Intracellular pH and free calcium changes in single cells using quene 1 and quin 2 probes and fluorescence microscopy

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Photometric fluorescence microscopy has been used to measure intracellular pH (pH_i) and free calcium concentrations ($[Ca]_i$) in individual mouse thymocytes and 2H3 rat basophil leukaemic cells containing indicators for pH (quene 1) or calcium (quin 2). The pH_i and $[Ca]_i$ measurements in individual 2H3 cells and mouse thymocytes and their responses to various stimuli were consistent with the corresponding data obtained from suspensions of these cells measured in a spectrofluorimeter. Photometric fluorescence microscopy of these indicators in individual cells provides a sensitive and fast method of following pH_i and $[Ca]_i$ responses in individual cells.

Free Ca^{2+} *Intracellular pH* *Thymocyte* *2H3 cell* *Antigenic stimulation* *Fluorescence microscopy*

1. INTRODUCTION

Changes in cytoplasmic pH (pH_i) and/or in free cytoplasmic Ca^{2+} concentration ($[Ca]_i$) are implicated in the responses of many eukaryotic cells to agents which control cellular functions or stimulate growth. Direct methods of measuring pH_i or $[Ca]_i$ have been limited mainly to large cells in which it is possible to insert microelectrodes or inject indicators (e.g., aequorin) into individual cells. Methods for measurement in small cells have mainly relied on indirect assays, for example the partition of 5,5-dimethyl oxazolidine-2,4-dione (DMO) to measure pH_i [1,2]. This has impeded analysis of the coupling of ionic signals to biological responses in many important cellular systems. New intracellular indicators for pH_i and $[Ca]_i$ have recently become available which can be used in a wide variety of cells without significantly perturbing cellular functions. The indicators, 'quene 1' and 'quin 2', are related to EGTA and are based on the quinoline reporter group [3,4]. Their fluorescence intensities can be used to determine the extent to which they are chelated with H^+ or Ca^{2+} and hence to calculate the free concentrations of these ions. The indicators diffuse into cells as the non-polar tetraacetoxymethyl ester derivatives and are then hydrolysed by endogenous esterases to the non-permeant tetracarboxylate anions which remain trapped in the cells. Thus far these indicators have been used to examine pH_i and $[Ca]_i$ in cell suspensions, which provide averaged ionic concentrations and responses to stimuli in large cell populations [4–7]. A detailed analysis of the relationship between ionic signals and cellular responses often requires these relationships to be defined in individual cells where, for example, the time courses of the signals and the responses can be directly compared.

The aim of this study was to determine whether

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fluorescence microscopy could be used to make photometric measurements on individual cells loaded with quene 1 or quin 2 with sufficient sensitivity to measure pH_i and $[Ca]_i$. We have explored the detection of fluorescence signals resulting from changes of ion concentration (H^+ and Ca^{2+}) in individual mouse thymocytes or 2H3 rat basophil leukaemic cells [8] and we report that changes in pH_i and $[Ca]_i$ can be readily detected. Specifically, we have characterised the responses to weak acids and bases and to ligands which have well-characterised effects on the cells (Concanavalin A on mouse thymocytes and antigen (ovalbumin) on 2H3 cells primed with IgE monoclonal antibody to the antigen). Such responses were generally similar to the corresponding responses of cell suspensions measured in a spectrofluorimeter. It is concluded that intracellular fluorescent indicators can be used with a fluorescence microscope to examine at high sensitivity and with excellent time resolution the coupling of ionic and metabolic responses in single cells in isolation, or as part of an interacting population of a small number of cells.

