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Cytoplasmic pH and Free Mg²⁺ in Lymphocytes

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ABSTRACT Measurements have been made of cytoplasmic pH, (pH_i) and free Mg²⁺ concentration, ([Mg²⁺]_i), in pig and mouse lymphocytes. pH_i was measured in four ways: by a digitonin null-point technique; by direct measurement of the pH of freeze-thawed cell pellets; from the ³¹P nuclear magnetic resonance (NMR) spectrum of intracellular inorganic phosphate; and by the use of a newly synthesized, intracellularly-trappable fluorescent pH indicator. In HEPES buffered physiological saline with pH 7.4 at 37°C, pH_i was close to 7.0. Addition of physiological levels of HCO₃⁻ and CO₂ transiently acidified the cells by ~0.1 U. Mitogenic concentrations of concanavalin A (Con A) had no measurable effect on pH in the first hour. [Mg²⁺]_i was assessed in three ways: (a) from the external Mg²⁺ null-point at which the ionophore A23187 produced no net movement of Mg²⁺ or H⁺; (b) by Mg-sensitive electrode measurements in freeze-thawed pellets; and (c) from the ³¹P nuclear magnetic resonance spectrum of the γ-phosphate of intracellular ATP. Total cell Mg²⁺ was ~12 mmol per liter cell water. The NMR data indicated [Mg²⁺]_i > 0.5 mM. The null-point method gave [Mg²⁺]_i ~0.9 nM. The electrode measurements gave 1.35 mM, which was thought to be an overestimate. Exposure to mitogenic doses of Con A for 1 h gave no detectable change in total or free Mg²⁺.

The concentration of H⁺ and free Mg²⁺ can powerfully influence many, probably most, intracellular processes. Knowledge of the normal cytoplasmic pH (pH_i) and free [Mg²⁺]_i is thus a prerequisite for setting conditions for investigation of intracellular mechanisms, from organelle function to enzyme kinetics. Furthermore, both these ions, though more often H⁺, have been implicated in various aspects of cell activation, especially cell growth, differentiation, and proliferation (e.g., references 3, 6, 13, 22).

One of the most widely studied model systems is mitogenic stimulation of mammalian lymphocytes by lectins such as concanavalin A (Con A). Many transducer systems are proposed as mediators of the effects of these surface ligands, including changes in ion flux and cytoplasmic ion composition (e.g., references 2, 3, 7, 25), and there has been considerable biochemical investigation into the processes of stimulation. Yet, reliable measurements of cytoplasmic pH are not available and there seem to be no data relating to free Mg²⁺. We therefore attempted to fill this gap, spurred on by a specific need for values for pH_i and [Mg²⁺]_i, to calibrate our fluorescent dye measurements of cytoplasmic free-[Ca²⁺]_i ([Ca²⁺]_i) (25).

Many of the available techniques seemed inappropriate for lymphocytes, which are too small and elusive for microinjection of indicators or impalement with ion-selective microelectrodes.

The equilibrium distribution of weak acids and bases gives not cytoplasmic pH, but some average intracellular pH for all the cell compartments (20). This method has given widely varying values in human peripheral lymphocytes, pH 6.8 to 7.4 with pH_o 7.4 according to the probe chosen (3, 29). Moreover measured changes in weak acid distribution (7) could reflect altered pH gradients across organelles rather than changing pH_i.

Methods more suitable for lymphocytes can be devised but each still has its own possible artefacts. We therefore assessed pH_i and [Mg²⁺]_i in several different ways, with satisfactory consistency of results.

Four methods were used for determining pH_i. (a) We adapted a procedure recently described for estimating [Ca²⁺]_i in liver cells (16). The external pH at which disruption of the lymphocyte plasma membrane by digitonin produced no shift in the external pH was taken as a measure of pH_i. (b) Lymphocytes were rapidly centrifuged, and then the pellet was freeze-thawed and its pH measured directly. Two further approaches gave signals from intact cells. (c) Fluorescent pH indicators were trapped in the lymphocytes by means of membrane-permeable, intracellularly hydrolyzable esters. Previously used compounds (23) proved unsatisfactory owing to excessive leakage and inappropriate pK_a, so novel derivatives

of fluorescein were synthesized. (d) We report briefly very preliminary estimates obtained from the ^{31}P nuclear magnetic resonance (NMR) measurements (15) of lymphocyte suspensions.

Three approaches were used for estimating $[\text{Mg}^{2+}]_i$. (a) In the absence of $[\text{Ca}^{2+}]_i$ gradients, the ionophore A23187 effects an electro-neutral exchange of 1 Mg^{2+} for 2 H^+ across the plasma membrane. The external $[\text{Mg}^{2+}]_o$ is found where A23187 does not produce net movement of Mg^{2+} or H^+ . At this null point $[\text{Mg}^{2+}]_o/[\text{Mg}^{2+}]_i$ should equal $([\text{H}^+]_o/[\text{H}^+]_i)^2$. By knowing also pH_i and pH_o , $[\text{Mg}^{2+}]_i$ can be calculated (4). (b) Free $[\text{Mg}^{2+}]_i$ was measured in rapidly freeze-thawed lymphocyte pellets. (c) The γ -P nuclear magnetic resonance from ATP, which is somewhat sensitive to $[\text{Mg}^{2+}]_i$ (9), was measured in dense suspensions of pig lymphocytes. Our NMR data are very incomplete but have been included mainly for purposes of comparison with other methods.

The effects of various experimental maneuvers, as well as basal readings, were observed, including altering pH_o , application of CO_2 and NH_3 , and elevation of $[\text{Ca}^{2+}]_i$ with ionophores. In view of the possible role of pH_i in mitogenesis and recent reports that intracellular pH shows a ~ 0.2 -U rise at ~ 6 h and ~ 48 h after application of Con A (7), we were interested to see whether pH showed any early (1–5 min) change along with the rise in $[\text{Ca}^{2+}]_i$ we have recently reported (25). Part of this work has been the subject of a brief communication (18).

