

# Calcium Homeostasis in Intact Lymphocytes: Cytoplasmic Free Calcium Monitored With a New, Intracellularly Trapped Fluorescent Indicator

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**ABSTRACT** A new, fluorescent, highly selective  $\text{Ca}^{2+}$  indicator, "quin2", has been trapped inside intact mouse and pig lymphocytes, to measure and manipulate cytoplasmic free  $\text{Ca}^{2+}$  concentrations,  $[\text{Ca}^{2+}]_i$ . Quin2 is a tetracarboxylic acid which binds  $\text{Ca}^{2+}$  with 1:1 stoichiometry and an effective dissociation constant of 115 nM in a cationic background mimicking cytoplasm. Its fluorescence signal (excitation 339 nm, emission 492 nm) increases about fivefold going from Ca-free to Ca-saturated forms. Cells are loaded with quin2 by incubation with its acetoxyethyl ester, which readily permeates the membrane and is hydrolyzed in the cytoplasm, thus trapping the impermeant quin2 there. The intracellular quin2 appears to be free in cytoplasm, not bound to membranes and not sequestered inside organelles. The fluorescence signal from resting cells indicates a  $[\text{Ca}^{2+}]_i$  of near 120 nM. The millimolar loadings of quin2 needed for accurately calibrated signals do not seem to perturb steady-state  $[\text{Ca}^{2+}]_i$ , but do somewhat slow or blunt  $[\text{Ca}^{2+}]_i$  transients. Loadings of up to 2 mM are without serious toxic effects, though above this level some lowering of cellular ATP is observed.  $[\text{Ca}^{2+}]_i$  was well stabilized in the face of large changes in external  $\text{Ca}^{2+}$ . Alterations of  $\text{Na}^+$  gradients, membrane potential, or intracellular pH had little effect. Mitochondrial poisons produced a small increase in  $[\text{Ca}^{2+}]_i$ , probably due mostly to the effects of severe ATP depletion on the plasma membrane. Thus intracellularly trapped chelators like quin2 offer a method to measure or buffer  $[\text{Ca}^{2+}]_i$  in hitherto intractable cell types.

Cytoplasmic  $\text{Ca}^{2+}$  has been proposed as a key regulator of numerous cellular processes and probably plays one or more important roles in every cell type. However, measurement of the key variable, intracellular free calcium concentration,  $[\text{Ca}^{2+}]_i$ , is always technically demanding, and in small cells has thus far been almost impossible. A novel method for monitoring and manipulating  $[\text{Ca}^{2+}]_i$  in large populations of small cells has now been provided by two recent chemical inventions (29, 30): (*A*) a new family of calcium chelators that shows high affinity for  $\text{Ca}^{2+}$ , very low affinity for  $\text{Mg}^{2+}$  and  $\text{H}^+$ , large absorbance and fluorescence changes resulting from  $\text{Ca}^{2+}$ -binding, and little or no detectable binding to the membranes; (*B*) a method for trapping such substances inside intact cells by means of nonpolar ester derivatives which cross the plasma membrane and are hydrolyzed intracellularly back to the parent membrane-impermeant polycarboxylate anions. We have

used a fluorescent quinoline  $\text{Ca}^{2+}$ -indicator (31), "quin2", whose structure is shown in Fig. 1, to obtain the first measurement of  $[\text{Ca}^{2+}]_i$  in lymphocytes, and to observe the responses to mitogenic doses of plant lectins. This paper further characterizes the loading process and the use of quin2 not only as an indicator but also as a buffer of  $[\text{Ca}^{2+}]_i$ . Homeostasis of lymphocyte  $\text{Ca}^{2+}$  was examined by testing the effects of a number of maneuvers which can change  $[\text{Ca}^{2+}]_i$  in other cells. The following paper (19) reports the use of quin2 in B-cells to analyze the relations between intracellular calcium and the binding and capping of anti-immunoglobulin.

