period following addition of nigericin, during which the potential did not change significantly, was deleted from the figure for illustrative purposes. Next, NaCl (30 mM) was added and recording was resumed immediately. ΔF/F is the fractional fluorescence change, where F is the fluorescence of the dye in the absence of cells. (B) Cells were pretreated with nigericin and amiloride (50 μM) for 5 min. Only the last 2 min are illustrated. Where indicated, 30 mM NaCl was added. (C) Cells were pretreated for 5 min with nigericin and ouabain (1 mM). Only the last 3 min are illustrated. Where indicated, 30 mM NaCl was added. The traces in A–C are representative of four similar experiments. The time and fluorescence scales apply also to B and C.

In summary, only comparatively small Eₘ changes were recorded during Na⁺⁺-induced recovery from acid-loading. The changes are not produced by current flowing through the exchanger, but rather can be accounted for by changes in [Na⁺]ᵢ and [Na⁺].

**Discussion**

**Buffering Power and pHᵢ of Thymocytes**

The three methods used for the determination of the cytoplasmic buffering capacity have limitations. The freeze-thawing procedure exposes compartments that may not be continuous with the cytoplasmic buffer, and therefore it tends
to overestimate the buffering power. The $K^+$-nigericin method involves determinations over comparatively long periods and assumes that other $H^+$ and $K^+$ transport pathways, including pH-regulatory systems, operate at a negligible rate. This can result in substantial overestimates of the buffering capacity. Probably the best method is $NH_4Cl$ titration, as it uses intact cells and the same detection method (DCF fluorescence) used for most of the experiments described. The $NH_4Cl$ method will overestimate the buffering power if a significant influx of the protonated base ($NH_4^+$) occurs. This factor, however, was minimized by graphical means (see Results). On the other hand, the presence of some extracellular dye may result in significant underestimations of $pHi$, particularly in heavily acid-loaded cells. This, in turn, would lead to an underestimation of the buffering power. The constancy of the buffering power in the $6.2-7.2$ range (Fig. 3) suggests that dye leakage was a minor factor in the determinations. Thus, the values obtained by the $NH_4Cl$ method are probably the most reliable and were therefore used for the calculation of $H^+$ extrusion rates. Because the buffering power was relatively constant in the $pH 6.0-7.0$ range, an average value of $25$ mmol-litrer$^-1$-pH$^-1$ was used for all the calculations. This number is on the lower end of the range reported for other tissues and summarized by Roos and Boron (1981).

The resting potential of thymocytes in choline$^+$ solution was $-76$ mV, as determined by "null-point" titration. This implies that a transmembrane $\Delta pH$ of $>1$ unit (acidic inside) would exist if $H^+$ were at electrochemical equilibrium. Two independent $pH$-measuring techniques indicate that this is not the case: both DCF fluorescence and $[^{14}C]$DMO partition experiments showed $pHi$ to be between $6.5$ and $7.1$ at a $pH_o$ of $7.2$. These values are in close agreement with determinations made in other types of lymphocytes by a variety of techniques (Deutsch et al., 1979, 1982; Rink et al., 1982; Gerson and Kiefer, 1982, 1983). Therefore, mechanisms for $H^+$ extrusion or consumption must exist. Because our experiments were carried out in nominally $HCO_3^-$-free media, exchange of this anion for $Cl^-$ is unlikely to play a major role. In contrast, evidence was found that a $Na^+$-driven $H^+$ extrusion may account for the maintenance of $pHi$.

Properties of the $Na^+*/H^+$ Exchange Process

Addition of $Na^+$ to acid-loaded cells induced $H^+$ extrusion (Fig. 2B). Cytoplasmic alkalinization was accompanied by net uptake of $Na^+$ and both processes were inhibited by amiloride. In line with previous reports (Aickin and Thomas, 1977; Kinsella and Aronson, 1981) and with the known properties of amiloride (Benos, 1982), these data are best explained by the operation of a $Na^+/H^+$ antiport. The stoichiometry of exchange, determined by measurements of net $Na^+$ uptake and $H^+$ extrusion, is not significantly different from $1:1$. This is supported by the apparent electroneutrality of the system (see below).