2. MATERIALS AND METHODS

2.1. *Cell preparation and loading*

Thymocytes were prepared from 4–6 week old BALB/c mice by teasing the thymus into RPMI 1640 medium (Gibco) buffered with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid), pH 7.3. After centrifugation at 500 × *g* for 5 min, the cells were resuspended at a density of 10^7/ml in RPMI 1640-HEPES and incubated with 1 μM quene 1 acetoxymethyl ester (AME) or 10 μM quin 2 AME added from 10 mM stock in dimethylsulphoxide (DMSO), for 30 min at 37°C [4,6]. After washing by centrifugation (500 × *g*, 5 min) and suspension in RPMI 1640-HEPES, the cells were incubated for a further 30 min at 37°C, during which time they settled on to glass coverslips in 60-mm Petri dishes. For microscopy the coverslips were immobilized on a small amount of silicone grease in 35 mm Petri dishes and covered with 3 ml of RPMI 1640-HEPES medium. Additions were made to the cells from a Finn pippette or 10 μl glass syringe as close to the coverslip as possible without perturbing the single cell in view.

2H3 cells from samples kindly donated by Dr R.P. Siraganian (NIH, Bethesda MD) were grown in Spinner culture in Eagle's Minimal Essential Medium with Earle's salts, 15% foetal calf serum (Gibco) in a 5% CO_2 incubator at 37°C, to a density of 5–7 × 10^5/ml. Cells were collected by centrifugation (245 × *g*, 10 min), suspended in fresh growth medium at a density of 5 × 10^5/ml and plated on to 13-mm siliconised glass coverslips in 16-mm Multi-dish wells 2–3 days prior to use. Cells used in stimulation experiments were preincubated overnight with 2 μl/ml medium of ovalbumin-specific mouse monoclonal IgE (Sera, Crawley). The 2H3 cells were loaded with the fluorescent dyes by replacing the growth medium with 1 ml of phosphate-buffered saline (PBS) (150 mM NaCl, 3.7 mM KCl, 3.0 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.45 mM $MgCl_2$, 5 mM glucose) containing 0.1% bovine serum albumin (BSA) and 10 μM quin 2 AME or 5 μM quene 1 AME added from 10 mM stock solutions in DMSO, for 60 min at 37°C. The cells were washed with PBS + 0.1% BSA before placing the coverslips in 35-mm Petri dishes with 3 ml of the same medium for microscopy. Inorganic salts, Con A (Miles Yeda, Rehovot) and aggregated ovalbumin (Mann Research, New York NY) were added from stock solutions made up in PBS; A23187 (Calbiochem) and valinomycin (Serva International) were added from stock solutions in DMSO and tetrachlorotrifluorobenzimidazole (TTFB, Sigma) was added from a stock solution in ethanol.

2.2. *Fluorescence microscopy*

Fluorescence from single cells was measured with a photon-counting fluorescence microscope [9] equipped with a 50 W high-pressure Hg arc lamp and a × 40 water immersion objective of numerical aperture 0.75. Excitation wavelengths of 339 nm (quin 2) or 366 nm (quene 1) were selected with interference filters; the barrier filter was 3 mm of Schott glass GG475 with a high-pass cut-off at 475 nm.

Fluorescence measurements on cell suspensions were made as described previously [4,6] on 2 ml of cells (10^7 thymocytes or 2 × 10^6 2H3 cells) in 1-cm pathlength cuvettes at 37°C in a Perkin Elmer Model 44B spectrofluorimeter. Excitation and emission wavelengths of 390 and 530 nm, respec-

tively, were used for quene 1 measurements and 339 and 492 nm for quin 2 measurements.

3. RESULTS

3.1. *pH_i Changes in single cells*

Single mouse thymocytes containing 0.2 mM quene 1 gave stable fluorescence signals 10 to 20-fold higher than the background from unloaded cells or an equivalent cell-free field. Addition of 5 mM NH_4Cl from a 1 M stock solution gave a transient increase in fluorescence and repeated additions gave progressively smaller transients (fig.1a). Similar fluorescence transients in the opposite direction were observed with serial additions of 5 mM Na acetate (fig.1c). In most experiments the resting fluorescence level decreased slightly after serial additions of either agent, although the external pH (pH_o) was constant (7.25 ± 0.02). Negligible changes were observed in the quene 1 fluorescence from single cells treated with serial additions of 5 mM NaCl from a 1 M stock solution, indicating that the responses to NH_4Cl or Na acetate were not due to osmotic shock.