## MATERIALS AND METHODS

### *Synthesis of Quin2 and Quin2/AM*

Quin2 was initially prepared as described in reference 29, in which it was referred to as ligand 3 b. The free acid form was precipitated from the final KOH

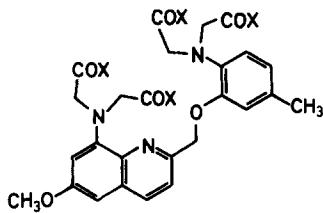


FIGURE 1 Structure of  $\text{Ca}^{2+}$ -indicator quin2 and its acetoxymethyl ester quin2/AM.

quin2 : X = O<sup>-</sup>

quin2/AM : X = OCH<sub>2</sub>OCOCH<sub>3</sub>

saponification solution by addition of an equivalent amount of HCl. The precipitate was filtered off, washed with water, and dried *in vacuo*. Re-esterification was done by the general procedure of reference 30 as follows. Ethyldiisopropylamine (Aldrich Chemical Co., Milwaukee, WI) was dried over CaH<sub>2</sub>, redistilled, and stored over sodium wire. Chloroform was freed from ethanol by passage through chromatographic alumina (Woelm, activity I). Acetoxymethyl bromide was prepared according to Neuenschwander and Iseli (15). Solid quin2 free acid was mixed with 6.5 equivalents of ethyldiisopropylamine, 4 ml chloroform per mmole quin2, and eight equivalents of acetoxymethyl bromide and stirred in a stoppered flask for 15 h. The solution was evaporated *in vacuo*, the residue taken up in benzene, filtered to remove ethyldiisopropylammonium bromide, and re-evaporated. This residue was extracted with multiple portions of dry diethyl ether (Aristar grade, British Drug Houses, Ltd., Poole, England). The extracts were evaporated *in vacuo*, finally at 0.1 mm Hg overnight. The yield from the free acid was 71%.

Most of the results were obtained with material obtained as above, but some of the later experiments used quin2/AM prepared by Dr. G. A. Smith (Biochemistry Department, Cambridge University) with extra steps to give a purer final product. Dr. Smith's modifications were as follows. The ethyl ester of quin2 (quin2/Et) was recrystallized from diethyl ether at -78°C instead of diisopropyl ether at room temperature or above. After saponification of quin2/Et, the free acid quin2 was precipitated at pH 2.5 with HCl. In the workup of the re-esterification reaction, the residue from benzene solution was dissolved in ethyl acetate, filtered, cooled at -78°C, and treated with diethyl ether at that temperature. The precipitate was filtered off, washed rapidly with cold petroleum ether, and dried. 250 mg of this material was dissolved in 10% ethyl acetate in benzene and chromatographed on 25 g of silica gel (CC-4, Mallinckrodt Inc., St. Louis, MO) with a solvent gradient reaching 50% ethyl acetate in benzene. The pooled central fractions were evaporated and the ethyl acetate-diethyl ether-78°C crystallization repeated, yielding 75 mg of a white powder. Thin-layer chromatography (silica gel eluted with 2% methanol in chloroform or 50% ethyl acetate in benzene, visualized with ultraviolet (UV) illumination) showed that this material lacked certain impurities detected in the earlier product. However we found that the two lots gave essentially identical fluorescence responses to  $\text{Ca}^{2+}$  in lymphocytes.

Stock solutions of quin2/AM were made up at 10 to 50 mM in dry dimethyl sulfoxide (DMSO) and were kept desiccated at -20°C between use. When such stock solutions are added to stirred cell suspensions, most of the ester seems to be rapidly absorbed onto or into the cells. If the aqueous medium contains no cells, solutions with final ester concentrations < 10  $\mu\text{M}$  look clear, while above 50  $\mu\text{M}$  concentration, a distinct haziness forms as if the solubility limit were exceeded. At higher ionic strengths as in artificial sea water, the ester is yet less soluble and tends to form macroscopic clumps; however such gross precipitates can be at least partially dispersed by sonication. By contrast to the hydrophobic ester, quin2 tetraanion or its Ca complex are sufficiently hydrophilic to be clearly soluble up to at least hundreds of millimolar.

