Intracellular pH of Stimulated Thymocytes Measured with a New Fluorescent Indicator*

(Received for publication, January 10, 1983)

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A new fluorescent intracellular pH indicator is described ("quene 1") which is related to the tetracarboxylate Ca++ indicator based on the quinoline fluorophor ("quin 2"). Quene 1 has excitation and emission maxima at 390 and 530 nm, respectively, and shows a 30-fold increase in fluorescence between pH 5 and 9 with a pK_0 of 7.3. The fluorescence is insensitive to Ca++ and Mg++ at free concentrations up to 10^-4 M and to the proportions of Na+ and K+ at total concentrations of Na+ and K+ from 100 to 200 mM. The indicator is loaded into thymocytes using the tetracarboxyethyl ester derivative which is hydrolyzed in the cells to give the tetracarboxylate anion. Intracellular pH can be measured at intracellular quene 1 concentrations of approximately 0.1 mM and quene 1 does not perturb glycolysis or the ATP level in resting cells at concentrations up to 0.8 mM. The intracellular pH of mouse thymocytes indicated by quene 1 is 7.16 ± 0.04 and it is insensitive to the concentration of Ca++ or Mg++ in the extracellular medium. The intracellular pH decreased when the pH of the medium was lowered by addition of HCl, but was insensitive to NaOH at extracellular pH values up to 8.0. Rapid transient changes in intracellular pH are induced by NHCl, NaCO3, CH3 or HCO3- /CO2. The thymocytes showed no early changes (within 30 min) in intracellular pH in response to mitogenic concentrations of lectins or 4β-phorbol-12-myristate-13-acetate.

An increase in pH, following an increase in [Ca], may occur during the stimulation of cell growth in a variety of cells. Within 2 min after fertilization of the sea urchin egg, a large transient increase in [Ca] is followed by an increase in pH, (1, 2). In fibroblasts, an early increase in pH, of about 0.2 unit has been reported in response to a synergistic mixture of growth factors, and the effect has been attributed to the stimulation of Na+/H+ exchange (3). From other studies, it has been suggested that stimulation of the Na+ flux in fibroblasts may be due to a prior increase in [Ca], (4). In lymphocytes, prolonged changes in pH, in response to the mitogenic lectin ConA have recently been described (5), with maximum increases in pH, in mouse spleenocytes at 6 and 40 h and a minimum pH, close to the value in quiescent cells, at 12 h. Using the fluorescent indicator quin 2, the [Ca], in thymocytes has been shown to increase by about 2-folds within 30 s of the addition of mitogenic concentrations of ConA (6). These observations suggest that there may be a common sequence of changes in [Ca], and pH, when different types of cells enter the cell cycle. Are these ionic changes obligatory for entry into the cell cycle and are they causally related to each other?

The current techniques available for the measurement of pH, in thymocytes and other small cells are unable to resolve the time course of any changes in pH, which may occur within 30 s of stimulation. We have synthesized a fluorescent pH indicator related to quin 2, designed to respond rapidly to changes in normal pH, values and to have negligible affinity for Ca++ at normal levels of [Ca]. We report the characterization of this new probe ("quene 1"), the measurement of pH, in mouse thymocytes, and the effect of varying external pH and cation concentrations on pH. We have also examined the effects of various mitogens on thymocytes to determine whether there are any changes in pH, correlated with the early changes reported in [Ca].