These responses were qualitatively similar to those observed in thymocyte cell suspensions (fig.1b,d) although larger in amplitude (as a percentage increase over the basal fluorescence) and faster in recovery [4]. Assuming that the pH_i in resting cells is about 7.15 (see below), the maximal increase in pH_i on addition of 5 mM NH_4Cl was 0.8 compared with 0.25 observed for thymocyte suspensions. In single cells the pH_i returned to the resting level within 3 min of addition of NH_4Cl, as compared with 12–15 min in cell suspensions. These quantitative differences in the responses in single cells may be attributable, at least in part, to the higher local concentration of added NH_4Cl, since the solution was unstirred in the single cell experiments to avoid movement of the cell under observation, whereas the cell suspensions were stirred continuously.

The calibration of pH_i in single cells requires the intensity of quene 1 to be titrated against pH in situ. A technique to achieve this was adapted from

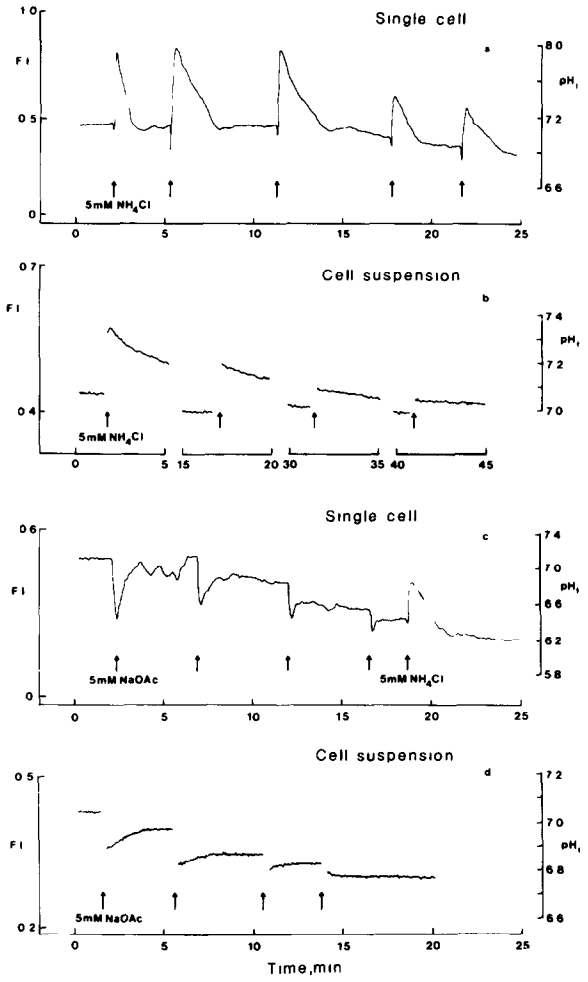

Fig.1. Effect of NH_4Cl and Na acetate (NaOAc) on pH_i of mouse thymocytes. Fluorescence measurements on single thymocytes and thymocyte suspensions were made as described in section 2. Fluorescence intensities (F.I.) in the thymocyte suspensions are in arbitrary units; in single thymocyte experiments 1.0 F.I. is equivalent to 4×10^5 photon cps. (In both types of experiment 1.0 F.I. corresponds to about the quene 1 fluorescence at pH 8.5, at which pH fluorescence of the indicator is maximal [4].) (a) Response of a single mouse thymocyte loaded with about 0.4 mM quene 1 to successive additions of 5 mM NH_4Cl (15 μl of 1 M NH_4Cl in PBS, pH 7.1) added to 3 ml of medium covering the cells. (b) Response of a suspension of mouse thymocytes loaded with 0.3 mM quene 1 to successive additions of 5 mM NH_4Cl (10 μl of 1 M NH_4Cl). (c) Response of a single mouse thymocyte to successive additions of 5 mM NaOAc (15 μl of 1 M NaOAc in PBS, pH 7.1) followed by 5 mM NH_4Cl (15 μl of 1 M NH_4Cl in PBS, pH 7.1). (d) Response of a mouse thymocyte suspension to successive additions of 5 mM NaOAc (10 μl of 1 M NaOAc in PBS, pH 7.1).

