pH Homeostasis in Human Lymphocytes:
Modulation by Ions and Mitogen

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ABSTRACT   Quiescent human peripheral blood lymphocytes have been shown to maintain a relatively constant intracellular pH of 7.0–7.2 over an extracellular pH range of 6.9–7.4. Two methods of measuring intracellular pH were used in these studies, ^{19}F nuclear magnetic resonance and [^{14}C]5,5-dimethyloxazolidine-2,4-dione (DMO) equilibrium distributions. When ATP levels were decreased in these cells, actively maintained pH regulation was abolished and cells exhibited a constant pH gradient of 0.2 pH unit (acid inside relative to outside). Possible mechanisms for pH regulation are discussed. The effects of the Na^+ and K^+ composition of the medium on pH regulation showed no correlation with their effects on mitogen-induced proliferative response, which we have previously determined (Deutsch, C., and M. Price, 1982, *J. Cell. Physiol.*, 111:73–79). In low-Na^+ mannitol medium, pH regulation was similar to that observed for lymphocytes in normal medium, whereas mitogen-induced proliferation was severely inhibited in low-Na^+ mannitol. In contrast, high-K^+, low Na^+ medium caused loss of pH homeostasis, whereas it restored the proliferative response. Loss of pH homeostasis was also observed on prolonged exposure of lymphocytes to mitogen (>6 h in culture). However, mitogen stimulation led to little or no change in intracellular pH in the first few hours of cell culture. Therefore, a shift in intracellular pH is not a necessary or general event in mitogen-stimulated proliferation of lymphocytes.

Gerson et al. (11) recently reported that an increase in intracellular pH accompanies mitogen-induced lymphocyte activation. Their suggestion that this increase is responsible for stimulated DNA synthesis was particularly intriguing because we have shown a requirement for Na early in the activation process (5), and Na/H exchange, which is known in a number of other cell systems (25), could be implicated in the rise in cell pH. To understand such processes more fully, however, it is necessary to characterize the regulation of cellular pH and show how such regulation is affected by mitogen stimulation. In this paper, we investigate (a) the regulation of the intracellular pH of resting peripheral blood lymphocytes, (b) the effect of varying the medium's ion content on this regulation, and (c) changes in this regulation accompanying mitogen stimulation.

Six methods are commonly used to determine transmembrane pH gradient: (a) digitonin-fractionation; (b) freeze-thaw; (c) pH microelectrodes; (d) pH-sensitive fluorescent dyes; (e) equilibrium intracellular/extracellular distribution of radioactively labeled weak acids and weak bases; and (f)

^{31}P nuclear magnetic resonance (NMR).[1] The potential artifacts associated with methods c–f are well known (29). Methods a and b present a more subtle problem: both methods require that the cells be washed and resuspended in high-K (140 mM) medium, and as we show (*vide infra*), the intracellular pH regulation can be altered by the ionic composition of the medium. We developed ^{19}F NMR probes of pH and have demonstrated both the accuracy and applicability of this approach to human blood lymphocytes (7, 35). A survey of weak acids and weak bases showed that ^{19}F NMR results were closely matched only by those obtained using the weak acid [^{14}C]5,5-dimethyloxazolidine-2,4-dione (DMO). The two methods of measuring intracellular pH, ^{19}F NMR and [^{14}C]-DMO equilibrium distributions, are based on different principles and have different advantages. [^{14}C]DMO can be used to measure pH at cell concentrations (cytocrits) used in cul-

ture, whereas NMR requires much higher cytocrits. However, the ^{19}F NMR pH indicators give separate resonances which indicate directly the cytoplasmic pH (pH_i) and the external pH (pH_e) (7, 35). (No other cellular compartment in lymphocytes has a sufficient volume and concentration of ^{19}F indicator to give a visible resonance under the conditions used.) ^{19}F NMR pH measurements have the additional advantages of not requiring cell water space determination, a source of experimental error in DMO determinations of pH, and not requiring that the indicator molecule be at equilibrium across the cell membrane. In this paper we use both [^{14}C]DMO and ^{19}F NMR to show that (a) quiescent human blood lymphocytes regulate intracellular pH, (b) this regulation is modified by the ion content of the extracellular medium and by mitogen, (c) upon mitogen stimulation, intracellular pH does not change in the first few hours of culture, and (d) a decrease in intracellular pH subsequently occurs over 2–3 d in culture.

MATERIALS AND METHODS

Preparation of Human Lymphocytes

Lymphocytes were prepared for both DMO measurements and NMR experiments either from plateletpheresis by-product or from whole blood freshly drawn from healthy donors. The lymphocytes were separated by gradient centrifugation, cultured, and assayed for DNA synthesis by [^3H]thymidine incorporation (5). Stimulated cultures contained either succinyl concanavalin A (Con A) (50 μg/ml) or phytohemagglutinin (PHA)-P (50 μg/ml). Unstimulated control cells were always from the same donor and preparation of cells. Lymphocytes used in NMR experiments were maintained for 5–20 min on ice as a 60% suspension. The pH of these suspensions was maintained between 7.0 and 7.2.

NMR Measurements

Cell suspensions of 20–25% cell volume were suspended in normal Hanks'-Hepes medium (142 mM Na, 5 mM K), low-Na medium (54 mM Na, 5 mM K, 176 mM mannitol), or high-K medium (54 mM Na, 88 mM K) containing 0.3–1 mM fluorinated amino acid. Cell-free solutions were pre-titrated to the appropriate pH in the presence of 20–30 mM Hepes. NMR experiments were carried out at 25°C; intracellular pH changed to the new value of pH_i within 5 min. Previous studies show that ^{19}F NMR and DMO methods give the same pH_i values at the same temperature (Fig. 4; reference 7). The NMR samples contained an internal standard for chemical shift measurement, 0.3–0.8 mM trifluoroacetate, and 10–12% D_2O for the field lock. D_2O at these low levels does not have any effect on mammalian cellular metabolism (18). Omission of the trifluoroacetate did not affect the measured pH values. Experiments with mitogen were carried out with suspension of 3% cell volume and 100–200 μg/ml succinyl Con A.

We obtained ^{19}F Fourier transform NMR spectra on a Bruker (Billerica, MA) CXP 200 instrument (Middle Atlantic Facility, University of Pennsylvania), applying broad-band proton irradiation and using pulse angles of ~40°, spectral accumulation times of 5–6 min, and a 10-mm probe with temperature control. For all NMR experiments with cells, we used a flow system that circulated the cell suspension continuously between the 10-mm sample tube in the NMR probe and an oxygenation chamber outside the magnet, thereby ensuring proper oxygenation of the cells during NMR measurements. The details of this chamber are given in Taylor and Deutsch (35). The ^{19}F chemical shifts are expressed with reference to trifluoroacetate, pH 7, in 90% D_2O; positive values are downfield from the reference compound.

Cell Water Measurements

Cell water was measured by assaying tritiated water and the extracellular marker, polyethylene glycol (4,000 mol wt), in the supernatant solutions and pellets of 400-μl samples were centrifuged through a single, silicone oil layer (30 μl, 1.03 g/ml), as described previously (3). The detailed investigation of the method used for cell water measurements has been reported by Deutsch et al. (2). Cell water and trapped extracellular water spaces were measured in every experiment (triplicate samples).