MATERIALS AND METHODS

Cell Preparation and Loading with Quene 1—Thymocytes were prepared from 4–6-week-old BALB/c mice by teasing the thymus into RPMI 1640 medium buffered with 10 mM Hepes, pH 7.3. The cells were centrifuged (500 × g, 5 min) and resuspended at 10^6/ml in a standard medium of the inorganic salts of RPMI 1640 without phenol red (103 mM NaCl, 5.6 mM NaHPO4, 2.54 mM KCl, 0.44 mM CaSO4, 7H2O, 0.42 mM Ca(NO3)2, 1.4M H2O) supplemented with 11 mM glucose and buffered with 10 mM Hepes, pH 7.3. The cells were incubated for 30 min at 37°C with 1 μM [3H]quene 1 acetoxymethyl ester (S48 Ci/mole) added from a stock solution in Me2SO (final concentration < 0.1%, v/v) before washing by centrifugation (500 × g, 5 min) and suspending the cells in standard medium. After a further 30 min at 37°C, the cells were washed as before and resuspended in standard medium at 5 × 10^6/ml for fluorescence measurements. Cell aliquots (20 μl) were equilibrated in 1-cm path length cuvettes for 10 min at 37°C in a Perkin Elmer Model 442 spectrophotometer before measurements of pH1 (excitation 390 nm, emission 530 nm). ConA, PHA, and WGA were obtained from Miles Yeda and were added from stock solutions in standard medium. FMA, TTFB (Sigma), and valinomycin (Serva International) were added in Me2SO (final Me2SO concentration less than 0.1%). The intracellular [H]quene 1 concentration was estimated by counting 3H in an aliquot of the cells, assuming an aqueous cell volume of 104 μm^3 determined from the [3H]H2O volume.

Synthesis of Quene 1—8-[bis(bisethoxyxycarbonyl methyl)amino]-6-methoxymethyl-2-(trans-2-bis(bisethoxyxycarbonyl methyl)amino)ethylquinoline was prepared by condensation of 6-methoxy-4-nitroquinoline with o-nitrobenzaldehyde followed by reduction to the diamine with stannous chloride. Alkylation of the bis amine with ethyl bromoacetate and hydrolysis of the ester gave quene 1 (Structure 1). Full details of the chemical synthesis of quene 1 and other new quinoline-based indicators will be published elsewhere. The preparation of quene 1 and [3H]quene 1 tetraacetoxymethyl esters were by the methods described previously for quin 2 (7).

RESULTS

pH and Cation Titrations of Quene 1 Fluorescence—The excitation and emission spectra of quene 1 acetoxyethyl
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Fig. 1. Fluorescence excitation and emission spectra of quene 1 and quene 1 acetoxymethyl ester. Excitation and emission spectra of 1 μM quene 1 and 1 μM quene 1 acetoxymethyl ester (dashed curves) in a medium of 150 mM KCl, 10 mM EGTA, 10 mM Hepes at 37 °C at the indicated pH values. Excitation spectra were recorded at 530 nm and emission spectra were excited at 390 nm. All spectra were recorded in the ratio mode and are uncorrected. Inset, pH dependence of quene 1 fluorescence intensity (excitation 390 nm, emission 530 nm).

Fig. 2. Effect of Ca²⁺ and Mg²⁺ on quene 1 fluorescence intensity. Fluorescence intensity (F.I.) in arbitrary units of 1 μM quene 1 in 150 mM KCl, 10 mM Hepes, pH 7.1, at 37 °C as a function of added CaCl₂ (△) or MgCl₂ (△) concentration.

In most experiments, the quene 1 fluorescence from cells loaded in the standard medium was stable for many minutes. Addition of 0.1 mM MnCl₂ to aliquots of cells at increasing times after the cells had been washed by centrifugation (500 × g, 5 min) to remove any external quene 1 indicated that leakage of quene 1 was insignificant over 30 min in stable preparations (<0.1%/min). Centrifugation of the loaded cells caused variable leakage of quene 1, detected from the immediate decrease in fluorescence from the resuspended cells on adding 0.1 mM MnCl₂. In some preparations, the fluorescence intensity from cells loaded with quene 1 decreased continuously for at least 20 min and there was also enhanced leakage of quene 1 from the cells (up to 5% in 10 min). We have been unable to determine why this occurs in some preparations, but noted that the downward drift in fluorescence could usually be stopped and the fluorescence intensity stabilized by the addition of 10 mM NaHCO₃/CO₂ (see below).