previous work [4] on thymocyte suspensions loaded with quene 1 and is illustrated in fig.2. The homeostatic pH_i mechanisms operating in thymocytes are overcome by suspending the cells in high K^+ medium and treating them with an uncoupling agent (TTFB) and valinomycin. The pH_i then follows changes in pH_o closely. The intracellular pH in single cells estimated in this way varied between 7.1 and 7.3, in reasonable agreement with the value obtained for thymocytes in suspension (7.15 ± 0.04) and from the accumulation of DMO of 7.18 [2]. It should be noted that pH_i cannot be calibrated in single cells by lysis and titration of the released quene 1 against pH_o [4].

The response of 2H3 cells loaded with 0.5 mM quene 1 to 5 mM NH_4Cl or 5 mM Na acetate was similar to that of thymocytes (not shown).

3.2. *$[Ca]_i$ Changes in single cells*

$[Ca]_i$ can be estimated by calibrating the fluorescence intensity of quin 2 in single thymocytes or 2H3 cells by addition of the Ca^{2+} ionophores A23187 or ionomycin to saturate the

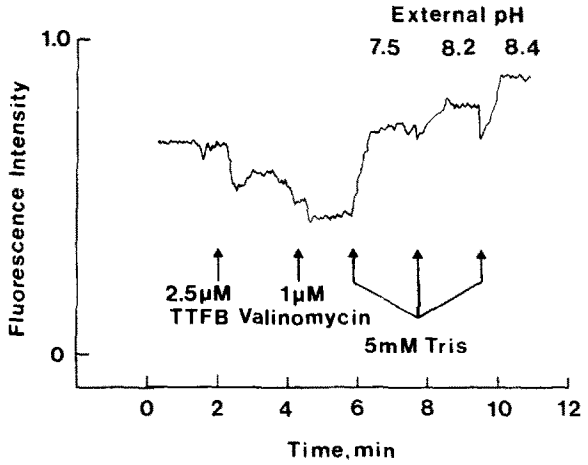

Fig.2. pH_i Calibration of quene 1 in a single mouse thymocyte. The relative fluorescence intensity (F.I.) of about 0.4 mM quene 1 in a mouse thymocyte in 3 ml of high K^+ medium (103 mM KCl, 5.6 mM NaCl, 10 mM HEPES, 0.42 mM $CaCl_2$, 0.41 mM $MgCl_2$, pH 7.2. Additions of TTFB (7.5 μl of 1 mM in ethanol), 1 μM valinomycin (3 μl of 1 mM in DMSO) and 5 mM Tris (15 μl of 1 M Tris in PBS) were made as shown. (1.0 F.I. ≡ 4 × 10^5 photon cps, and corresponds to about the maximal fluorescence of quene 1 as in fig.1.)