DMO Measurements

The general protocol for DMO measurements involved the addition of DMO (6–12 μM) to triplicate samples of cell suspension 10 min before

sampling; DMO distributions, water spaces, and the pH of the suspensions were measured simultaneously in replicate samples. DMO distributions were determined using the method described for determination of cell water. DMO was measured (1.3-ml aliquots) at various pHs in cell suspensions containing 1.7–4.6 × 10⁶ cells/ml in 1.5 ml of Eagle's minimal essential medium (MEM) (HCO_3^--free) and 20 mM HEPES, which had been incubated in a gently shaking 37°C water bath for 2 h. These cells were also assayed for stimulated growth in culture. When agents such as DIDS (10^{-4} M, 4,4-diisothiocyanostilbene-2,2-disulfonic acid), SITS (10^{-4} M, 4-acetamido-4-isothiocyanostilbene-2,2-disulfonic acid), ouabain (10^{-4} M), KCN (0.5 mM), and iodoacetate (1 mM) were added to cells incubating in MEM of various extracellular pH, these agents were added to the cells 2 h before sampling. Cell viability was assessed by trypan blue exclusion, always determined at the end of this 2-h period. Cell counts were determined for the DMO experiments. In addition, cell water measurements were made in triplicate along with each DMO measurement, i.e., at each pH_e, under every condition of added reagent or mitogen. There was no significant cell death (i.e., viability ≥95%) and no loss of cells (cell count in sample suspension within 97% of cell count in original suspension).

DMO Measurements in Cells Cultured for 48 h

Cells were cultured in MEM containing either 20 mM HEPES or 26 mM $NaHCO_3$ at 2.5–3.5 × 10⁶ cells/ml in either an air incubator or a 5% CO_2 incubator, respectively, at 37°C for up to 48 h. At the appropriate times, culture tubes (1.5-ml suspensions) were removed from the incubator and decanted carefully with a modified 1-ml pipette, leaving 50 μl of supernatant above the cells. 0.5 ml of fresh MEM (20 mM HEPES, HCO_3^--free), which contained the concentrations of K^+ and Na^+ that had been in the culture medium, was adjusted to the appropriate pH and gently added back to each test tube. The presence of mitogen in the replacement media did not affect measurements; the same results were obtained whether or not mitogen was in the replacement media. However, most experiments were conducted so that cells cultured for 48 h in the presence of succinyl con A (50 μg/ml) were placed in a medium that did not contain succinyl Con A. The tubes were placed in a shaking water bath at 37°C and incubated for 2 h.

In the case of the experiments that were carried out under 5% CO_2/air, a glove-bag enclosed the pH meter, centrifuge, and sampling racks, and all manipulations were performed in this enclosure. The MEM that replaced the culture MEM contained HCO_3^- concentrations that varied by more than an order of magnitude from 2 to 142 mM, thereby varying the extracellular pH (pH_e) from 6.8 to 8.2. In experiments carried out directly on mitogen-stimulated and unstimulated culture samples, [^{14}C]DMO (10 min incubation), [^3H]H_2O, and [^{14}C]PEG (<1 min incubation) were added directly to triplicate samples at times between 30 min and 70 h and worked up as described above. [^3H]-Thymidine incorporation was assayed in parallel culture samples throughout this period.

DMO Concentration and Uptake into Lymphocytes

Studies were carried out over a range of DMO concentrations from 5 to 30 μM, and the calculated intracellular pH was found to be independent of the DMO concentration throughout this range. Thus, there were no low- or intermediate-affinity DMO binding sites in this range: we were, in fact, measuring the distribution of DMO between the intracellular and extracellular water spaces. We therefore used 5–7 μM DMO (7 μCi/ml). The results also appear to be independent of cell concentration over the range from 2 × 10⁶ to 30 × 10⁶ cells/ml (35; this paper).

Cells, either stimulated in culture or nonstimulated and noncultured, as described above, were suspended in MEM containing 20 mM HEPES (2.10–3.8 × 10⁶ cells/ml) with [^{14}C]DMO (6–12 μM) and incubated in a gently shaking water bath at 37°C for intervals ranging from 0 to 40 min. The time course of DMO uptake is shown in Fig. 1 for unstimulated lymphocytes suspended in MEM containing 20 mM HEPES at 37°C. DMO equilibrates within 10 min and remains constant up to at least 2 h; there appears to be no metabolism of the ^{14}C probe. Similar studies were reported previously (2) for suspensions incubated at 25°C. Equilibration occurs within 5 min. Mitogen-stimulated lymphocytes, cultured for 6–69 h, show in all cases a similar rapid uptake of DMO, complete within 10 min, which suggests that there is no metabolism of the probe (Fig. 1), despite the fact that stimulated cells are metabolically more active than control cells (22). The different levels to which cell suspensions accumulated DMO are set by the transmembrane pH gradient, which, as this paper shows, is dependent on the pH of the extracellular medium (see also reference 7).

DMO determinations of intracellular pH were made in stimulated cells under mitogenic, culture conditions. This is shown for sample data in Table I
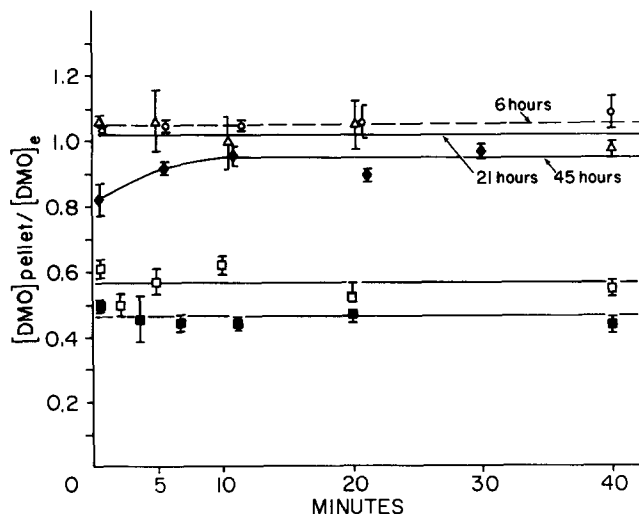
FIGURE 1 Time course of the change in the ratio of cellular [DMO] to extracellular [DMO] in human blood lymphocytes. Cells were cultured (37°C, 5% CO_2 incubation) at 2–5 × 10^6 cells/ml in normal MEM plus 10% mixed human serum in the absence (□, ■) and presence of 50 μg/ml succinyl Con A (◆, ○, △) for the hours indicated. [^{14}C]DMO (6 μM) was added to one set of cultures, and [^{3}H]H_2O and [^{14}C]PEG to another set of cultures. Samples were simultaneously assayed at the times shown. Values are given as means ± SD for triplicate samples.

for various volumes of culture suspension and various cell concentrations. Cultures grown at 2–3 × 10^6 cells/ml exhibited stimulated thymidine incorporation that was ~50% that found in cultures grown under optimal conditions of 0.5 × 10^6 cells/ml. When cells were cultured in 1.5-ml suspensions, the stimulated thymidine incorporation was 85–90% that found in 1-ml cultures. Measurements of cell water space and cellular DMO were neither accurate nor precise in 1-ml cultures at 0.5 × 10^6 cells/ml. However, reasonably good precision and accuracy (±0.05–0.1 SD) could be obtained using 1.5-ml cultures. It is clear that the range of cell culture suspensions in which mitogen-stimulated thymidine incorporation is observed is 0.5–8.8 × 10^6 cells/ml (0.02–0.5% cell volume percent). At higher cell density and/or suspension volume, early events (changes in ion transport, protein phosphorylation, and phospholipid turnover) may be expressed but not later events (stimulated DNA synthesis and mitosis). For example, we observe similar mitogen-induced increases in intracellular [Na^+] at both low and high cell densities (5), and a mitogen-induced Ca influx at high cell densities (4).