### Calibration Solutions

Calcium-magnesium buffers for calibration were made up with Ca-Mg-EGTA recipes calculated on the basis of apparent dissociation constants for Ca-EGTA and Mg-EGTA of 214 nM and 8.96 mM, respectively at pH 7.05, 37°C. The apparent dissociation constants were calculated from the absolute stability constants and enthalpies tabulated in Martell and Smith (10). The series of spectra shown in Fig. 2 was obtained from a solution of 20  $\mu\text{M}$  quin2 in 115 mM KCl, 20 mM NaCl, 10 mM 4-morpholinepropanesulfonate (MOPS), 2.223 mM EGTA, and 1.223 mM MgCl<sub>2</sub>, titrated with KOH to pH 7.05 at 37°C, to which were then made sequential additions of 500 mM K<sub>2</sub>CaEGTA from an Agla micrometer syringe (Burroughs Wellcome Ltd., London, England), except for the final curve ("100  $\mu\text{M}$   $\text{Ca}^{2+}$ ") which was obtained by addition of CaCl<sub>2</sub>. The K<sub>2</sub>CaEGTA solution was prepared from CaCO<sub>3</sub>, EGTA, and KOH, following the procedure of Moisescu and Pusch (11) to equalize Ca and EGTA contents. In view of the great sensitivity to pH of Ca-EGTA and Mg-EGTA equilibria, the pH was maintained constant to  $\pm 0.005$  throughout, monitored by an unusually stable

pH electrode (GK2321C, Radiometer, Copenhagen, Denmark) calibrated at 37°C with buffers freshly made up from National Bureau of Standards recipes.

### Preparation of Cells

Lymphocytes from the mesenteric nodes of pigs slaughtered at the local abattoir were teased out into RPMI-1640 medium and purified on Ficoll-metrizamide gradients as previously described (7). Lymphocytes from different animals were kept apart. The same purification procedure was used for mouse spleen cells. Thymocytes were teased out into RPMI-1640 medium from the thymus glands of 6 to 8-wk-old Balb/c mice. Tissue fragments were removed by either sedimentation or filtration through nylon mesh. The suspension was centrifuged at ~1,000 g for 3–4 min and the cells resuspended in fresh medium. Cells were counted in a hemocytometer, and viability was assessed by eosin exclusion. In all acceptable preparations the viability exceeded 95% and the red cell contamination was <1%. Intracellular volume was taken as 1.7  $\mu\text{l}$  per  $10^7$  pig cells (S. Felber and M. Brand, *personal communication*) and 1.1  $\mu\text{l}$  per  $10^7$  mouse thymocytes. The cells were handled throughout in plastic tubes and with plastic pipets until the fluorescence measurements in quartz vessels, some of which were siliconized and some not. In practice siliconization made no apparent difference.

### Solutions for Cells

Cells were isolated and stored in RPMI-1640 medium, usually without bicarbonate but buffered to pH 7.4 at 37°C with 20 mM HEPES. Occasionally bicarbonate-buffered medium was used. Solutions were freshly filtered through 0.45  $\mu\text{m}$  filters, and usually 10  $\mu\text{g}/\text{ml}$  gentamycin was added. Sometimes, as specified in the text, CaCl<sub>2</sub> or K<sub>2</sub>H<sub>2</sub>EGTA was added to alter the  $\text{Ca}^{2+}$  concentration from the normal 0.5 mM.