The estimated intracellular pH was 7.15 ± 0.04 (S.D., n = 9) when the extracellular pH was 7.3 throughout the stages of cell preparation, loading with quene 1, and assay. This pH value is uncorrected for the small quenching effect of a free intracellular Mg²⁺ concentration of approximately 9.8 mM (12), equivalent to an offset of approximately +0.04 unit estimated from the data in Figs. 1 and 2. It should be noted that the technique is very sensitive to relative changes in pH, (±0.01 unit) in contrast to the reproducibility of absolute pH measurements. The replacement of Na⁺ by K⁺ in the medium and the subsequent addition of up to 10 mM NaCl had no effect on quene 1 fluorescence. However, when 10 mM NaCl was added to cells in medium in which Na⁺ was replaced by

ester are shown in Fig. 1. On hydrolysis to quene 1, the emission maximum shifts from 490 to 530 nm and is independent of pH. The fluorescence intensity of quene 1 increases by more than 30-fold from pH 5 to 9 and an apparent pKₐ of 7.30 was derived from the titration shown in Fig. 1 (inset). Quene 1 in total concentrations of sodium and/or potassium chloride from 100 to 200 mM gave the same fluorescence intensity at constant pH and, therefore, the indicated pH is unaffected by any changes which may occur in Na⁺ or K⁺ concentrations in the cells. Both Ca²⁺ and Mg²⁺ ions quench quene 1 fluorescence at pH 7.1 at concentrations above 10⁻⁴ M (Fig. 2). This concentration of Ca²⁺ is much higher than normal [Ca²⁺] values (~10⁻⁷ M), but free intracellular Mg²⁺ concentrations of up to 1 mM will slightly reduce intracellular quene 1 fluorescence by an amount equivalent to an offset for pH, calibration of up to +0.03 unit.

Measurement of pH, in Thymocytes—The measurement of pH, is illustrated in Fig. 3. The fluorescence from thymocytes loaded with quene 1 is calibarated by the addition of 0.05% Triton X-100 in 0.5 mM EGTA + 0.5 mM EDTA to lyse the cells and release quene 1 into medium containing free Ca²⁺ and Mg²⁺ concentrations well below the levels which affect quene 1 fluorescence (Fig. 2). Serial additions of 1 mM Tris and 0.5 mM HCl solutions calibrate the fluorescence of the released quene 1 as a function of pH. Complete release of quene 1 from the cells was demonstrated by the addition of 0.1 mM MnCl₂ which quenches quene 1 fluorescence immediately by more than 99% to give a background signal which is the same as from cells without quene 1. The emission of quene 1 was the same whether loaded inside the cells or added externally to unloaded cells. Addition of Triton X-100 to unloaded cells in the presence of external quene 1 did not affect the fluorescence intensity under the conditions defined in the legend to Fig. 1; therefore, the calibration is not affected by changes in light scattering or auto fluorescence of the cells on addition of Triton X-100.

In most experiments, the quene 1 fluorescence from cells loaded in the standard medium was stable for many minutes. Addition of 0.1 mM MnCl₂ to aliquots of cells at increasing times after the cells had been washed by centrifugation (500 × g, 5 min) to remove any external quene 1 indicated that leakage of quene 1 was insignificant over 30 min in stable preparations (<0.1%/min). Centrifugation of the loaded cells caused variable leakage of quene 1, detected from the immediate decrease in fluorescence from the resuspended cells on adding 0.1 mM MnCl₂. In some preparations, the fluorescence intensity from cells loaded with quene 1 decreased continuously for at least 20 min and there was also enhanced leakage of quene 1 from the cells (up to 5% in 10 min). We have been unable to determine why this occurs in some preparations, but noted that the downward drift in fluorescence could usually be stopped and the fluorescence intensity stabilized by the addition of 10 mM NaHCO₃/CO₂ (see below).