Cellular Sodium and Potassium Measurements

SODIUM MEASUREMENTS: Cells were suspended in the appropriate MEM medium and carefully layered on a multilayer column of various liquid in a 0.4-ml polypropylene centrifugation tube and sodium analysis was carried out as described in reference 5.

POTASSIUM MEASUREMENTS: Cell potassium was measured as described in reference 6. The assay of cellular potassium in cells suspended in medium containing high extracellular potassium was carried out by using the multilayer technique described above for sodium.

MEASUREMENTS OF THE ADENINE NUCLEOTIDES: The cells were incubated under appropriate conditions in an oxygen atmosphere with rapid shaking. At the end of the incubation period, the cells were quenched by the addition of ice-cold perchloric acid (4% final concentration) and the suspension was centrifuged. The perchloric acid supernatants were neutralized with 3 M K_2CO_3/0.5 M triethanolamine base and aliquots of the supernatant were used for the assay of ATP and ADP. ATP was measured by the method of Lamprecht and Trautschold (20) and ADP by the procedure of Jaworek et al. (17).

Materials

Hanks' balanced salt solution, Ca^{2+}- and Mg^{2+}-free 20 mM Hepes, was obtained from Grand Island Biological Company (Grand Island, NY); Ficoll from Pharmacia Fine Chemicals (Piscataway, NJ); hypaque (sodium salt, 50% solution) from Winthrop Laboratories (New York); PHA-P from Difco Laboratories Inc. (Detroit, MI); succinyl con A from Polysciences, Inc. (Warrington, PA) and Vector Inc. (Burlingame, CA). [^{3}H]Thymidine, [^{14}C]DMO, [^{14}C]PEG, and [^{3}H]H_2O from New England Nuclear (Boston, MA); trifluoroacetic acid

and D_2O from Aldrich Chemical Co., Inc. (Milwaukee, WI); α-(difluoromethyl)-alanine and α-(trifluoromethyl)-alanine methyl esters from Lee's Bio-organic Laboratories (Marcus Hook, PA).

RESULTS

ATP Requirement for pH Regulation by Lymphocytes

When freshly isolated lymphocytes are incubated in normal culture medium at either 25 or 37°C at various extracellular pHs, they regulate intracellular pH (7). Fig. 2 (solid symbols) shows that at 2 h there is a reversal in the sign of the pH gradient, with zero gradient occurring between 7.0 and 7.1.

TABLE I
Stimulated DNA Synthesis

	Cell concentration	Volume	Peak [^{3}H]thymidine incorporation	
	10^6 cells/cc	ml	cpm	
PHA	0.5	1.0	71,400 ± 5,729	(100%)
	2.9	1.0	43,382 ± 1,018	(60%)
	4.9	1.0	11,041 ± 3,950	(16%)
	8.8	1.0	3,867 ± 358	(5%)
Succinyl Con A	0.5	1.0	54,320 ± 1,601	(100%)
	0.5	1.5	48,170 ± 2,215	(87%)
	0.5	2.0	39,401 ± 5,635	(73%)
	1.0	1.0	37,758 ± 5,492	(70%)
	2.0	1.0	24,088 ± 7,839	(44%)
	4.0	1.0	6,568 ± 741	(12%)
	5.0	1.0	6,002 ± 200	(11%)

Cells were cultured in normal MEM in the presence of succinyl Con A (50 μg/ml) and PHA (25–50 μg/ml) at the concentrations and volumes indicated. Peak [^{3}H]thymidine incorporation was assayed on day 3 of cultivation. Controls without mitogen were always <1,000 cpm. Values are means ± SD for triplicate samples.
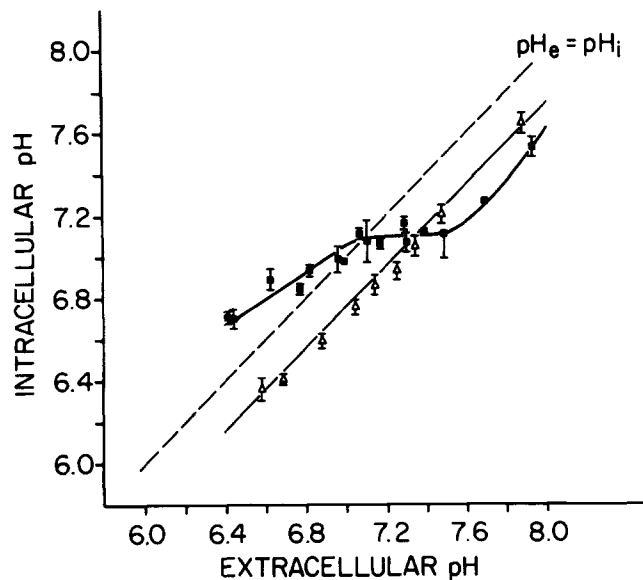

FIGURE 2 Intracellular pH vs. extracellular pH in ATP-depleted human peripheral blood lymphocytes. Cells (2.2 × 10^6/ml) were incubated at 37°C for 2 h in the presence (△) and absence (■) of 0.5 mM KCN and 1.0 mM iodoacetate, respectively, in MEM-HEPES media of the appropriate pH. Cells were suspended at 2–5 × 10^6 cells/ml in Hanks-HEPES medium at the appropriate pH and incubated at 37°C for 2 h. [^{14}C]DMO (6 μM) was added to one set of cultures, and [^{3}H]H_2O and [^{14}C]PEG to another set of cultures. After 10 min, samples were simultaneously assayed for DMO accumulation and cellular and trapped extracellular water spaces. Values are given as means ± SD for triplicate samples.

We observed the same pH profile whether cells were exposed for 10, 15, 30, 60, or 120 min to media of altered extracellular pH. Furthermore, DMO and NMR experiments both showed that, whether cells were suspended at a given pH_e or the pH_e was allowed to change as a result of cellular metabolism, the same pH_i was observed.