The fluorescence experiments were usually performed in a simplified saline rather than RPMI-1640 medium, since the latter contains phenol red which could filter the excitation and emission wavelengths, as well as pyridoxine and riboflavin which contribute considerable fluorescence of their own. The simplified medium consisted of (mM): NaCl, 145; KCl, 5; Na<sub>2</sub>HPO<sub>4</sub>, 1; CaCl<sub>2</sub>, 1; MgSO<sub>4</sub>, 0.5; glucose, 5; HEPES, 10; titrated with NaOH to pH 7.40 at 37°C. In Ca-free solution the CaCl<sub>2</sub> was simply omitted, and the contaminating [Ca<sup>2+</sup>] was usually found to be ~10  $\mu\text{M}$ . Additions of Ca<sup>2+</sup> were made from either 1.00 M or 0.2 M CaCl<sub>2</sub> stock and EGTA from 0.2 or 0.4 M K<sub>2</sub>H<sub>2</sub>EGTA. Adjustments of the pH of cell lysates was done with 1 M HCl or 1 M Tris base. All solutions were made with water purified to >15 MΩ·cm by a Millipore system (Millipore Corp., Bedford, MA).

### Quin2 Loading

Unless stated otherwise, cells were loaded by incubation with quin2/AM in HEPES-buffered RPMI-1640 at 37°C. The final concentration of DMSO from the quin2/AM stock did not exceed 0.5% (vol/vol). Our usual procedure was to add 50  $\mu\text{M}$  quin2/AM to a cell suspension containing ~10<sup>6</sup> cells/ml and incubate for 20 min, then dilute tenfold and continue incubating for a further 40–60 min. The resulting quin2 loading was typically 2 mmol per liter of cells; higher or lower loadings were achieved by altering the ester concentration. After loading, the cells were centrifuged at 1,000 g for 3–4 minutes and resuspended in fresh RPMI-1640 at ~1.5 × 10<sup>7</sup>/ml and kept at room temperature. For measurement of fluorescence, 1 ml of this stock suspension was centrifuged as briefly as possible at 14,000 g in a microcentrifuge (model 320, Jobling Ltd., Stone, Staffordshire, England) and the cells resuspended in 2 ml simplified medium at 37°C and transferred to the cuvet, the whole resuspension procedure taking about 1 min. This final step minimizes the carry-over of external quin2.

### Fluorescence Measurements

Fluorescences were recorded with Perkin-Elmer MPF-44A and MPF-44B spectrophotometers in ratio mode, except for excitation spectra, for which energy mode was preferred. Standard monochromator settings were 339-nm excitation with 4-nm slits; 492- or 500-nm emission, 10-nm slits. 339 nm was chosen as excitation wavelength because quin2 and its Ca and Mg complexes adsorb equally there; longer wavelengths would decrease the sensitivity to Ca<sup>2+</sup>-binding, whereas shorter wavelengths excite more cell autofluorescence. Samples were contained in thermostatted 1-cm square quartz cuvets or round quartz test tubes of 0.9 cm ID. Interruptions of the traces show where the sample compartment was opened either to add reagents or stir the suspensions with a plastic pasteur pipet. Emission polarization measurements were made with square cuvets and sheet polarizers by the general procedure of Chen and Bowman (4).

### Membrane Permeabilization with High-voltage Discharges

The apparatus was loosely based on that of Baker and Knight (2). The sample chamber was a U-shaped poly(methyl methacrylate) spacer, 0.5-cm thick, sand-