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choline (choline medium), there was a small increase in fluorescence equivalent to an increase in pH of approximately 0.02 unit.

Lowering the external Ca**2+ and Mg**2+ concentration in the medium to \( \text{<}10^{-6} \text{ M} \) by the addition of 0.5 mM EGTA + 0.5 mM EDTA had no immediate effect on quene 1 fluorescence from the cells. The intracellular pH, therefore, appears to be insensitive to the extracellular concentrations of Ca**2+ and Mg**2+ for at least several minutes.

In previous studies \( ^{7} \), we have found that intracellular quin 2 causes increased lactate production at intracellular concentrations between 0.1 and 1 mM, and depletes cellular ATP levels at concentrations above 0.8 mM. In contrast, quene 1 had no significant effect on lactate production or the ATP level (\( \text{<}10\% \) decrease) at intracellular concentrations up to 0.8 mM (Fig. 4). It is therefore unlikely that the intracellular concentration of quene 1 required for fluorescence measurements (0.1 to 0.5 mM) causes any change in pH as a result of metabolic perturbation.

**Effect of Varying the pH of the Medium with Strong Bases or Acids**—Raising the extracellular pH from 7.3 to 8.0 by serial additions of 0.5 mM NaOH to the medium had no effect on quene 1 fluorescence in the cells over at least 10 min (Fig. 5a). Centrifugation of the cells in medium at pH 7.3 and resuspension at pH \( \text{<}8.0 \) also had no effect on the indicated pH. In contrast, additions of 0.5 mM HCl caused a slow decrease in pH over several minutes to a new stable level (Fig. 5b). Subsequent additions of 0.5 mM NaOH did not affect the quene 1 fluorescence, indicating that the additions of HCl did not release quene 1 from the cells. Centrifugation of the cells in medium at pH 7.3 and resuspension at pH 7.3 to 6.8 caused a decrease in pH similar to that due to the addition of HCl. The response to additions of HCl, therefore, is not attributable to damage to the cells by transient exposures to low pH during mixing. The pattern of responses to additions of HCl or NaOH were similar in medium containing 10 mM HCO**3/CO**2 and, therefore, is not due to the absence of physiological buffer in

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**Fig. 3.** pH calibration of quene 1 in thymocytes. The fluorescence intensity (arbitrary units) of 0.3 mM quene 1 in thymocytes (10⁶ cells in 2 ml) in standard medium prepared as described under "Materials and Methods." Arrows indicate additions of (i) 0.05% Triton X-100 with 0.5 mM EGTA and 0.5 mM EDTA (10 μl of 10%; Triton X-100 + 10 μl of 0.1 mM EGTA + 10 μl of 0.1 mM EDTA); (ii) 2.5 mM Tris (5 μl of 1 M Tris); and (iii) sequential additions of 1.25 mM HCl (5 μl aliquots of 0.5 M HCl).

**Fig. 4.** Effect of quene 1 on cellular ATP content and lactate output. Thymocytes were loaded with various concentrations of quene 1 by using quene 1 acetoxymethyl ester concentrations from 1 to 50 μM, as described under "Materials and Methods" for fluorescence measurements and resuspended in standard medium at 5 × 10⁶ cells/ml at 37 °C. ATP content (□) and lactate output (■) were measured after incubation for 1 h at 37 °C as described previously \( ^{7} \). In control cell samples without quene 1, the ATP content was 500 fmol/cell (±5 mM, 100%) and lactate output was 2-3 fmol/cell/h. Data taken from Ref. 7 for ATP content (□) and lactate production (□) for cells loaded with quen 2 under the same conditions are included for comparison.