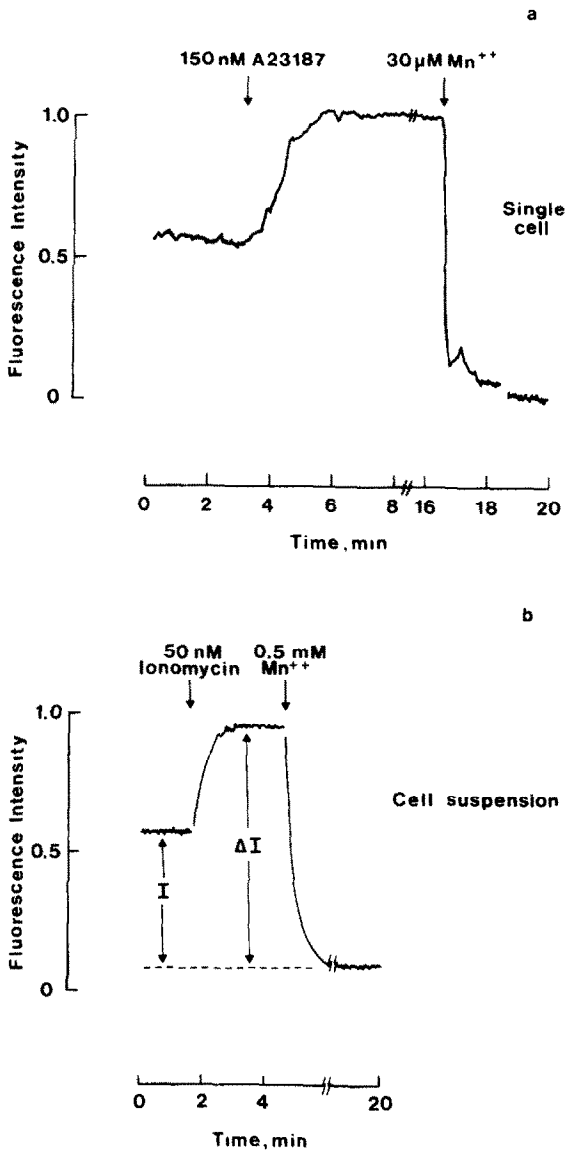

Fig.3. $[Ca]_i$ Calibration of quin 2 in a single mouse thymocyte. (a) Relative fluorescence intensity of a mouse thymocyte loaded with about 2 mM quin 2 and treated with 150 nM A23187 (3 μl of 150 μM A23187 in DMSO) followed by 30 μM $MnCl_2$ (9 μl of 10 mM $MnCl_2$). 1.0 F.I. ≡ 10^5 photon cps. The percentage saturation of Ca-quin 2 was calculated from:

$$\frac{(I - 0.16\,\Delta I)}{0.84\,\Delta I} \times 100 \qquad [6]$$

with the values of I and ΔI defined as shown in (b), after correction for the fluorescence of A23187. (b) $[Ca]_i$ calibration for quin 2 loaded mouse thymocytes in suspension [6].