MATERIALS AND METHODS

Cell Preparation

Lymphocytes were obtained from pig mesenteric lymph nodes, as previously described (10) or teased from the thymus glands of 6 to 8-wk-old BALB/c mice. Cells were isolated and stored in RPMI 1640 medium (Flow Laboratories Inc., Rockville, MD) lacking bicarbonate and buffered with HEPES or MOPS. The suspensions were usually kept at room temperature until required for experimentation. Cell "viability," assessed by exclusion of eosin, was $>90\%$ in those preparations reported here. Sometimes batches were kept overnight at 4°C with no obvious effects on the variables measured in this study.

Solutions

The basic physiological saline contained 145 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 0.5 mM MgSO_4 , 1 mM phosphate, 5 mM dextrose, 10 mM HEPES, pH 7.4 at 37°C . This was modified as appropriate for the different measurement procedures. For the digitonin pH null-point procedure and for the final resuspension just before pelleting and freeze-thawing, the medium usually had 140 mM KCl, 20 mM NaCl, 0.5 mM MOPS, 1 mM MgSO_4 , and 0.1 mM $\text{K}_2\text{H}_2\text{EGTA}$ to mimic intracellular cationic conditions. In fact, no differences in results were found with medium containing 145 mM NaCl and 5 mM KCl, or without the EGTA. For the $[\text{Mg}^{2+}]_o$ null-point determinations the medium contained 140 mM NaCl, 5 mM KCl, 1 mM phosphate, 0.1 mM K_2EGTA , 5 mM dextrose, 5 mM glucose, and 25 mM MOPS. MgSO_4 was added as required. For determining the effect of added A23187 on external pH the medium was: 150 mM NaCl, 5 mM KCl, 0.1 mM K_2EGTA , 0.5 mM MOPS, and the required amount of MgSO_4 . Unless otherwise stated all experiments were done at 37°C . pH was measured with Radiometer (Copenhagen) combination electrodes calibrated at 37°C against standard buffers prepared as specified by the National Bureau of Standards.

pH_i Measurement by Digitonin Null-point

Cells were first incubated, at $\sim 2 \times 10^7$ per ml, in RPMI-1640 medium buffered with 25 mM MOPS, at 37°C and at the required pH_o , for at least 45 min. Aliquots were then washed and resuspended in the 140 mM K, lightly buffered medium to a concentration of near 10^8 cells/ml; the cell concentration was the same throughout any one experiment. With the suspension continually stirred, pH_o was monitored and recorded on a pen recorder, and adjusted to the required value with addition of 0.1 M HCl or NaOH. Digitonin was then added from a DMSO stock solution to give a final concentration of 50 or 100 μM . The final DMSO concentration never exceeded 2% (vol/vol), which in itself had no effect on the pH. The maximum pH shift in the first 10 s after adding the digitonin was noted. The cells were in the 140 mM K medium for only 2–3 min.

pH of Freeze-thawed Pellets

About 200 μl of packed cells were centrifuged from the incubation medium, rapidly washed in lightly buffered, high K medium, and then pelleted at 14,000 g for 30 s. The supernatant was removed and the tube was plunged into liquid nitrogen. As the pellet thawed out in the 37°C water bath the pH electrode was immersed in the disrupted cells and the pH recorded.

pH_i Measurement with Trapped Fluorescent Indicators

The procedures were similar to those described for the Ca^{2+} indicator quin2 (17, 25, 26). Cells were loaded by incubating them with 0.1 to 0.5 μM of the permeant esters for ~ 30 min. The cells were then centrifuged and resuspended in the standard solution to give a stock suspension. The centrifuged cells were now clearly yellow. As required, 1 ml of this stock was briefly centrifuged at 14,000 g and resuspended in 2 ml medium in a fluorescence cuvette. Thus any leaked dye was removed just before experimental observation. The fluorescence signal was recorded in a thermostated cell holder in a Perkin-Elmer MPF 44A fluorimeter (Perkin-Elmer Corp., Norwalk, CT). Details of the recording conditions and subsequent experimental procedures are given in the Results section. Dye leakage was assessed by centrifuging the cuvettes, which were round test tubes, at 9,000 g and recording supernatant fluorescence.

Carboxyfluorescein acetate (23) was a gift from Dr. G. A. Smith, Department of Biochemistry, University of Cambridge. The novel indicator bis(carboxyethyl)carboxyfluorescein, BCECF, whose structure is shown in Fig. 2, was synthesized as follows.

Ethyl 3-(2,4-dihydroxyphenyl)-propionate was prepared by atmospheric pressure hydrogenation of 7-hydroxycoumarin (Aldrich Chemical Co., Milwaukee, WI) in absolute ethanol containing 5% palladium on charcoal catalyst and a little trifluoroacetic anhydride. When hydrogen uptake was complete, the catalyst was filtered off and the solvent evaporated *in vacuo*, leaving behind an oil that readily crystallized. It was recrystallized from 10% ethanol in toluene.

2 mmol of this ester plus 1 mmol trimellitic anhydride (purified by sublimation at 220 – 230°C , 0.5 torr) plus 1 mmol anhydrous zinc chloride were heated 4 h under nitrogen by immersion of the reaction tube in an 180°C oil bath. The cooled melt was digested with 3 ml 3 M KOH at 100°C for 20 min, cooled, filtered to remove white inorganic salts, and acidified with 0.5 ml 12 M HCl followed by dropwise titration with 1 M HCl, ~ 1.5 ml, until the precipitate no longer increased in volume. The brown precipitate was filtered off, and recrystallized from boiling water with charcoal. Eventually a brick red powder of BCECF was deposited, which when collected and dried weighed 39 mg (8% yield). The NMR spectrum in K_2CO_3 - D_2O showed phthalic ring signals at $\delta = 8.3$ – 7.8 and a doublet at 7.34, total 3 protons; a singlet at 7.04, 2 protons; a doublet at 6.62, 2 protons; and a pair of multiplets at 2.73–2.60 and 2.37–2.27, each 4 protons. BCECF was esterified with acetoxymethyl groups by standard procedures using acetoxymethyl bromide and diisopropylethylamine (24).

Total Cell Mg

The suspension was centrifuged at 14,000 g, in the presence of [^3H]inulin to measure trapped medium in the pellet. The supernatant was removed and 50 μl of 4% perchloric acid was added. The tubes were vigorously shaken, and left for 15 min and then 1 ml of deionized water was added. After centrifugation at 14,000 g, samples of supernatant were taken for measurement of inulin by liquid scintillation counting and of Mg by atomic absorption spectrophotometry.