**Fig. 5.** Effect of strong and weak acids and bases on pH. Cells were loaded with 0.2 to 0.4 mM quene 1 and prepared for fluorescence measurements (10⁶ cells in 2 ml) in standard medium as described under "Materials and Methods." The range in pH values in the untreated cell samples in the various experiments represents the variation in pH in resting cells indicated by quene 1. a, Additions (▲) of 1.25 mM NaOH (5 μl of 0.5 M NaOH); b, additions (●) of 1 mM HCl (20 μl of 0.1 M HCl); c, cells in high K⁺ medium (103 mM KCl, 5.6 mM NaCl, 10 mM Hepes, 4.2 mM CaCl₂, 0.41 mM MgCl₂, pH 7.26) were treated as indicated with 25 mM TTFB (5 μl of 1 M in ethanol), 1 mM valinomycin (5 μl of 1 M in MeSO₄), 1.25 mM Tris (5 μl of 0.5 M Tris, pH 7.30), and 1.25 mM HCl (5 μl of 0.5 M HCl, pH 7.18). No leakage of quene 1 from the cells could be detected at the end of the experiment by the addition of 0.1 mM MnCl₂ (not shown). d, Additions (□) of 5 mM NH₄Cl (10 μl of 1 M NH₄Cl) without change in pH (±7.27). e, Additions (■) of 10 mM NaCO₃/CH₃COOH (20 μl of 1 M NaCO₃/CH₃COOH) without change in pH (±7.27). f, Additions (△) of 10 mM NaHCO₃/CO₂ (20 μl of 1 M NaHCO₃, saturated with CO₂).
the medium.

The pH can be made to respond rapidly and reversibly to changes in external pH by Tris (or NaOH) and HCl by treating the cells with the mitochondrial uncoupler TTFB and 1 µM valinomycin in a high K⁺ medium containing 103 mM KCl and 5.6 mM NaCl instead of the standard Na⁺, K⁺ concentrations (Fig. 5c).

**Effect of Weak Bases and Acids—**Addition of 5 mM NH₄Cl to the medium at constant pH (7.27 ± 0.02) caused a rapid transient increase in pH (Fig. 5d). Further additions of 5 mM NH₄Cl caused progressively smaller responses. Addition of 10 mM sodium acetate to the medium caused transient decreases in pH, and similar effects were observed on addition of 10 mM NaHCO₃/CO₂ from a stock solution of 1 mM NaHCO₃ saturated with CO₂ (Fig. 5, e and f). From these data, the buffering capacity of the cell is estimated as equivalent to approximately 3 fmol of base/cell compared with 0.01 to 0.1 fmol of quene 1/ cell (≈0.1 to 1 mM intracellular quene 1) (8).

**Effects of Mitogens—**In many experiments, mitogenic and stimulatory concentrations of ConA (1 µg/ml and 3 µg/ml) or PHA (1 µg/ml and 2 µg/ml) had no effect on quene 1 fluorescence in the first 5 min after addition of the mitogens to the cells, irrespective of the medium used or the intracellular concentration of quene 1. No effect was observed with the nonmitogenic WGA at concentrations which cause early metabolic stimulations and increases in [Ca] similar to those produced by ConA or PHA (7). PMA had no significant effect on pH at concentrations from 10⁻⁶ to 10⁻³ M, either alone or with 1 µg/ml of ConA.