To determine whether the regulation of pH required cytosolic ATP, we assessed the pH profile in ATP-depleted lymphocytes. Cells were incubated in normal MEM in the absence and presence of 0.5 mM KCN to inhibit oxidative phosphorylation, and in 1 mM iodoacetate to inhibit glycolysis. Samples were assayed for ATP and ADP at 30, 90, and 120 min, after inhibition. Within the first 30 min ATP levels in CN iodoacetate-incubated cells were found to be 1.2 mM, 25–30% of the ATP levels in control cells. Negendank and Shaller (24) have shown that depletion of ATP to these levels in human lymphocytes leads to a gain in cell Na^+ and a decrease in cell K^+ in the first 2–3 h; i.e., ion gradients are dissipating when ATP decreases to these levels. At low ATP, membrane integrity was maintained to better than 96% at 2 h, which is identical to that of control cells (measured by trypan blue exclusion). Cell counts and viability determinations, done concurrently with pH measurements, showed unimpaired viability and no loss of cells in CN iodoacetate samples compared with controls. The ATP-depleted lymphocytes, however, exhibited a markedly altered dependence of intracellular pH on extracellular pH, as shown in Fig. 2 (open triangles). They maintained a constant pH gradient, 0.2 pH unit more acid inside with respect to outside, over the extracellular pH range of 6.6–7.9. This was in contrast to the control cells, which changed the magnitude and direction of the pH gradient, in order to maintain a constant intracellular pH. It appears that an ATP-dependent system is required to maintain a constant intracellular pH, while external pH varies. Cells whose membranes were deliberately damaged by direct addition of acid or base to cell suspensions showed no

regulation, but exhibited intracellular pH equal to extracellular pH (data not shown).

Effects of Extracellular Na^+, K^+ Levels on Intracellular/Extracellular pH Profile

Stimulated DNA synthesis is significantly inhibited when cells are cultured with mitogen in low-Na^+ mannitol medium, but is unaffected in low-Na^+, high-K^+ medium (3). To study pH regulation relevant to stimulated growth, we therefore examined the effects of these low-Na^+ media on intracellular pH. When freshly isolated lymphocytes were incubated in low-Na^+ mannitol at 37°C, their intracellular/extracellular pH profiles were similar to cells in normal medium (Fig. 3, A and B). In contrast, lymphocytes suspended in low-Na, high-K MEM maintained values of intracellular pH closer to the extracellular pH (Fig. 3 C). In the low-Na^+, high-K^+ medium, the cells appear to have a reduced ability to maintain a constant intracellular pH. These observations were confirmed by ^{19}F NMR measurements of lymphocytes suspended in normal, low-Na^+ mannitol, and low-Na^+, high-K^+ Hanks'-HEPES medium at 25°C (Fig. 3, a–c). When cells were suspended in media of a different pH, the intracellular pH reached its new steady state value within 5 min, as observed by ^{19}F NMR.

The metabolic energy status and stimulated growth characteristics of cells incubated in normal high-Na^+ MEM, low-Na^+ mannitol MEM, and low-Na^+, high-K^+ MEM have been reported in a previous publication (3). The intracellular concentrations of Na^+ and K^+ in lymphocytes incubated in each of these media are shown in Table II. In low-Na^+ media, regardless of the K^+ concentration, intracellular [Na^+] decreased compared with that of cells incubated in normal MEM. In contrast, intracellular [K^+] was constant at ~140 mM in all cases.

Intracellular [Na^+] per se did not correlate with the ability to maintain pH. pH homeostasis either continued to exist (in
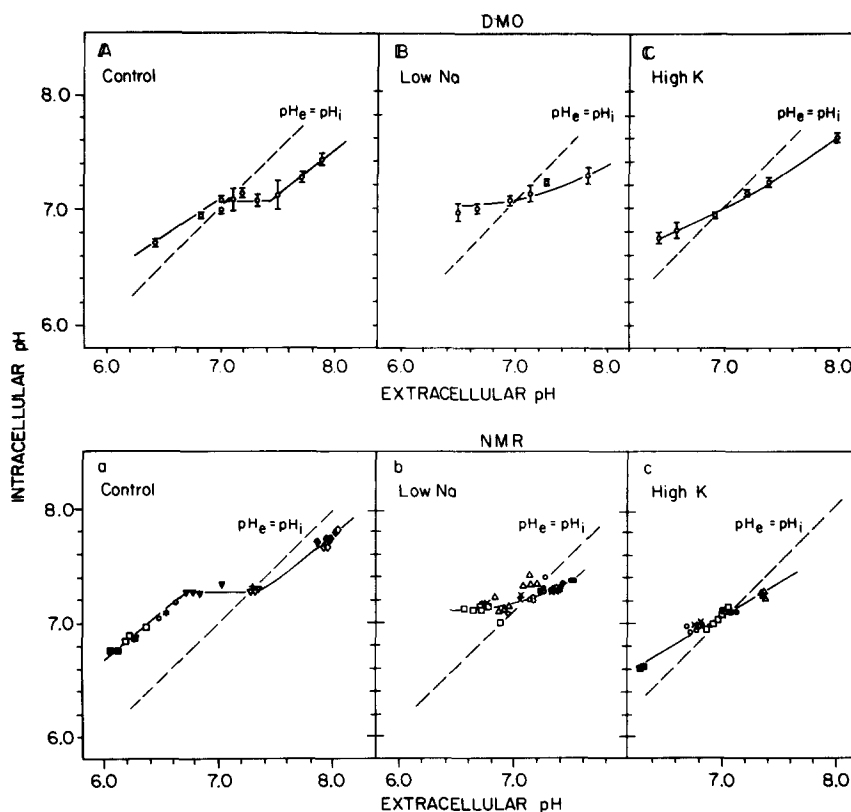

FIGURE 3   Intracellular pH as a function of extracellular pH in resting human peripheral blood lymphocytes. A, B, and C are DMO experiments. The procedure for the determination of pH_i was identical to that described in Fig. 2. Values are given as means ± SD for triplicate samples. The bottom panel (a–c) shows ^{19}F NMR pH measurements at 25°C, 188.2 MHz. Lymphocytes were suspended at 20–25% cytocrit in the indicated media, which also contained 12% D_2O, 1–3 mM trifluoroacetate (internal standard), and 0.3–1 mM ^{19}F pH indicator (α-difluoro- or α-trifluoro-methyl alanine methyl ester). Cells were oxygenated as described in Materials and Methods. Different symbols represent independent experiments.

low-Na$^+$ mannitol) or was lost (in low-Na$^+$, high-K$^+$), when intracellular [Na$^+$] decreased (Fig. 3, B and C, respectively, and Table II). Furthermore, after a 2-h exposure to 0.1 mM ouabain, cell sodium increased to 50.2 ± 5 mM ($n = 4$), while cell potassium decreased to 112.8 ± 14 mM ($n = 4$), but the lymphocytes still exhibited pH homeostasis.

Effects of HCO$_3$ Concentration, DIDS, and SITS on pH Profile

To assess the dependence of the pH profile on external HCO$_3$ concentration, lymphocytes were cultured in MEM media, and at 48 h the medium was replaced with MEM that contained HCO$_3$ at concentrations from 2 to 142 mM, thereby varying pH$_e$ from 6.8 to 8.2. The intracellular pH of lymphocytes suspended in bicarbonate/CO$_2$-buffered medium was similar to the pH profile for cells in normal MEM-HEPES-buffered medium (Fig. 4). The Hepes medium contained no added HCO$_3$; the maximum [HCO$_3$] is therefore ~0.2 mM at pH 7.3, because of dissolved CO$_2$. These observations suggest that HCO$_3$ is not mandatory for pH regulation in lymphocytes.