Free Mg^{2+} by A23187 Null-point

(a) Mg movements. Cells, at 2×10^7 /ml, were incubated in the well-buffered, "simplified" medium at pH 7.05, 37°C , and different $[\text{Mg}^{2+}]_o$. 20 μM A23187 was added from DMSO stock and after 45 min the total cellular Mg was measured as described above. (b) H^+ movement. Cells were prepared as for pH_i measurement except that they were washed and resuspended in lightly buffered NaCl medium. pH_o was initially adjusted to above 7.10. There was a steady decline from that value owing to metabolic acid production. As pH_o fell past 7.05, 20 μM A23187 was added and the subsequent change in the rate of change of pH_o measured.

Free Mg^{2+} in Freeze-thawed Cell Pellets

The procedure was essentially that described for pH measurement of freeze-thawed pellets, except that the electrode was a glass micropipette containing the liquid Mg^{2+} sensor, recently described by Lanter et al. (12). Electrode construction and signal recording were similar to that previously described for Ca-sensitive electrodes (27). The reference electrode was a blunt microelectrode filled with 3 M KCl.

The selectivity of the Mg electrode over K and Na is not great (12), so that in the presence of intracellular concentrations of these ions the response to Mg in the millimolar range is small. We therefore calibrated each electrode in solutions containing K and Na in concentrations matching those found in pig lymphocytes by use of K-selective electrodes and flame photometry. These calibration solutions contained 140 mM KCl, 20 mM NaCl, 5 mM MOPS, pH 7.0, and the required amount of MgSO₄. In these solutions the electrodes gave only 2–3 mV between 0.5 and 1.0 mM [Mg²⁺] and 3–4 mV between 1.0 and 2.0 mM [Mg²⁺]. The electrodes did not respond to alteration of pH and [Ca²⁺] in the range expected in cytoplasm.

NMR

The methods previously described for obtaining ³¹P spectra from perfused organs (11) were adapted for use with pig lymphocytes suspended at ~7% cytochrome in basic incubation solution with extra pH buffering, i.e., with 40 mM HEPES and only 125 mM NaCl. 9 ml of suspension was continually stirred in a plastic chamber inside the magnet, and oxygenated by blowing 100% O₂ over the surface.

RESULTS

pH_i Measured by Digitonin Null-point and in Freeze-thawed Pellets

Fig. 1 shows data from an experiment in which digitonin disrupted the membranes of pig lymphocytes suspended in lightly buffered KCl media of different pH_o. The pH_o null-point for cells preincubated for 1 h at 37°C in RPMI 1640 at pH 7.4 indicated a pH_i of 7.05. For cells incubated at pH_o of 7.05 the null-point indicated a pH_i of 6.95. Similar values were found for other batches of cells treated in this way, and also

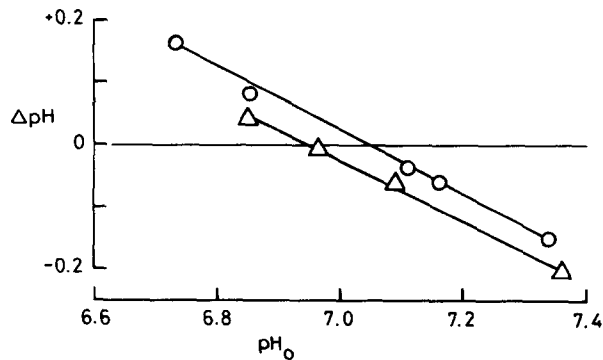


FIGURE 1 pH_o null-points for digitonin permeabilization of the plasma membrane of pig lymphocytes. Vertical axis shows the change in pH of lightly buffered medium on addition of 100 μM digitonin. Circles (○) represent data from cells preincubated in HEPES-buffered RPMI medium at pH 7.40 and triangles (Δ) show cells kept at pH 7.05. The lines were fitted by eye.

for mouse thymocytes. No measurable difference was found if cells were permeabilized in NaCl medium, or in media containing contaminating Ca²⁺ levels, 10–20 μM, not chelated by EGTA. At the lower temperature of 20°C, pig cells preincubated for 1 h at external pH 7.4 had a pH_o null-point of 7.35.

Addition of aliquots of 0.1 M NaOH before and after addition of digitonin permitted an estimate of the H⁺ buffering of the cell cytoplasm that came to ~30 slykes (30 meq base required per liter of cells to increase pH by 1 U) in this bicarbonate-free medium. This value is similar to that in various other mammalian cells (20). In vivo, with a pCO₂ of ~40 mm Hg and intracellular bicarbonate ~11 mM the buffering power would be greater.

pH measurements in freeze-thawed pellets, taken as soon as possible after thawing out to 37°C, gave values between 6.95 and 6.90. The readings, however, were steadily falling, presumably owing to metabolic acid production, and therefore represent lower limits for pH_i.

pH_i Measured with Trapped Fluorescent Indicators

6-Carboxyfluorescein can be incorporated into intact cells by uptake of its diacetate with subsequent intracellular hydrolysis regenerating the less permeable original compound (21). It was recently shown (23) that the spectral signal from the dye trapped in Ehrlich ascites tumor cells at room temperature could report pH_i. Unfortunately, in lymphocytes at 37°C, this indicator had serious drawbacks. The apparent pK_a of ~6.3 at 37°C is rather low for pH_i near 7.0, and some 30–40% of the dye leaked out of the cells in the first 10 min after washing. We then synthesized and tested an analogue with one extra carboxyl group, 5,6-dicarboxyfluorescein, but its pK_a proved indistinguishable from that of 6-carboxyfluorescein and its leakage rate only slightly better. Better results were eventually obtained with yet another analogue, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein, BCECF, which links two extra carboxylate groups via short alkyl chains to carboxyfluorescein, as shown in Fig. 2A.