The thymocyte antiport has an apparent $K_m$ for $Na^+$ of $59$ mM and is inhibited by $H^+_o$ with an apparent $K_i$ of $3 \times 10^{-8}$ M and by amiloride with an apparent $K_i$ of $2.5 \times 10^{-6}$ M. Inhibition by the diuretic is completely reversible and purely competitive, whereas that by $H^+_o$ is not. Somewhat similar properties have been reported for $Na^+/H^+$ antiporters of other systems: the apparent $K_m$ for $Na^+_o$ at
physiological pH was found to be 6–7 mM in renal brush border vesicles (Kinsella and Aronson, 1980, 1981), 11.3 mM for the apical membrane of amphibian gallbladder (Weinman and Reuss, 1982), ~5 mM in neuroblastoma cells (Moolenaar et al., 1981), 25 mM in skeletal muscle cell cultures (Vigne et al., 1982), 38 mM in S3T3 cells (Schuldiner and Rozengurt, 1982), and 42 mM in MDCK cells (Rindler and Saier, 1981). The inhibitory effect of amiloride has been found to be competitive in most cases, but the concentrations required for half-maximal inhibition vary by over two orders of magnitude (e.g., compare Vigne et al., 1982, with Weinman and Reuss, 1982). In the cases where it has been studied, $H_\text{\textsuperscript{+}}$ inhibits $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ exchange with half-maximal effects in the $10^{-7}$–$10^{-8}$ M range, but some disagreement exists regarding the nature of the inhibitory effect (compare Rindler and Saier, 1981, with Vigne et al., 1982). On the other hand, the thymocyte system is clearly different from the $Na_\text{\textsuperscript{+}} + HCO_\text{\textsuperscript{3}}/Cl_\text{\textsuperscript{-}}$ exchanger of squid axons and other invertebrate cells (Roos and Boron, 1981) in that it can operate in the virtual absence of $HCO_\text{\textsuperscript{3}}$ and is insensitive to disulfonic stilbene derivatives.

The $[H_\text{\textsuperscript{+}}]$ dependence of $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ exchange in thymocytes does not follow simple Michaelis-Menten kinetics. This characteristic has also been reported for brush border vesicles and, as pointed out by Aronson et al. (1982), could be accounted for either by a greater than first-order dependence on $[H_\text{\textsuperscript{+}}]$ or by the existence of a modifier site. The coupling ratio of $Na_\text{\textsuperscript{+}}$ and $H_\text{\textsuperscript{+}}$ is ~1.0 and exchange obeys Michaelian kinetics with respect to $Na_\text{\textsuperscript{+}}$, which strongly suggests that only one $Na_\text{\textsuperscript{+}}$ (and therefore one $H_\text{\textsuperscript{+}}$) is transported per cycle. Thus, a higher than first-order dependence on $[H_\text{\textsuperscript{+}}]$ is unlikely and the existence of a $H_\text{\textsuperscript{+}}$-sensitive modifier site must be considered. In this context it is interesting to note that several authors have reported very large rates of $Na_\text{\textsuperscript{+}}/Na_\text{\textsuperscript{+}}$ exchange in thymocytes with presumably normal pH (Lichtman et al., 1972; Averdunk, 1976). It is therefore conceivable that activation of the modifier site by a slight acidification increases the affinity of the internal transport site for $H_\text{\textsuperscript{+}}$ and converts the $Na_\text{\textsuperscript{+}}/Na_\text{\textsuperscript{+}}$ exchanger into the $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ mode. This possibility is being presently investigated.

Depletion of intracellular $Na_\text{\textsuperscript{+}}$ activated $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ exchange (Fig. 7). This effect is most likely competitive, but graphical analysis of the data was precluded by the complexity of the pH dependence. A similar activation probably exists in other systems, where $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ exchange could only be observed after depletion of $Na_\text{\textsuperscript{+}}$ (Moolenaar et al., 1981; Vigne et al., 1982; Paris and Pouyssegur, 1983). In these systems, as well as in thymocytes (Fig. 7), $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ exchange persists even at normal values of pH. Under these circumstances, the cells are compensating for the lowered $[Na_\text{\textsuperscript{+}}]$ at the expense of the internal pH. Within limits, the latter will change little, in view of the buffering power of the cells. Thus, the $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ antiport can operate as a $[Na_\text{\textsuperscript{+}}]$-regulatory mechanism.

Effects of $Na_\text{\textsuperscript{+}}/H_\text{\textsuperscript{+}}$ Exchange on $E_m$