Incubating cells with 100 μM DIDS or SITS in the medium for 2 h did not alter pH regulation. Cells were cultured in the presence of DIDS and SITS at concentrations ranging from 10^{-6} to 10^{-4} M with no impairment of stimulated DNA synthesis. Although we observed no effect on pH regulation or stimulated DNA synthesis at a concentration of 100 μM of these anion transport inhibitors, the possibility exists that at higher stilbene sulfonic acid concentrations inhibition would be observed. For instance, Sarkadi et al. (30) report that 200 μM DIDS, after a 20-min preincubation, inhibits the volume-induced Cl efflux. Radiolabeled anion flux studies would be required before any definitive conclusion could be reached.

Short-term Effects of Mitogen Stimulation on Intracellular pH, Na$^+$ and K$^+$

When cells were stimulated with succinyl Con A (50 μg/ml) in culture, there was no change in the pH profile within the first 1–2 h, as measured by DMO (Fig. 5). We also measured intracellular pH in control and succinyl Con A-stimulated lymphocytes by ^{19}F NMR (at lymphocyte concentrations of 3–6% cells/ml), and the NMR results duplicate the DMO results.

Although these conditions are not ideal for stimulated growth, activity does occur as manifest in early events such as changes in ion transport. We observe similar mitogen-induced increases in intracellular [Na$^+$] at cell densities that range over an order of magnitude (cf. reference 5 and this paper, Table II).

Neither NMR nor DMO pH measurements provide evidence for a significant difference in intracellular pH between control and mitogen-stimulated lymphocytes within 2 h after stimulation. At a cell concentration of 3–6% cells/ml, the NMR pH data presently have relatively poor signal-to-noise and yield pH$_i$ values for mitogen-stimulated cells that vary from 0 to 0.08 pH unit lower than the pH$_i$ of the control cells. However, these data rule out a lowering of internal pH of >0.1 pH unit and certainly rule out an alkaline shift of pH at early times after mitogen addition. More precise NMR data, obtained from samples that had been incubated under culture conditions for 2 h in the presence and absence of mitogen, and collected and concentrated for NMR measurements, also showed no alkaline shift and were identical to the DMO data shown in Fig. 5.

Lymphocytes were stimulated with 50 μg/ml succinyl Con A in culture and intracellular [Na$^+$] and [K$^+$] measured within 1–2 h (Table II). Mitogen treatment left intracellular [K$^+$] unchanged. Mitogen clearly caused elevation of intracellular [Na$^+$] in cells incubated in normal MEM, and [Na$^+$] elevation is possibly present in cells incubated in low-Na$^+$ mannitol MEM with mitogen. In low-Na$^+$, high-K$^+$ MEM, there was no significant difference in intracellular [Na$^+$], compared with unstimulated cells. These results are consistent with Segel et al. (32), who also observed an increase in intracellular [Na] in human lymphocytes stimulated with PHA in high-Na$^+$ media.

Long-term Effects of Mitogen on pH Regulation

To investigate pH regulation in both stimulated and unstimulated cells in culture, particularly at the time of peak

TABLE II

Intracellular Concentrations of Na and K

| | Time | Unstimulated | | Stimulated/control | | | |
		[Na]$_i$	[K]$_i$	[Na]$_i$ ratio	P values	[K]$_i$ ratio	P values
	min	*mM*	*mM*				
Normal MEM							
	60	19.7 ± 3.0 (6)	140.0 ± 10.8 (6)	1.42 ± 0.15 (4)	$P \leq 0.05$	0.94 ± 0.04 (4)	NS
	100	21.4 ± 4.0 (5)	138.6 ± 15.6 (5)	1.42 ± 0.09 (4)	$P < 0.01$	0.87 ± 0.07 (4)	NS
Low-Na mannitol							
	60	14.6 ± 1.8 (5)	146.0 ± 5.4 (5)	1.33 ± 0.16 (4)	NS	0.96 ± 0.03 (4)	NS
	100	16.0 ± 1.5 (5)	141.0 ± 6.6 (5)	1.37 ± 0.17 (4)	NS	0.87 ± 0.03 (4)	$P \leq 0.01$
High K, low Na							
	60	13.4 ± 1.2 (5)	144.0 ± 8.9 (5)	1.06 ± 0.10 (5)	NS	0.96 ± 0.06 (5)	NS
	100	12.0 ± 1.8 (4)	147.5 ± 9.6 (4)	1.23 ± 0.10 (4)	NS	0.97 ± 0.07 (4)	NS

Cells were suspended at 1–2 × 10^6 cells/ml in normal MEM (142 mM Na), MEM in which Na had been partially replaced with mannitol (63 mM Na), and MEM in which Na had been partially replaced with K (63 mM Na, 79 mM K). Control and succinyl Con A-stimulated cells were incubated at 37°C in a 5% CO$_2$/air incubator and sampled at the indicated times as discussed in the Materials and Methods. Each experiment contained triplicate samples for each ion determination and cell water determination. Values are given as means ± SEM for the number of experiments (different donors) in parentheses.
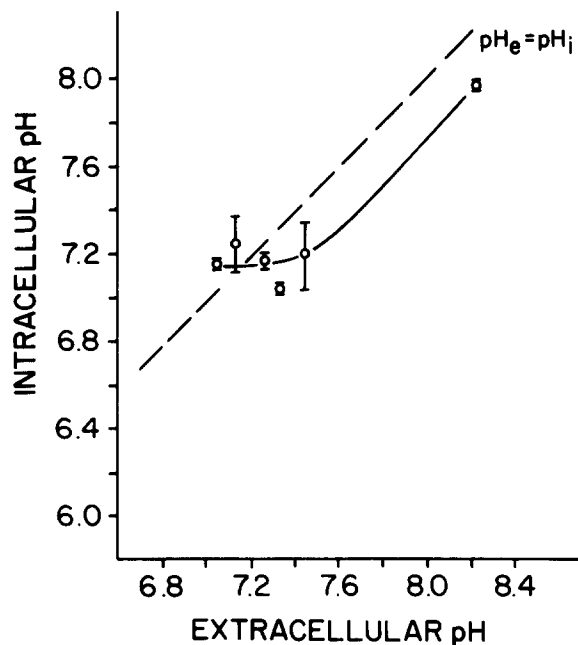
FIGURE 4 Intracellular pH vs. extracellular pH as measured with DMO in 48-h-cultured resting lymphocytes. Cells were cultured at 2-4 × 10⁶ cells/cc for 48 h in normal MEM (142 mM Na). After 48 h the cultures were transferred to a 5% CO_2-air glove-bag and the supernatant was decanted. The corresponding MEM-HCO_3 medium of appropriate pH was added, and the pH was assayed as described in Fig. 2. All manipulations were carried out in the glove-bag. Values are means ± SD for triplicate samples.

DNA synthesis (that is, 48-72 h), we performed the following experiments. Lymphocytes were cultured under normal conditions and stimulated with succinyl Con A. After 40-48 h, cells were suspended (by decanting culture medium and replacing with appropriate MEM) in MEM-HEPES medium at various pHs and the intracellular pH was measured after the cells had equilibrated in the fresh medium. As shown in Fig. 6, A and C, unstimulated lymphocytes incubated at 37°C for 48 h in normal MEM or in high-K^+ MEM behave similarly to those incubated for only 2 h (Fig. 3, A and C): unstimulated cells cultured and subsequently incubated in normal MEM are still able to maintain a constant intracellular pH (pH homeostasis) after 48 h in culture, and cells cultured in and subsequently incubated in high-K^+ MEM still show little pH homeostasis. However, unstimulated cells incubated in low-Na mannitol (Fig. 6 B) show less pH homeostasis at 48 h in culture as compared with 2 h. Lymphocytes stimulated with succinyl Con A under all culture conditions used thus far show little or no pH homeostasis, and no crossover (Fig. 6, a-c). These results with 48-h cultures of mitogen-stimulated cells were the same regardless of the HCO_3^- content (Fig. 6a, open triangles).