Fig. 2B shows excitation and emission spectra from 70 nM BCECF at different pHs in a calibration solution containing 130 mM KCl, 20 mM NaCl, and 1 mM Mg²⁺. Increasing pH causes a further small shift in excitation peak, but its major effect is to increase the intensity of the fluorescence. A Hill plot of these data indicates a pK_a under these conditions of 6.97, nearly ideal for reporting pH_i. BCECF was loaded into the lymphocytes by permeation and subsequent hydrolysis of

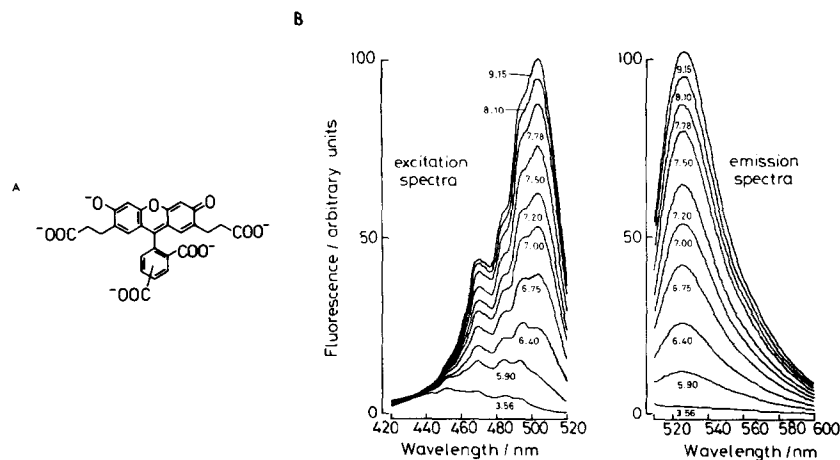


FIGURE 2 (A) Structure of bis(carboxyethyl)carboxyfluorescein (BCECF). (B) Excitation and emission spectra of 70 nM BCECF at the indicated pHs. The calibration solution contained 130 mM KCl, 10 mM NaCl, 1 mM MgSO₄, and 10 mM Na MOPS. pH was altered by addition of 1 M HCl. Excitation spectra recorded at 530 nm in "energy" mode. Emission spectra recorded with excitation at 500 nm, in "ratio" mode. Hill plots of excitation and emission signal at 500 and 530 nm, respectively, give apparent pK_as of 6.96 and 6.98. The spectra have not been corrected for the Xenon lamp or photomultiplier characteristics. In particular, the bumps in the excitation spectra between 450 and 495 nm are due to peaks in the Xenon lamp output spectrum.

the acetoxymethyl ester, a procedure introduced for intracellular trapping of carboxylate Ca^{2+} indicators (24). This indicator was better retained than carboxyfluorescein, although the loss over 10–20 min was still typically 10%.

Fig. 3 shows fluorescence signals from lymphocytes containing $\sim 10 \mu\text{M}$ BCECF. The simplest calibration is done by releasing the dye into the medium by lysing the cell and then recording the fluorescent signal at known pHs. This procedure is satisfactory unless the dye behaves differently in the cells than it did in physiological saline. In fact, the excitation peak of intracellular dye did turn out to be $\sim 5\text{-nm}$ red-shifted, and such a shift would slightly reduce the signal for any given pH at our chosen wavelengths. An appropriate correction or offset for the calibration scale could be made if one could measure dye fluorescence while imposing known pH values inside the cell. This was attempted in the procedure of Thomas et al. (23), which sets $[\text{H}^+]_i/[\text{H}^+]_o = [\text{K}^+]_i/[\text{K}^+]_o$ with the H^+/K^+ antiporter nigericin. The correction factor thus obtained was $+0.1\text{--}0.15$ U, so that an apparent basal pH_i of 6.85 obtained from the simple lysis calibration would represent a true pH_i of ~ 7.0 . In one batch of BCECF-loaded cells we found that pH_i determined with this correction matched within 0.05 U the pH_i of those same cells determined by digitonin null-point. Since the size of the correction needed with this calibration was fairly small and since the main purpose of using BCECF was to

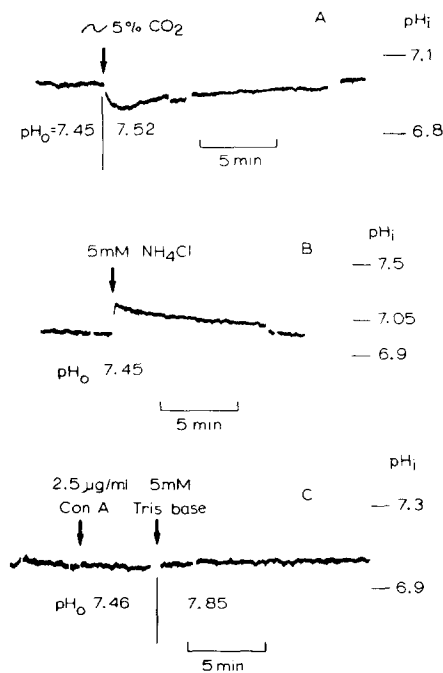


FIGURE 3 Fluorescence signals from pig lymphocytes loaded with $\sim 10 \mu\text{mol}$ BCECF/liter cells. Excitation, 500 nm; emission, 530 nm; slit widths, 4 and 10 nm. Temperature, 37°C . Cell density in the cuvette was $1.3 \times 10^7/\text{ml}$. The calibration scales were obtained by lysing the cells with 0.1% Triton X-100 and then measuring the signal from the released dye at known pH. A correction offset has been applied to compensate for the slightly reduced signal from intracellular dye, as described in the text. (A) Effect of raising pCO_2 by adding 25 mM $\text{NaHCO}_3/\text{CO}_2$ buffer from 0.75 M NaHCO_3 stock saturated with CO_2 . Judged by the measured pH_o , pCO_2 stayed nearly constant for many minutes following the addition of NaHCO_3 . (B) Effect of 5 mM NH_4Cl . (C) Effects of Con A and alteration of pH_o with Tris base. Gaps in the traces indicate periods for addition of reagents or stirring.

detect changes in pH_i , we have not analyzed the effect in greater detail, though the calibrations shown in Fig. 3 have been corrected.