**DISCUSSION**

Many of the properties of quene 1 are well suited to its use as an intracellular indicator of pH. The apparent pKₐ of quene 1 of 7.3 is close to the estimated pH of thymocytes and other cells (9, 10). Autofluorescence from the thymocytes at 530 nm when excited at 390 nm is very small compared with the fluorescence of intracellular quene 1 at concentrations above 0.1 mM. Leakage of quene 1 was negligible (<10%) in cell preparations in which quene 1 fluorescence was stable. The fluorescence intensity and emission maximum were the same for quene 1 both within cells and in media which simulated the intracellular concentrations of cations. No significant effects of quene 1 on ATP level or lactate production which might affect pH were detected at intracellular concentrations below 0.8 mM. This observation is consistent with previous data which indicated that metabolic stimulation by intracellular cation chelators is correlated with their affinity for Ca²⁺ (or other M³⁺ ions). Quene 1 has an affinity for Ca²⁺ of pKₐ,Ca = 2.70 compared with 7.05 for quen 2 (7). An adverse feature of quene 1 as a pH indicator is that it is necessary to correct the pH calibration for the concentration of free Mg²⁺ in the cell by about +0.05 pH unit for thymocytes, and changes in intracellular Mg²⁺ levels of greater than 2-fold could interfere with pH measurement by quene 1. In the present experiments, however, external Mg²⁺ concentrations from 10⁻⁴ to 10⁻³ M had no effect on quene 1 fluorescence over several minutes, or on the pattern of responses to acids and bases shown in Fig. 5. It should be possible to design derivatives of quene 1 with pKₐ,Mg⁺ well below 2, which would be insensitive to normal intracellular free Mg²⁺ concentrations.

The indicated pH values of 7.15 ± 0.04 is close to the value of 7.18 obtained from the uptake of DMO into mouse spleen lymphocytes (5). However, the two probes may report on different intracellular environments. The distribution of DMO gives some weighted, average intracellular measure of pH including the intra-organelle environment (11) whereas, using image intensified microscopy, quene 1 appears to be uniformly distributed throughout the cells with no observable concentration into organelles. Furthermore, quene 1 is not accumulated by isolated thymocyte mitochondria, but we cannot exclude the possibility that there may be significant uptake into other organelles (e.g. lysosomes or endoplasmic reticulum).

In a recent paper, an intracellular indicator based on fluorescein was used to measure pH in pig lymphocytes (12). The pH value was approximately 7.0, which is lower than that reported here using quene 1, but the general pattern of responses obtained with strong bases and weak acids and bases was similar to that described here.

The insensitivity of pH to the concentrations of Ca²⁺ and Mg²⁺ in the extracellular medium suggests that neither of these ions are directly coupled to the systems which regulate pH. The pH is normally stable without added HCO₃⁻/CO₂, but the stabilizing effect of low concentrations of HCO₃⁻/CO₂ on preparations in which the pH drifts downwards may indicate a physiological role for bicarbonate in pH regulation in these cells, as found in other systems (13). This observation and the small increase in pH in response to Na⁺ in the choline medium require further evaluation.

The sensitivity of the indicator to changes in pH is demonstrated by the rapid transient responses to additions of NH₄⁺, CH₃COO⁻ and HCO₃⁻/CO₂ ions, and the titrations of pH₁ by Tris and HCl in the presence of TTFB and valinomycin in high K⁺ medium. In normal medium, the cells are insensitive to increase in external pH by strong bases, but respond to HCl with a slow decrease in pH₁. This decrease in pH₁ is not due to damage to the mechanisms of pH₁ regulation since similar effects are observed when the cells are resuspended in medium at lower pH with or without HCO₃⁻/CO₂. Treatment of the cells with HCl, Tris, or NaOH under the conditions described did not affect the [Ca] in resting cells indicated by quen 2 or the increase in [Ca] in response to ConA, confirming that the functions of the plasma membrane proteins are not generally degraded by additions of acid or base.

The absence of any effect on pH₁ of the mitogens ConA and PHA which increase [Ca], or of PMA which decreases [Ca], indicates that the free concentrations of Ca²⁺ and H⁺ in the cytoplasm can be altered independently in the thymocyte. Furthermore, any pH changes which may be necessary or result from, mitogenic stimulation occur at least 30 min after the early increase in [Ca] observed within 30 s of the addition of ConA. Using quene 1, we have observed no change in pH₁ over the first 12 h after stimulation of thymocytes by ConA, in marked contrast to the data reported for mouse splenocytes using DMO to measure pH₁ (5).

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Intracellular pH of stimulated thymocytes measured with a new fluorescent indicator.
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J. Biol. Chem. 1983, 258:5994-5997.

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