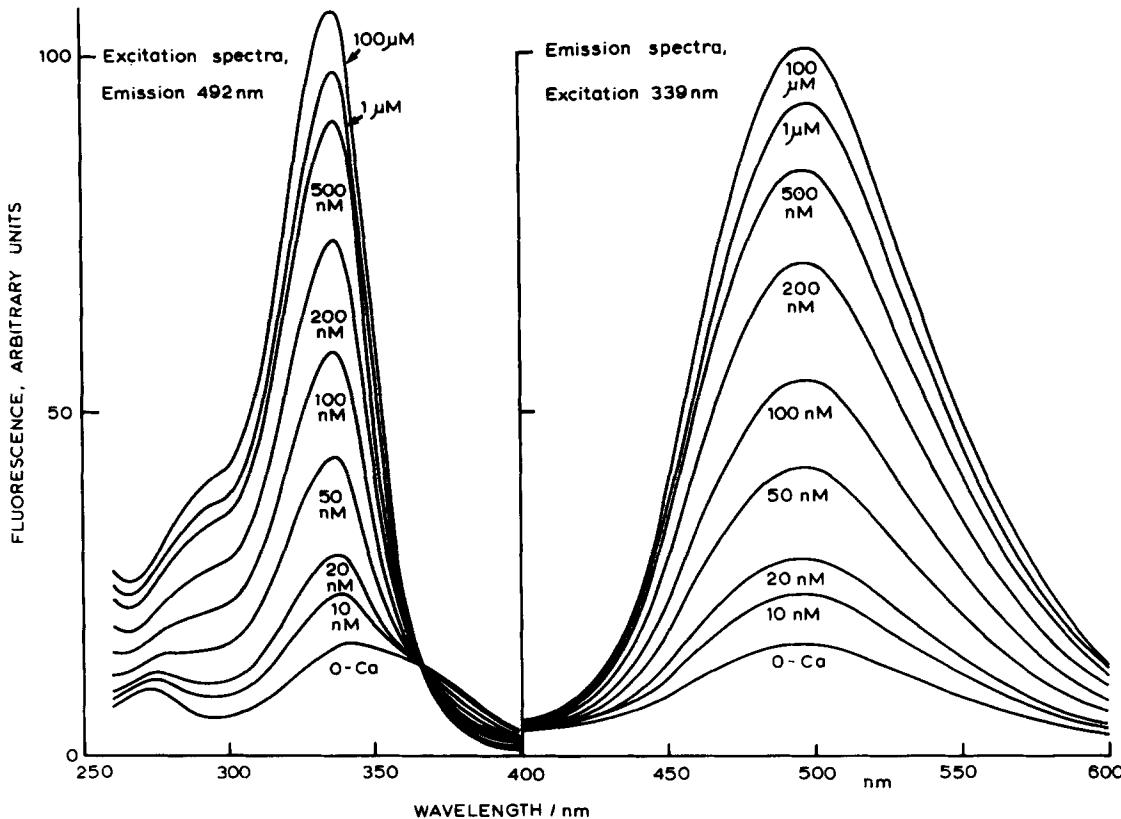


FIGURE 2 Excitation and emission spectra of 20- $\mu$ M quin2, with varying  $[Ca^{2+}]$  as shown against an ionic background of 120–135 mM  $K^+$ , 20 mM  $Na^+$ , 1 mM free  $Mg^{2+}$  and pH 7.05 at 37°C. Further details are given in Materials and Methods. Excitation and emission bandwidths were 4 nm and 10 nm.

wiched between two platinum foil plates. Each shock was delivered by charging a 2  $\mu$ F capacitor to 1.8 kV then discharging it through the sample chamber, giving an initial electric field of 3.6 kV/cm decaying in a few microseconds.

#### Assays for ATP, Total Ca, and Enzymes

ATP was assayed by the luciferase method (20). Total calcium of cells was measured by resuspending a 14,000 g cell pellet with ice-cold Ca-free medium containing 0.1 mM EGTA, immediately recentrifuging, and carefully removing the supernatant. The pellet of  $4.5 \times 10^7$  cells was dissolved in 4% perchloric acid, then diluted to 1 ml with water and fed into a Perkin-Elmer 380 atomic absorption spectrophotometer. Lactate dehydrogenase was assayed by the change in absorbance with NADH and excess pyruvate. Glutamate dehydrogenase was analogously assayed with NADH and  $\alpha$ -ketoglutarate at pH 8.