Fig. 3A shows the expected rapid acidification following an elevation of pCO_2 to the physiological level. As in various other preparations (20) pH_i recovers nearly to the initial value over the next several minutes. Fig. 3B shows the transient rise in pH_i seen on addition of NH_4Cl . These changes show that the fluorescent signal does reflect internal pH because the external pH either stayed constant or changed slightly in the opposite direction. In contrast there was very little effect during the minutes following changes of up to ± 1 U in external pH, produced by nonpermeating acid or base (see, e.g., Fig. 3C). The changes in pH_i produced by changing CO_2 or adding NH_4Cl can be used to estimate intracellular buffering capacity as discussed by Roos and Boron (20). The values calculated from experiments like those shown in Fig. 3A and B came to ~ 50 slykes, but the method tends to give overestimates (20). Thus the ~ 30 slykes found by titration of freeze-thawed pellets may be more correct for CO_2 -free cells.

Some factors that might influence pH_i were examined. Suspending the cells in isotonic KCl solution had no effect on the measured pH_i . Gramicidin 10^{-8} M added to a suspension in the standard medium had no effect at all, although this would both depolarize the membrane potential and replace internal K by Na (19). (With this concentration of gramicidin the conductance to H^+ is presumably so small that electrogenic flux of H^+ through the gramicidin channels is negligible.) Transmembrane gradients of Na and potential do not, therefore, appear to have any short-term influence on pH_i . There has recently been discussion of possible interactions between pH_i and cytoplasmic free-Ca concentration $[\text{Ca}^{2+}]_i$, (see, e.g., reference 1) and so the effects of elevating $[\text{Ca}^{2+}]_i$ with the Ca ionophores A23187 and ionomycin were tested. Concentrations of these agents between 10 and 50 nM that elevate $[\text{Ca}^{2+}]_i$ by 10- to 50-fold (25, 26) had no measurable effect on the BCECF signal.

We also made 18 tests (6 with dicarboxyfluorescein) of the effect of mitogenic doses ($2\text{--}5 \mu\text{g}/\text{ml}$) of Con A in pig cells and mouse thymocytes. Fig. 3C shows the invariable result; over many minutes there were no measurable effects (<0.05 U change) on pH_i . These levels of Con A gave within 1 to 2 min sustained elevations of $[\text{Ca}^{2+}]_i$ (27). In two experiments cells were double-labeled with the calcium indicator quin2 (25, 26) and BCECF, and the $[\text{Ca}^{2+}]_i$ rise was verified in the very same cells in which BCECF fluorescence showed no change in pH_i after addition of Con A.

pH_i by NMR

NMR spectra at 20°C showed the expected peaks due to sugar and nucleotide phosphates. The chemical shift of inorganic phosphate corresponded to ~ 7.3 . However, NMR measurements at 37°C were not successful: at the high cytochromes necessary to get decent signals, acid production lowered external pH more than 0.5 U during the 15–30 min required to set up the machinery and acquire the spectra.

Total Mg

Total Mg in lymphocytes incubated in RPMI medium was 1.99 ± 0.12 (SE $n = 12$) $\text{nmol}/10^6$ cells. Given a value of $0.17 \mu\text{l}$ cell water/ 10^6 cells (Felber, S., and M. Brand, personal communication) this represents 11.7 mmol Mg/liter of cell

water. Externally bound Mg assessed as that removed by a brief exposure to Mg-free medium appeared to account for a small proportion of the total. It was a nearly linear function of $[Mg^{2+}]_o$ with ~ 0.1 nmol bound/ 10^6 cells for each 1 mM $[Mg^{2+}]_o$. Thus at the normal 0.5 mM $[Mg^{2+}]_o$ only some 0.05 nmol/ 10^6 cells or 2.5% of the total was readily removable from the surface of the cells. The total Mg of cells incubated with mitogenic levels of Con A 5 mg/ μ l was not significantly different from that of the control.

Some idea of the rate at which Mg may enter or leave resting cells was gained from incubation in low or high Mg solutions. With 5 mM $[Mg^{2+}]_o$ the Mg content increased by only 0.11 nmol/ 10^6 cells/h. In Mg-free solution the loss in the first hour was 0.21 nmol/ 10^6 cells, which represents a fractional loss of 0.11/h or a net efflux across the plasma membrane of ~ 0.03 pmol/ cm^2 /s.

$[Mg^{2+}]_i$ by A23187 Null-point for Mg Movement

Fig. 4A shows the time course of loss and gain of cellular Mg in Mg-free and 5 mM Mg solutions, in the presence of 20 μ M A23187. Much of the Mg movement was over in 15 min

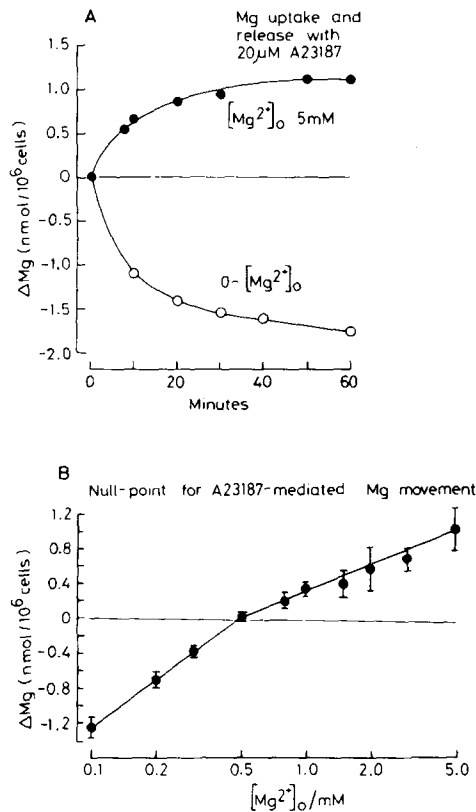


FIGURE 4 A23187-induced Mg movements. (A) Time course of uptake and release of Mg from pig lymphocytes treated with 20 μ M A23187 in high, 5-mM Mg or Mg-free solution. The vertical axis shows the gain, or loss, of cell Mg compared with the value for cells, in the appropriate medium, before addition of ionophore. The points show the mean of duplicate measurements from one batch of cells. (B) $[Mg^{2+}]_o$ null-points for A23187-mediated Mg movements. The vertical axis shows gain or loss of cell Mg from the value in cells without ionophore, after incubation for 45 min at the various $[Mg^{2+}]_o$ and pH 7.05 in the presence of 20 μ M A23187. The values have been corrected for the different external binding of Mg at different $[Mg^{2+}]_o$; the points and bars represent the mean and SE of from 2 to 8 determinations. The lines were fitted by eye.