The experiments presented above addressed the question of regulation and the ionic modulation of regulation. However, it is of major concern whether or not the intracellular pH is altered during mitogen-stimulated proliferation, and whether such a change is a critical determinant in the activation process. We therefore measured intracellular and extracellular pH in culture samples directly, at intervals of ≤10 h, on parallel cultures of stimulated and unstimulated cells. These measurements were carried out both under CO_2/HCO_3^- buffering conditions and in a HEPES-buffered medium. We also replaced culture media at the time of pH measurement with fresh MEM at approximately the same pH. The results, in all cases, indicate that there is little or no shift in intracel-

lular pH in stimulated as compared with unstimulated cells in the first 6 h (Fig. 7).

After the first few hours, the metabolic activity of the cells leads to a progressive acidification of the culture medium. Since the regulation of intracellular pH has been altered in mitogen-activated cells compared with control cells, as illustrated in Fig. 6A, the stimulated cells become noticeably more internally acidic than unstimulated cells at long culture times (Fig. 7A). The difference in internal pH in stimulated cells with respect to internal pH in unstimulated cells is time dependent, as indicated in Fig. 7A. Initially, there is no difference between stimulated and unstimulated cells in intracellular pH. However, with increasing time in culture, this difference increases because the pH_i of stimulated cells drops to a greater extent in the same period of time. After long periods in culture, pH_i approaches pH_e for stimulated cells, because the pH regulatory mechanism(s) has changed from that able to maintain pH homeostasis (exhibited by unstimulated or 2-h stimulated cells [Figs. 5 and 6A]), to that giving rise to a state in which pH_i approximately equaled pH_e, exhibited by 48-h-stimulated cells (Fig. 6a). The consequence of this transition is that the pH_i of stimulated lymphocytes acidifies in a fashion identical to pH_e, as pH_e becomes progressively more acid throughout the culture period (Fig. 7B).

DISCUSSION

pH Regulation: General Comments

Two independent methods show that human peripheral blood lymphocytes exhibit pH homeostasis over a wide range of extracellular pH (7, 35). Lymphocyte pH regulation exhibits several characteristics. First, quiescent lymphocytes in normal or low-Na^+ mannitol medium are able to maintain a relatively constant intracellular pH of 7.0-7.2 over an extracellular pH range of 6.9-7.4. Second, the observed pH gradient changes sign at pH 7.1: the gradient is acid inside with respect to outside for $pH_e > 7.1$, and is alkaline inside with respect to outside for $pH_e < 7.1$. Third, neither protons nor hydroxyl ions are in electrochemical equilibrium anywhere in the regulated region: for the measured value of the membrane potential (2, 3, 8, 9, 19, 26, 33, 36), the lymphocyte maintains pH_i such that the pH and electrical gradients are not equal.

The present work shows that metabolic energy is required for pH regulation. When ATP levels are decreased in these cells (Fig. 2), actively maintained regulation is abolished and cells exhibit a constant pH gradient of 0.2 pH unit (acid inside
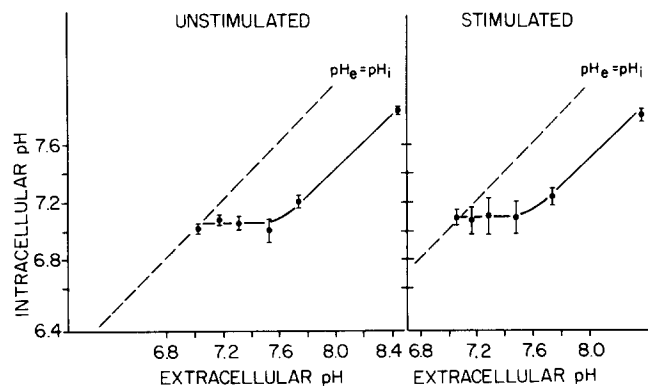

FIGURE 5 Intracellular pH vs. extracellular pH as measured with DMO in mitogen-treated human peripheral blood lymphocytes. Cells (2.7 × 10⁶/ml) were incubated at 37°C for 2 h in the presence and absence of succinyl Con A (50 µg/ml) in MEM-HEPES media of the appropriate pH and assayed as described in Fig. 2. Values are given as means ± SD for triplicate samples.

relative to outside). The constant pH gradient observed in ATP-depleted cells could result from a constant Donnan potential of -18 mV, a residual membrane electrical potential caused by undissipated ion gradients, or a combination of these. Ouabain incubation for 2 h inhibits the Na^+-K^+ ATPase in lymphocytes, and intracellular Na^+ falls while intracellular K^+ rises. However, since ouabain incubation did not alter the pH profile of the normal lymphocytes, the effects of CN^- iodoacetate on pH regulation cannot be ascribed to decreased flux through the Na^+-K^+ ATPase, nor can the Na^+-K^+ ATPase itself be a major factor in pH regulation, either in terms of its

electrogenicity or its ATP-driven fluxes. The behavior of ATP-depleted cells shows that some other ATP-dependent transport system(s)—direct or indirect—is present in human peripheral blood lymphocytes. Direct involvement of energy may be mediated by, for example, a H^+-ATPase, and indirect involvement may be mediated by an ATP-dependent gradient(s) of ions that exchange with H^+, HCO_3^-, or OH^-.

The important site of this pH regulation is in the plasma membrane. The regulation cannot be explained solely by intracellular buffer capacity (physiocochemical and biochemical reactions) or subcellular compartment proton uptake.
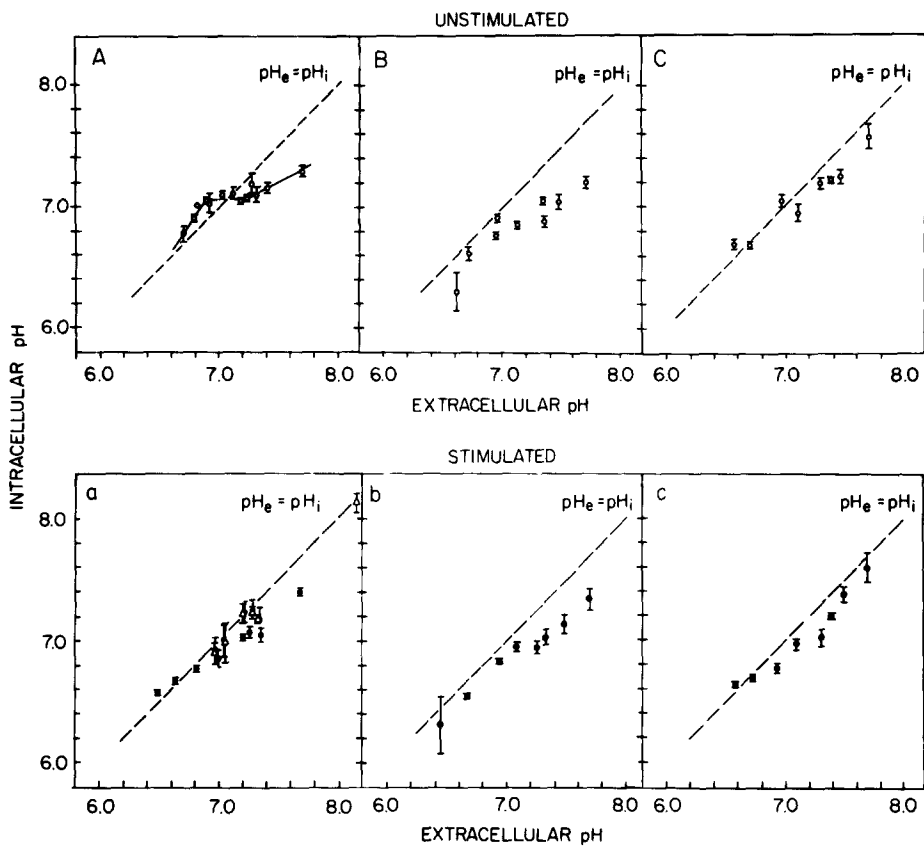