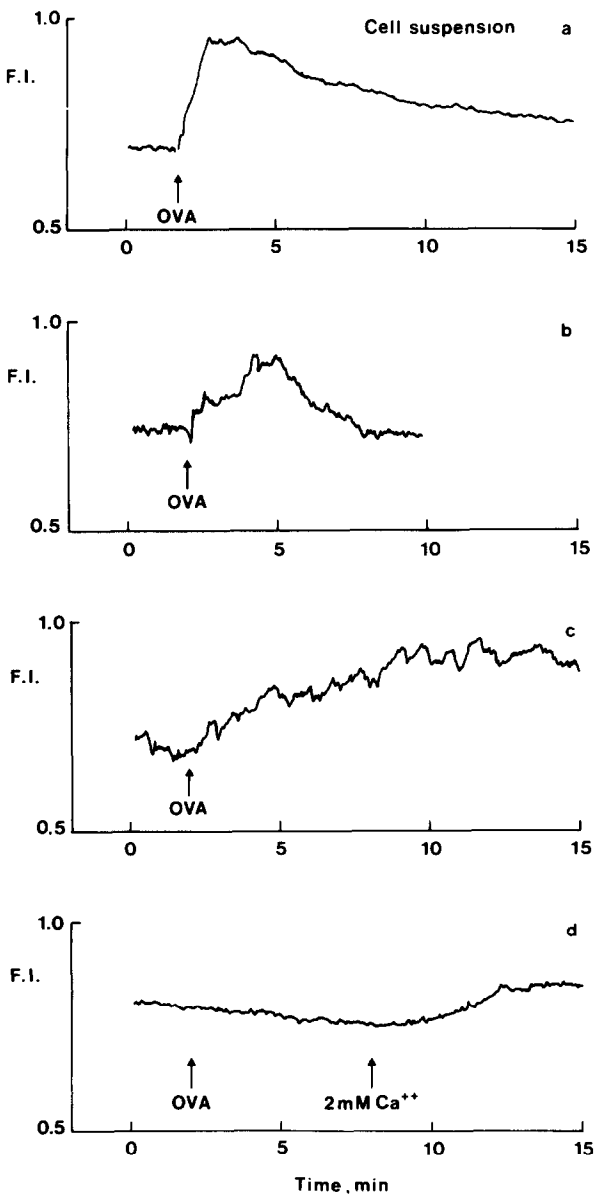

Fig.4. Response of quin 2-loaded 2H3 cells to ovalbumin. 2H3 cells were primed with mouse monoclonal IgE specific for ovalbumin and loaded with quin 2 as described in section 2. (a) F.I. (arbitrary units) of 2H3 cells in suspension treated with 2.5 μg/ml of ovalbumin (OVA) (5 μl of 1 mg/ml of aggregated ovalbumin in PBS). (b,c) Fluorescence responses of individual 2H3 cells (in 3 ml of PBS + 0.1% BSA, pH 7.1) to the addition of 20 μg/ml ovalbumin (OVA) (6 μl of 10 mg/ml of aggregated ovalbumin in PBS). 1.0 F.I. $\equiv 10^4$ photon cps. (d) Fluorescence response of a single 2H3 cell (in 3 ml of Ca^{2+}-free PBS + 0.1% BSA, pH 7.1) to 20 μg/ml of ovalbumin (OVA) (6 μl of 10 mg/ml aggregated ovalbumin in PBS) followed by the addition of 2 mM Ca^{2+} (6 μl of 1 M $CaCl_2$ in PBS, pH 7.1). 1.0 F.I. $\equiv 10^4$ photon cps.

intracellular quin 2 with Ca^{2+}, followed by the addition of Mn^{2+} which enters via the ionophore and completely quenches quin 2 fluorescence. The calibration of $[Ca^{2+}]_i$ in a single thymocyte is compared with the corresponding calibration for thymocytes in suspension in fig.3 [6]. It is noted that A23187 is less suitable than ionomycin because of its intrinsic fluorescence, but ionomycin is no longer available. A correction for the A23187 fluorescence has been estimated by adding the same concentration of A23187 to single cells without quin 2 under the same conditions (see legend to fig.3). The percentage saturation of quin 2 with Ca^{2+} was calculated as 48 ± 5% which corresponds to a $[Ca]_i$ of 97 ± 12 nM. This is in reasonable agreement with the corresponding $[Ca]_i$ value for cell suspensions of 110 ± 15 nM [6].

The responses of single 2H3 cells and thymocytes to ligands which increase $[Ca]_i$ in suspensions of the same cells loaded with quin 2 were variable. Part of this variability is attributable to the relatively low sensitivity of quin 2, compared with quene 1. The lower excitation (339 nm) and emission (492 nm) wavelengths and the lower fluorescence intensity of quin 2 compared with quene 1 gave a signal to background ratio of fluorescence for single cells loaded with about 1 mM quin 2 of 4–5:1 for 2H3 cells and 2–3:1 for thymocytes. Single thymocytes showed no consistent changes in fluorescence with 1 μg/ml Con A in more than 20 separate experiments, compared with an average increase in quin 2 fluorescence intensity of about 20% in thymocyte suspensions where the corresponding signal to background ratio is greater than 10:1. In some experiments in which thymocytes were treated with 20 μg/ml Con A a large increase in fluorescence intensity of nearly 2-fold was observed, consistent with complete saturation of the intracellular quin 2 with Ca^{2+}. This may correspond to the cytotoxic action of high Con A concentrations on mouse thymocytes.

The response to antigen (aggregated ovalbumin)

of single quin 2-loaded 2H3 cells primed with IgE is similar to that in 2H3 cell suspensions although more variable in magnitude and time course. In a typical 2H3 cell suspension experiment the fluorescence signal rises to a maximum 2–3 min after addition of ovalbumin, before declining towards the original level over 30 min (fig.4a). Two single cell experiments are shown on fig.4b,c in which the onset of the Ca signal in response to ovalbumin is slower than in the cell suspension. Removal of Ca^{2+} from the medium, however, completely blocks the Ca^{2+} signal in single 2H3 cell experiments (fig.4d), as in experiments on cell suspensions, and the signal is restored by readdition of Ca^{2+} in either type of experiment.