and the levels had almost stabilized after 1 h. As a compromise between waiting for a steady state and prolonged exposure to A23187, a 45-min period was chosen for the null-point experiments. Fig. 4B shows collected data from four batches of cells which indicate a $[Mg^{2+}]_o$ null-point of 0.5 mM. The incubation medium pH, 7.08, was chosen in an attempt to minimize the transmembrane pH gradient. At this pH_i normal cells had a pH_i of 6.95. We used the digitonin method to measure pH_i in cells incubated for 45 min at the $[Mg^{2+}]_o$ null-point, with 20 μ M A23187 and found a pH_i of 6.96. Exposure to ionophore therefore appeared not to have altered pH_i. With 0.5 mM or greater $[Mg^{2+}]_o$ cell viability was not affected by the A23187 but Mg depletion produced by the ionophore at low $[Mg^{2+}]_o$ was associated with some loss of viability and decreased ATP levels. Note that in these experiments the external Ca was kept very low, <0.1 μ M, by the 0.1 mM EDTA in the solutions, and so Ca transport via A23187 was expected to be minimal.

From the $[Mg^{2+}]_o$ null-point of 0.50 mM, and taking pH_i to be 6.95, $[Mg^{2+}]_i$ is calculated to be 0.80 mM for pig lymphocytes. Similar values were obtained in two batches of mouse thymocytes.

$[Mg^{2+}]_i$ by A23187 Null-point for H⁺ Movement

The null-point measurements described above involved long incubations with 20 μ M A23187 that might have altered various cytoplasmic factors, though at the null-point neither viability nor ATP content was altered. However, it seemed worthwhile using a method that allowed a rapid measure of the ionophore-evoked Mg^{2+} /H⁺ exchange. The inset to Fig. 5 shows three traces from an experiment in which the pH of a lightly buffered cell suspension was recorded and the effects of A23187 added when pH_o was 7.05, observed at different $[Mg^{2+}]_o$. The initial decline in pH is due to metabolic acid products. At 0.2 mM $[Mg^{2+}]_o$ there was a marked reduction in the pH change, at 2 mM $[Mg^{2+}]_o$ an increase, and at 0.5 mM $[Mg^{2+}]_o$ no detectable change. The main part of Fig. 6 shows changes in slope of records of this kind plotted against $[Mg^{2+}]_o$, and gives a null-point of 0.58 mM. The $[Mg^{2+}]_i$ calculated from this data is 0.95 mM. Similar values were obtained in two other experiments.

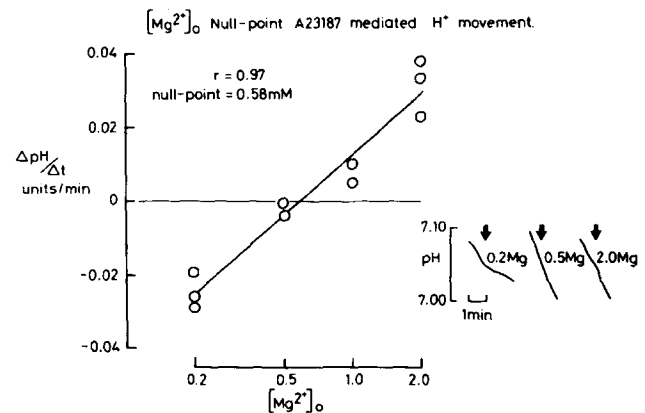


FIGURE 5 $[Mg^{2+}]_o$ null-point for A23187-mediated H⁺ movements. The vertical axis shows the change in the rate of acidification of lightly buffered medium immediately following addition of 20 μ M A23187 $[Mg^{2+}]_o$ is plotted on a logarithmic axis. The inset shows sample traces. The record before addition of ionophore shows progressive acidification due to metabolism, and a change in slope after the addition (indicated by an arrow) is attributed to superimposed A23187-mediated Mg^{2+} /H⁺ exchange.

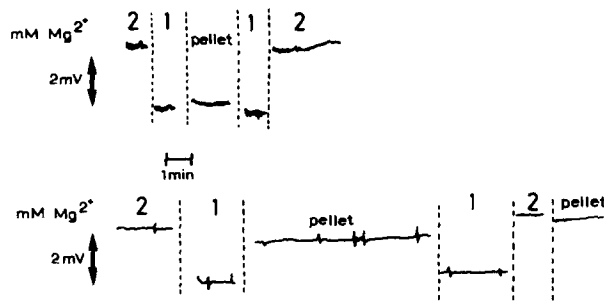


FIGURE 6 Mg^{2+} electrode readings in freeze-thawed pellets of pig lymphocytes. Tracings of two different determinations are shown. The numbers above the traces denote the mM of $[Mg^{2+}]$ in calibration solutions. It can be seen that the readings in the pellet fall between 1 and 2 mM, ~ 1.1 mM in the upper trace and ~ 1.7 mM in the lower. The calibration solutions contained 140 mM KCl, 20 mM NaCl, 0.1 mM K_2 EGTA, 5 mM MOPS, pH 7.0 at $37^\circ C$, and the indicated $[Mg^{2+}]$.

A useful check on this technique came from measurement of the effect of A23187 with $[Mg^{2+}]_o$ 0.5 mM but pH_o 7.55. A23187 then produced a large increase in H^+ release comparable to that seen at pH 7.05 and 5.0 mM $[Mg^{2+}]_o$. This is expected with a 1 Mg^{2+} for 2 H^+ exchange in which a 0.5-U pH shift should be equivalent to a 10-fold change in $[Mg^{2+}]_o$.

$[Mg^{2+}]$ from Freeze-thawed Pellets

Measurements were made in freeze-thawed pellets from four batches of pig cells. As discussed in Materials and Methods, the electrodes were operating quite near their Mg detection limits and, not surprisingly, the results were somewhat scattered, but all lay between 1.1 and 2 mM free- $[Mg^{2+}]$. Fig. 6 shows traces from two of these determinations. The mean value for seven determinations was 1.35 ± 0.11 mM.