FIGURE 6   Intracellular pH vs. extracellular pH as measured with DMO in 48-h-cultured lymphocytes. Cells were cultured at 2-4×10^6/cc for 48 h in the presence (●) and absence (○) of succinyl Con A (50 μg/ml) in: (A, a) normal MEM (142 mM Na); (B, b) low-Na mannitol MEM (63 mM Na); and (C, c) low-Na, high-K MEM (63 mM Na, 79 mM K). After 48 h the cultures were decanted, the corresponding MEM-HEPES medium of appropriate pH was added, and the pH was assayed as described in Fig. 2. Open triangles in a are experiments carried out in a CO_2 atmosphere using suspensions containing varying $[HCO_3]$. Values are means $\pm$ SD for triplicate samples.
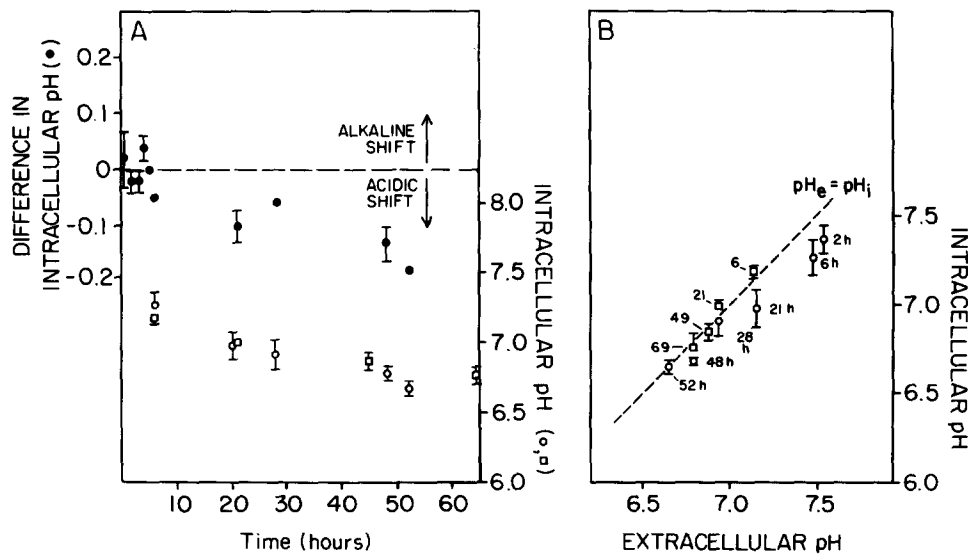

FIGURE 7   Measurements of intracellular pH in human peripheral blood lymphocytes directly in culture. (A) Cells were cultured at 2-3×10^6/ml (1.5 ml) and measured at the indicated times by addition of [^{14}C]DMO (6 μM), ^3H_2O, and [^{14}C]-PEG directly to individual culture samples without having altered the samples in any way. [^3H]Thymidine incorporation was monitored in parallel samples and showed 50–70% stimulated DNA synthesis. The difference in intracellular pH between paired control and stimulated cells as a function of time is shown by closed circles according to the left-hand ordinate and each time point represents four to five experiments (different donors) each of which was carried out using triplicate sampling. Values are given as means $\pm$ SEM for four to five experiments. All positive values represent an alkaline shift of pH$_i$ in stimulated relative to the corresponding control lymphocytes, whereas negative values represent an acidic shift. The absolute value of the intracellular pH as a function of time is shown by the open symbols according to the right-hand ordinate. Each time point represents a single donor and is the mean $\pm$ SD for triplicate samples. Two (○, □) out of six such experiments are shown here. All experiments showed acidification of pH$_i$ with time. (B) Same experiments shown in A by the open symbols, plotted as a function of extracellular pH.

Both DMO and NMR data showed that the steady state pH_i was established within 5 min and maintained for hours, as cells continued to produce acid. In all experiments described here (except for cells poisoned with CN^- iodoacetate), the lymphocytes were producing acid, which acidified the external medium against an electrochemical gradient (there is no net consumption of protons by biochemical reactions). At the same time, for external pH_e <7.1, the intracellular pH was alkaline relative to the extracellular pH.

If this pH gradient were due to simple physicochemical buffering, then eventually the cell buffer capacity would be saturated as the cell continued to produce acid, and with time the pH gradient would decline. In fact, both DMO and NMR results demonstrated that the steady state pH_i was reached within 5 min of suspending cells at a new pH_e, and there was no time-dependent dissipation of the pH gradient as the cells continued to produce acid. Furthermore, the CN^- iodoacetate-incubated cells should have similar physicochemical buffers, but the pH profiles were very different. Thus, the intracellular pH cannot be accounted for by cell buffer capacity.

Another possibility for maintaining an alkaline-inside pH gradient in a cell secreting protons against an electrochemical gradient is that protons may be taken up by a subcellular compartment. If a subcellular compartment were taking up protons, the electrochemical driving force for protons to move into the cell would be increased; as protons move in, the extracellular pH should become alkaline with time. In fact, only acidification of the external media was observed. Therefore, there must exist an energy-dependent mechanism for transport of proton equivalents across the plasma membrane.

Ion Control

Cells have been shown to extrude H^+, or its equivalent, by Na^+/H^+ exchange, K^+/H^+ exchange, HCO_3^-/Cl^- exchange, Na^+-HCO_3^- entry and H^+-ATPases (1, 16, 21, 28, 29, 31). In the studies reported here, lowering $[HCO_3^-]$ to submillimolar concentrations (HEPES experiments) or varying $[HCO_3^-]$ from 2 to 142 mM had no effect on the lymphocytes' ability to regulate their internal pH over a wide range of external pH. If HCO_3^-/Cl^- exchange were involved in pH regulation in these cells, then the apparent K_m for bicarbonate would appear to be much lower than the K_m reported for other systems (2–10 mM in the presence of >400 mM Na^+), which suggests that this scheme is unlikely.