4. DISCUSSION

The main aim of this study was to assess the use of photometric fluorescence microscopy of single cells containing pH and Ca indicators. The sensitivity of fluorescence detection is limited ultimately by the statistical noise inherent in the signal. Other limitations arise from background fluorescence and bleaching of the fluorochrome. In the experiments reported here the major source of signal noise was arc-lamp instability, causing signal fluctuations (± 3%) several fold greater than those expected from the statistical noise of the relatively high photon count rates that were obtained. Background fluorescence increases as the excitation wavelength falls further into the UV and arises from many sources, including the microscope objective itself. The background fluorescence may be reduced by using quartz optics in place of glass. The use of a pin-hole in the image plane of the objective [9] is essential and without it the fluorescence signal from a single cell is swamped by background.

The comparison between the fluorescence responses in single cells loaded with quene 1 or quin 2 and in suspensions of the same cell systems demonstrates that reliable assays of pH_i and $[Ca]_i$ changes can be made in single cells. Rapid fluorescence responses within a few seconds are detectable by the simple protocols described here and at appropriate light intensities single cells can be monitored continuously for many minutes without photobleaching of the indicators. The sensitivity of the quene 1 indicator is sufficient to allow further development of accurate in situ calibration techniques in single cells. The sensitivity of quin 2 is lower than that of quene 1 in the cell systems used here and the failure to observe any consistent $[Ca]_i$ changes with quin 2 in thymocytes with Con A is at least partly due to the low signal to background ratio in these experiments, where the expected increase in signal intensity of about 10% is close to the limit of detection. However, variability in the responses of single thymocytes may also reflect the heterogeneity of the cells in the system (only 30–60% of mouse thymocytes are usually stimulated to DNA synthesis after 48 h exposure to optimal mitogenic concentrations of Con A). The use of new Ca^{2+} indicators based on phenanthridine as the fluorophor, that have longer excitation and emission wavelengths and higher fluorescence intensities than quin 2, may overcome the problem of the insensitivity of quin 2 in very small cells. It should be noted that the signal to background ratio with quin 2 was higher in the larger 2H3 cells than in thymocytes, and it was relatively easy to detect a Ca^{2+} signal in response to the ovalbumin antigen. The development of precise in situ calibration techniques for $[Ca]_i$ would be facilitated by the availability of a non-fluorescent Ca^{2+} ionophore.

The techniques for pH_i and $[Ca]_i$ measurements in single cells should be applicable to the wide variety of cells that can be loaded with quene 1 or quin 2 without loss of their biological responses. We have selected lymphocytes and the 2H3 cell line for further development of the single cell pH_i and $[Ca]_i$ assays since a biological response can also be assayed in individual cells. The mitogenic stimulation of thymocytes (e.g., by Con A) can be detected by autoradiography of the [^{3}H]thymidine-labelled cells, and the response of single 2H3 cells to antigen can be monitored by histamine release (3–6 fmol/cell). Finally we note that the use of a modified fluorescent cell sorter in the analytical mode should enable $[Ca]_i$ or pH_i to be monitored in a number of individual cells simultaneously. This presents the interesting possibility of examining ionic interactions between heterogeneous cell populations, for example within lymphocyte subpopulations stimulated with antigen.

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REFERENCES

[1] Schuldiner, S. and Rozengurt, E. (1982) Proc. Natl. Acad. Sci. USA 79, 7778–7782.
[2] Gerson, D.F., Kiefer, H. and Eufe, W. (1982) Science 216, 1009–1010.
[3] Tsien, R.Y. (1980) Biochemistry 19, 2396–2404.
[4] Rogers, J., Hesketh, T.R., Smith, G.A. and Metcalfe, J.C. (1983) J. Biol. Chem. 258, 5994–5997.
[5] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 295, 68–71.
[6] Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V. and Metcalfe, J.C. (1983) J. Biol. Chem. 258, 4876–4882.
[7] Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) J. Cell Biol. 95, 189–196.
[8] Barsumian, E.L., Isersky, C., Petrino, M.G. and Siraganian, R.P. (1981) Eur. J. Immunol. 11, 317–323.
[9] Johnson, P. and Garland, P.B. (1982) Biochem. J. 203, 313–321.