Two tests were done to try to validate the method. First the free-Mg concentration was measured in lysate of oxygenated human erythrocytes. Values between 0.3 and 0.5 mM were obtained, which agree well with values previously obtained using an A23187 null-point method (4). Secondly, the electrodes were used to measure $[Mg^{2+}]$ in pellets of lymphocytes either partially depleted of Mg or loaded with Mg by exposure to A23187 in Mg-free or 5 mM Mg solution. Values around 0.5 and 2.5 mM, respectively, were found showing that the electrodes could detect the expected changes. Readings obtained from cells preincubated with 5 $\mu g/ml$ Con A for 1 h lay within the normal resting range.

$[Mg^{2+}]_i$ by NMR

The peaks from ATP were prominent and the chemical shift from the γ -phosphate corresponded to $>90\%$ bound to Mg^{2+} . Assuming there is an effective dissociation constant of $\sim 50 \mu M$ for Mg ATP (9), the ATP signal gives $[Mg^{2+}]_i > 0.5$ mM.

DISCUSSION

These results give resting values at $37^\circ C$ for lymphocyte cytoplasmic pH and free Mg^{2+} near 7.0 and 1 mM, respectively. Four methods were used for pH_i and three for $[Mg^{2+}]_i$. Although none is foolproof, the possible artefacts are different in each of them so that the general agreement gives some confidence in the numbers.

Cytoplasmic pH

The digitonin method gave accurately reproducible results for the pH_o null-point. This method, as with all the methods we used, gives an average value for the whole population, which might conceal differences between subpopulations of cells. The same pH null-point was found for the mixed lymphocyte population from pig mesenteric nodes as for the T-cell preparation from mouse thymus, suggesting that there may be little difference between different kinds of lymphocytes. There are, however, a number of possible artefacts to consider before accepting the measured null-point as a reasonable value for pH_i . (a) Dissipation of intracellular pH gradients could give an error in the estimated pH_i . For example, mitochondria have an alkaline matrix pH, but digitonin is chosen for its lack of effect on the mitochondrial membrane (30). Moreover, given a buffering capacity in mitochondria similar to that of cytoplasm (14), a pH gradient across the mitochondrial membrane usually < 1 pH U, and that mitochondria constitute only some 5% of lymphocyte volume, the maximum error from this source would be ~ 0.05 pH U. Disruption of lysosomes would release their acid contents, producing a spuriously low pH null-point, but the volume of lysosomes in lymphocytes seems to be exceedingly small and it can be shown in cell types much richer in lysosomes that digitonin leaves these organelles largely intact, as judged by retention of acid phosphatase and the ability to accumulate aminoacridine dyes (Pozzan, T., in preparation). Possible errors of this type could come from other organelles, but there is no expectation of significant intracellular pH gradient other than those mentioned above. This source of error seems likely to be very small. (b) The existence of a Donnan potential between the medium and digitonin-treated cells would mean that the null-point would give the electrochemical equilibrium value for the suspension pH, and not a pH equal to pH_i . Digitonin released lactate dehydrogenase from the lymphocytes so that the organic phosphates and soluble proteins that presumably carry most of the "fixed negative charge" in the cell do equilibrate with the medium. But the residual macromolecular matrix of the cell could still bear a net charge and produce a Donnan potential. However, we found no exclusion or accumulation of a tetra-anionic dye, quin2, in digitonin-treated lymphocytes (26), so any such effect appears to be very small. (c) An error could occur if the release and dilution of the soluble cytoplasmic components in some way altered the pK_a of major buffers. We found no way of testing this point directly.

This digitonin null-point technique seems thus to be a useful way of assessing pH_i for cells in suspension. It generates very much better data than those obtained when attempting to measure $[Ca^{2+}]_i$ in a similar manner, for three main reasons. First, even at relatively low cytochromes the buffering capacity of the cells can be made large relative to that of the suspending medium. Second, the changes in suspension pH necessary to move either side of the null-point are close to the physiological range for pH. By contrast the null-point $[Ca^{2+}]_i$ is very far from normal external $[Ca^{2+}]_o$, and it is almost impossible to get below the null-point in unbuffered solution. Third, commercial pH electrodes can give accurate, reliable, and simple measurements in the necessary range, whereas measurement of calcium in unbuffered solutions in the submicromolar range is relatively difficult.

Measuring the pH of freeze-thawed cell pellets is crude but simple. The values will be perturbed by disruption of intracellular pH gradients and probably show lower limits for pH_i .

owing to continued acid production by glycolysis. The problems of possible Donnan potentials and dilution artefacts discussed above should, however, not be present. These readings came within 0.1 U of the null-point values and we cannot see why they should be far wrong.

Disadvantages of these methods are that they (a) are one-shot, destructive, and (b) require tens or hundreds of microliters of cells. Additionally, the null-point method does not work in CO₂/bicarbonate buffered solutions.

³¹P NMR should be an ideally nonperturbing technique. At present, though, for this kind of work it too has drawbacks. Several milliliters of quite dense cell suspensions are required that need a lot of material and present problems with gas exchange and external pH control inside the relatively inaccessible sample compartment (8). The effect of pH on the chemical shift may not be the same inside the cells as it is in calibration conditions (5) and it may be difficult to assign a particular peak to the cytoplasm as opposed to inside an organelle (5, 8). The time resolution is limited by the period needed to collect spectrum—usually tens of seconds to minutes (5, 8), and the absolute accuracy of measurement is not expected to be better than ± 0.1 pH U (5). The necessary apparatus is complex and expensive and available in only very few centers. Our better data were obtained with cells at 20°C where the pH_i indicated from the chemical shift of inorganic phosphate was ~7.3, in reasonable agreement with the null-point value at that temperature. This method will doubtless become increasingly popular as the machinery is refined and becomes more widely available.

Intracellularly trapped pH indicators such as BCECF have some distinct advantages for measuring pH_i in vitro: unsurpassed time resolution in a continuous record, high sensitivity to small changes in pH, small number of cells required, and compatibility with almost any extracellular medium. Known limitations include a small but definite leakage and the tendency for fluorescein and its derivatives to show slight spectral shifts, indicative of minor binding or other perturbations, when inside cells. BCECF probably reports pH in the cytoplasm. We have not yet rigorously excluded dye uptake into organelles, though it appears not to be accumulated in either mitochondria or lysosomes. No evidence has been seen of any toxicity. The typical content of 10 μM could not itself directly influence pH_i. "Cell viability" was unaffected by BCECF loading and there was no effect on [Ca²⁺]_i measured with quin2 either at rest or after Con A stimulation. We have found that BCECF can also be readily loaded into blood platelets and isolated toad bladder epithelial cells and it seems a promising addition to the available methods for measuring pH_i.