If the charge of either $Na_\text{\textsuperscript{+}}$ or $H_\text{\textsuperscript{+}}$ were crossing the membrane without compensation by counterflux of another cation (or co-transport of anions), a change in $E_m$ would be predicted. The magnitude of the latter can be roughly estimated
from the value of membrane resistance and the measured fluxes and by making assumptions of the possible stoichiometry. To our knowledge the resistance of the thymocytes membrane has not been reported, but that of the red cell membrane ($\sim 10^6 \, \Omega \cdot \text{cm}^2$; Cala, 1980) can be taken as a guideline. Using the fluxes reported in Figs. 4-7 and considering a mean cellular area of 113 $\mu$m$^2$, coupling ratios of $\text{Na}^+/\text{H}^+$ of 2:1 or 3:2 would generate potentials of the order of volts. Therefore, exchange is probably electroneutral. This was confirmed by direct $E_m$ determinations using diS-C$_5$(5). Only changes of a few millivolts were recorded upon activation of the exchanger. Moreover, the biphasic $E_m$ change could be attributed: (a) to a direct depolarizing effect of $\text{Na}^+$, observable in cells where $\text{Na}^+/\text{H}^+$ exchange was not operating either because of the elevated $pH_i$ or because of addition of amiloride, and (b) to a secondary activation of the $\text{Na}^+$ pump in response to elevated cytoplasmic $[\text{Na}^+]_c$. Data obtained in media with varying $[\text{K}^+]$ or in the presence and absence of ouabain provided evidence for the electrogenicity of the $\text{Na}^+\text{-K}^+$ pump in thymocytes.

**Physiological Significance of $\text{Na}^+/\text{H}^+$ Exchange**

Short-term challenges to the stability of $pH_i$, like those imposed by acute removal of $\text{Na}^+$ from the medium, are largely neutralized by the considerable buffering power of the cells. However, the maintenance of an electrochemical disequilibrium with respect to $\text{H}^+$(OH$^-$) in the steady state requires the continued operation of a regulatory system involving, directly or indirectly, expenditure of energy. The $\text{Na}^+_i\text{/H}^+_i$ antiport is ideally suited for this function for several reasons. (a) The system is very active when the cytoplasm is acidic but quiescent at $pH_i \geq 6.9$, i.e., when the regulatory set-point is reached. (b) The system is fully activated at $\text{Na}^+_o = 140$ mM, the approximate concentration normally found in the extracellular fluid. (c) The exchanger operates adequately at normal $pH_i$ values and even in somewhat acidic media, ensuring intracellular pH regulation under moderate acidosis.

For an electroneutral one-for-one $\text{Na}^+/\text{H}^+$ exchanger, thermodynamic equilibrium will be attained when $H^+/H_o^+ = (Na^+_i/Na^+_o \cdot K_e$, where $K_e$ is the equilibrium constant for the exchange reaction. Assuming that the system is symmetric (i.e., $K_e = 1$), and considering that $[\text{Na}^+]= 140$ mM and $[\text{Na}^+]_i = 14$ mM, it is clear that the exchanger could drive $pH_i$ up to 1 unit above $pH_o$. Therefore, sufficient energy for the maintenance of $pH_i$ is indirectly provided by the $\text{Na}^+$ pump and is stored as a $\text{Na}^+$ chemical gradient. In fact, a mechanism must exist to prevent the system from reaching thermodynamic equilibrium, a situation that would excessively alkalinize the cytoplasm. This purported kinetic modifier is also suggested by the sharp $pH$, activation threshold (see above) and has been proposed for other cell types (Aronson et al., 1982; Boron and Boulpaep, 1983).

The results with $\text{Na}^+$-depleted cells point to an additional function of the $\text{Na}^+/\text{H}^+$ antiport. At normal $pH_i$ levels the antiport will function as a $\text{Na}^+$-regulatory system. Regulation of $\text{Na}^+$ will occur at the expense of a cytoplasmic alkalinization, but the buffering power will minimize the $pH_i$ changes. Indeed, cells completely depleted of $\text{Na}^+$ could be replenished to normal levels through the exchange system with a $pH$ change of only $\sim 0.5$ unit. If other $pH$-regulatory
systems, such as a HCO₃⁻/Cl⁻ exchange, are also operating, the pH change would be further reduced.

Finally, modulation of Na⁺/H⁺ exchange by hormones and intracellular messengers may be physiologically significant. In the case of lymphoid cells, pH has been reported to change during the course of mitogenic activation (Gerson and Kiefer, 1982, 1983). Cells in the S-phase are ~0.3 pH unit more alkaline than their G₀-G₁ counterparts, and the alkalinization has been suggested to be the trigger for nucleic acid replication (Gerson and Kiefer, 1983). Our observations in thymocytes were performed with unfractionated populations. The results reported here most likely reflect the behavior of G₀-G₁ cells, inasmuch as they comprise ~85% of the unfractionated population (unpublished observations) and because DCF-loading was nonselective (Fig. 1). Fractionation experiments are underway to analyze the attractive possibility that in S-phase cells the Na⁺/H⁺ exchanger has an altered pH dependence. A shift in the pH vs. H⁺ extrusion-rate relationship could be the mechanism underlying mitogen-induced alkalinization.

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