Na^+/H^+ exchange remains a possibility, because even in our low-Na medium or ouabain-incubated cells, the remaining $[Na^+]_e/[Na^+]_i$ ratio would be 6.3 in the former case and 3.2 in the latter (compared with a ratio of 7 for lymphocytes in normal medium), i.e., sufficient to drive Na^+/H^+ exchange; however, the apparent K_m for external Na^+ would have to be significantly less than 50 mM.

Experiments were not done with much lower Na^+ or Na^+-free media because we were interested in investigating pH regulation relevant to stimulated growth; i.e., when significant inhibition of stimulated DNA synthesis is observed, is pH regulation altered? The choice of Na^+ content of the medium was thus dictated by the conditions under which the ion-dependent proliferative response was studied. The interesting point, in the context of these studies, is the lack of correlation between media effects on pH regulation (Fig. 3, a–c) and media effects on lymphocyte activation (3).

The loss of pH homeostasis caused by the high-K^+, low-Na^+ medium could arise in either of two ways. First, high-K^+ medium, by causing depolarization of the cells, could reduce

any membrane potential-dependent component of H^+ extrusion, leaving the cells with only electroneutral H^+ transport paths. Second, external high K^+ could compete effectively for external Na^+-binding sites on a Na^+/H^+ exchange system, thus effectively raising the apparent K_m for external Na^+.

Our observations are consistent with a pH-regulating mechanism that has two components: one sensitive to voltage, and the other insensitive to voltage and therefore electroneutral. A voltage-sensitive mechanism could account for the diminished pH homeostasis exhibited by low-Na^+, high-K^+ cells. Control and low-Na^+ mannitol cells have similar membrane electrical potentials, whereas low-Na^+, high-K^+ cells are markedly depolarized (3, 26). The displacement of the high-K^+ pH profile from the CN^- iodoacetate pH profile (the CN^- iodoacetate-poisoned cells showed an equilibrium ΔpH of 0.2, and the high-K cells show a ΔpH of ~0) suggests that an active electroneutral proton extrusion is still present in high-K cells, assuming that high K^+ has completely depolarized the cells. If K^+/H^+ exchange is important in maintaining the pH gradient, then the near-zero transmembrane proton gradient, which we observed in high-K cells, is expected because the ratio $[K^+]_i/[K^+]_o$ is greatly reduced.

A previous study (27) has concluded that neither Na^+ gradients nor membrane potential gradients have any short-term influence on intracellular pH, based on experiments at a single extracellular pH, presumably ~7.3. However, intracellular pH measurements at a single external pH in the region of 7.2–7.3 would not detect the difference between the pH profiles of, for example, Fig. 3, A and C; in this region, the difference in pH_i between normal and low-Na^+, high-K^+ cells is small and is less than or equal to the precision of the measurement of pH_i.

Our determinations of pH_i in resting lymphocytes suspended at an extracellular pH of 7.2–7.4 are in agreement with previously reported values obtained by using a variety of experimental techniques (2, 7, 10, 23, 27, 37). In addition, our results indicate a high degree of pH homeostasis. This pH homeostasis may be typical of free-living cells and has recently been reported for a number of such organisms: *Escherichia coli* (34), *Saccharomyces cerevisiae* (12), *Tetrahymena pyriformis* (13), Chinese hamster ovarian cells (15), and Ehrlich ascites tumor cells (14).

Mitogen Effects

Mitogen-stimulated cells showed a time-dependent change in pH regulation but not an ion-dependent change. Mitogen, in cell suspensions of various cation contents and concentrations, induced a similar loss of pH homeostasis, as did high-K^+ medium, and this induction took >6 h to become manifest in our measurements.

Within the first hour or two of exposure to mitogen there was no difference between unstimulated or stimulated cells with respect to the steady state pH_i achieved in response to pH_e. Hence, we suspected that similar behavior would be exhibited by unstimulated and stimulated culture samples, which had been placed in culture (CO_2 incubator, 26 mM HCO_3^-) at an extracellular pH 7.4–7.2 and allowed to grow. In fact, we observed no change in intracellular pH over at least the first 6 h, and no difference between stimulated and unstimulated cells, although the extracellular pH was decreasing. Over the next 2 d acidification of the external milieu continued, particularly in cultures of stimulated cells (Fig. 7 B). Unstimulated cell cultures acidified according to the control curve (Figs. 3 A and 6 A), while stimulated cells behaved according to the curve in Fig. 6 a, the non-homeo-

static curve where $pH_i = pH_e$, and acidified to a greater extent in the same time.

Rink et al. (27) also report no change in pH_i at an extracellular pH of 7.4 when lymphocytes isolated from pig mesenteric lymph nodes and mouse thymus were treated with Con A for 75 min. Our data disagree, however, with the results obtained by Gerson et al. (11) and Gerson and Kiefer (10), who presented data showing that mitogenic stimulation of mouse spleen lymphocytes results in two sequential intracellular alkalinizations. The first reaches a maximum (0.2 pH unit shift) at 10 h, and the second reaches a second maximum (0.3 pH unit shift) at 48–60 h after mitogen stimulation. We observed no short-term effect of mitogen stimulation, and the only shift in pH_i that we observed at longer times was an acid shift.

The discrepancies between our results and those of Gerson et al. most probably derive from differences in tissue type and experimental techniques. The various tissues from which lymphocytes are derived (spleen, thymus, mesenteric lymph nodes, blood) have (a) different distributions of cell types and (b) significant differences in numbers of cells that have been activated in vivo. Consequently, the cell suspensions isolated from the various sources differ markedly in metabolic activity, size and number of mitochondria per cell, and percent viable cells. For example, the fraction of B cells in the mouse spleen cells used by Gerson et al. (10, 11) was ~0.5, whereas for human peripheral blood lymphocytes the B cell fraction was ~0.10. The cell viability of the spleen cells was also much lower: 41% of the cells were dead after 26 h in culture (D. F. Gerson, personal communication), whereas human peripheral blood lymphocyte viabilities, determined by cell counts as well as trypan blue exclusion, were >95% throughout the culture period (3, 5). There is no simple way to correct cell water space data for large numbers of heterogeneous dead cells. Such differences can account for the discrepancies among the reported effects of mitogen on intracellular pH in various lymphocyte preparations.

In summary, we have shown that the intracellular pH regulation displayed by quiescent human blood lymphocytes was modified by the ion content of the extracellular medium and by mitogen. We have also shown that mitogen stimulation led to little or no change in intracellular pH in stimulated lymphocytes in the first few hours of culture, and that intracellular pH decreased, rather than increased, over the subsequent days in culture. Therefore, a shift in intracellular pH is not a necessary or general concomitant of mitogen-stimulated proliferation in lymphocytes.

Interpretation of the observations and assignment of the specific changes to mechanisms of the pH regulation will require characterization of the membrane potential, ion concentrations, and proton fluxes in cells that have been stimulated with mitogen for 48 h (the time at which we observed changes in regulation) or less. Presumably, at some time between 2 h and 48 h after stimulation in culture, pH regulation is altered. The precise time course is currently being determined.

Note Added in Proof: Grinstein et al. (S. Grinstein, S. Cohen, and A. Rothstein, 1984, *J. Gen. Physiol.*, in press) have recently demonstrated the presence of a Na^+/H^+ antiporter in rat thymic lymphocytes that have an acidic intracellular pH (pH 6.9).

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