The resting pH_i of lymphocytes, near 7.0 with external pH 7.4, is similar to that in various mammalian cells for which reliable data is available (20). This value happens to be quite close to that calculated from DMO distribution at that particular pH_o but not for values obtained from the distribution of weak bases (3). We found that over tens of minutes pH_i was well stabilized against changes in external pH at constant, low pCO₂ (see, e.g., Figs. 1 and 3). This behavior seems to be typical of most cell types (16), though it was not what was found from measurements of DMO distribution in human lymphocytes where ΔpH appeared to be constant (3). An increase in pCO₂ to normal in vivo levels gave the expected initial change in pH_i which was followed by quite rapid recovery to near the initial level. This again is behavior typical of many cell types (20). The mechanism of this pH recovery remains to be investigated, though it did still occur after

addition of gramicidin and so may not depend on either sodium or potential gradients across the membrane. These results are important in showing that steady state pH_i is not critically dependent on pCO₂, excusing the common but unphysiological practice of incubating cells in air-equilibrated, HEPES-buffered solution. As lymphocytes have a membrane potential of ~-60 mV (19), H⁺ is not at electrochemical equilibrium and so the cells must maintain a steady state by pumping H⁺ out or OH⁻ or HCO₃⁻ in. The finding that, at 20°C with external pH 7.4, pH_i rose to ~7.35, is yet again similar to observations in other preparations (20) and underlines the importance of working with mammalian cells at normal body temperature. The change might reflect a different Q₁₀ for H⁺ transport and acid production.

Perhaps the most important observation was that mitogenic doses of Con A had no measurable effect on pH_i during the time in which elevation of [Ca²⁺]_i and membrane hyperpolarization are observed (25). Our limit of detection was probably ~0.05 U and our observations were limited to the first hour or so after application of Con A, so that we say nothing about possible later changes (7). But the data argue against H⁺ as an early messenger.

Cytoplasmic Free Mg²⁺

The values obtained here in pig lymphocytes, with different techniques, range from 0.8 to 1.35 mM. The latter value, obtained with Mg²⁺-electrodes in freeze-thawed cell pellets, is probably an overestimate because the readings seem to rise somewhat during the time taken to get a steady recording. This may be due to progressive acidification changing the Mg buffering properties. The A23187 null-point experiments gave more consistent data, though both the Mg²⁺ and H⁺ movement methods have potential drawbacks. Mg²⁺ movements were measured in cells that had been incubated with ~10⁻⁷ M [Ca²⁺]_o and a high ionophore concentration for 40 min. This might have altered various factors, including free Mg²⁺. There was, however, no loss of viability or fall in ATP levels in cells treated with 20 μM A23187 at the [Mg²⁺]_o null-point, nor was pH_i different from that in control cells. Only in the presence of high external Ca²⁺ or low [Mg²⁺]_o does A23187 show overtly toxic effects. The possibility of Mg²⁺ redistribution across organelles needs considering, though we know of no evidence for intracellular Mg gradients.

The [Mg²⁺]_o null-point for H⁺ movement is obtained with cells exposed to ionophore for only seconds, and so effects of prolonged action of ionophore become irrelevant. This method, however, involves a kinetic measurement, the initial rate of ionophore mediated H⁺/Hg²⁺ exchange, rather than the equilibrium (or approach to equilibrium) method of the [Mg²⁺] measurements. There was no prior expectation that ΔpH/dt should have been a linear function of log[Mg²⁺]_o, but the data of Fig. 5 are fairly well fitted thus. The close correspondence of the null points obtained with these two different A23187 techniques gives some confidence in the method. NMR is nondestructive and more direct but is difficult to make accurate since [Mg²⁺] is so much greater than the dissociation constant for Mg ATP. Although the NMR data did indicate [Mg²⁺]_i in the millimolar range, the precision of the determination was less than for other methods used here. The only way we anticipate getting better data on lymphocyte [Mg²⁺]_i is to find an intracellularly trappable [Mg²⁺] indicator.

Finding that only a small proportion of the total cell [Mg²⁺]

is in the free form in lymphocyte cytoplasm is in line with the results reported in various other tissues. Lymphocytes contain ~5 mM ATP, which can account for almost half the bound $[Mg^{2+}]_i$. It has been shown in liver and kidney cells (28) that various cell proteins can also bind Mg and these could account for much of the remaining bound fraction in lymphocytes. The actual value of $[Mg^{2+}]_i$ assessed in various other cell types by many different methods has varied rather widely, according to investigation and technique. For instance, in striated muscle values ranging from 0.6 mM (9) to 8.9 mM (12) are reported.

Our results appear to determine lymphocyte $[Mg^{2+}]_i$ within fairly narrow limits and suggest that a value near 1 mM is a reasonable approximation to the true normal $[Mg^{2+}]_i$. This level is far from the electrochemical equilibrium value of 50 mM, given $[Mg^{2+}]_o$ 0.5 mM and a membrane potential of -60 mV. Since the membrane does allow measurable flux of Mg^{2+} there is presumably an outwardly directed Mg^{2+} pump, but its nature awaits investigation.

The presence of large amounts of bound Mg^{2+} with a relatively high free concentration implies very effective buffering. Considering also the influence of Mg^{2+} on so many ongoing cellular processes, we would guess that free $[Mg^{2+}]_i$ is not a likely candidate as a cellular regulation, but rather a factor that the cell is at some pains to hold steady. It contrasts with Ca^{2+} , which is present at much smaller total content and very much (10^4 times) lower free concentration (26) and which is probably not strongly buffered in the relevant submicromolar range. Ca^{2+} is well set up as a regulator ion: translocation of small amounts producing large proportional changes in free concentration. In accordance with these notions we find rapid changes in T-cell $[Ca^{2+}]_i$ in response to plant lectins (25) but no measurable alteration in cell Mg^{2+} .

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