#### RESULTS

##### Quin2 as a Fluorescent Indicator of Free $Ca^{2+}$

Fig. 2 shows the excitation and emission spectra of quin2 as a function of the free  $Ca^{2+}$  concentration, varied from "0" ( $<1$  nM) to a saturating level,  $\sim 100$   $\mu$ M. The solutions were Ca-EGTA buffers with the free concentrations of other cations chosen to mimic the intracellular ionic environment, 125 mM  $K^+$ , 20 mM  $Na^+$ , 1 mM  $Mg^{2+}$ , and pH 7.05 at 37°C (21). With excitation at 339 nm, the intensity of fluorescence increases about sixfold over the full range of  $[Ca^{2+}]$ , though the wavelength of the emission peak remains nearly constant at 492 nm. (If the emission spectrum is corrected for the spectral sensitivity of the monochromator and photomultiplier, the true peak is found at 525 nm (29), but for biological experiments it is convenient to fix the emission monochromator at the wavelength of the uncorrected maximum.) Obviously the fluorescence amplitude is a sensitive measure of free  $[Ca^{2+}]$  in the range of tens to hundreds of nanomolar, fortunately encompassing the expected resting level of  $[Ca^{2+}]_i$ . However the fluorescence response is nearly saturated for  $[Ca^{2+}]$  above 1  $\mu$ M, which is reached on strong stimulation of other cell types. The dependence of fluorescence at 492 nm on free  $Ca^{2+}$  is

quantitatively analyzed in Fig. 3 A, the circles corresponding to the emission spectra of Fig. 2. An excellent fit is obtained to the theoretical behavior (smooth curve) for simple 1 dye:1  $Ca^{2+}$  binding with an effective dissociation constant  $K_d$  of 115 nM. The equation which predicts fluorescence as a function of  $[Ca^{2+}]$  is:

$$F = F_{\min} + (F_{\max} - F_{\min}) ([Ca]/K_d)/(1 + [Ca]/K_d),$$

where  $F_{\min}$  and  $F_{\max}$  are the fluorescences at very low and very high  $[Ca^{2+}]$  respectively. Conversely, if we know  $F$ ,  $F_{\min}$ , and  $F_{\max}$ , we can calculate  $[Ca^{2+}]$  from the inverse equation:

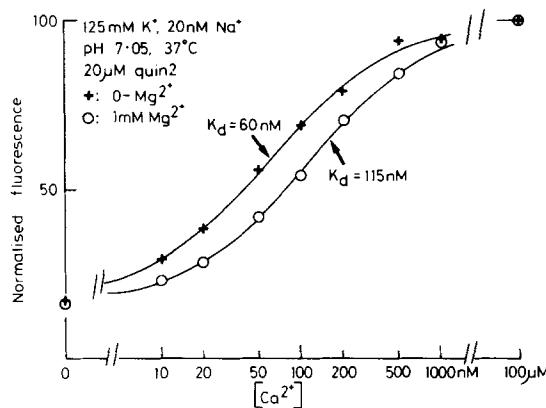
$$[Ca] = K_d (F - F_{\min})/(F_{\max} - F).$$

Parallel measurements of the excitation and absorbance spectra confirm the 1:1 stoichiometry and the value of  $K_d$ .

Fig. 3 A also shows the influence of the 1 mM free  $Mg^{2+}$ . Without  $Mg^{2+}$  the crosses are obtained, corresponding to an effective dissociation constant of 60 nM. This shift in effective dissociation constant is the only significant effect of  $Mg^{2+}$ , which hardly influences  $F_{\min}$  and  $F_{\max}$ . An example of the effect of pH is given in Fig. 3 B. Again the spectra at very low and very high  $[Ca^{2+}]$  are unaffected by acidification from pH 7.05 to 6.75, but at an intermediate  $[Ca^{2+}]$ , acidification slightly inhibits the fluorescence enhancement. The obvious interpretation is that a proton can bind (with  $pK_a < 7$ ) to the nitrogen on the benzene ring, blocking part of the binding site for  $Ca^{2+}$  but not directly affecting the quinoline system which is the fluorescent chromophore. Raising the pH above the 7.05 would raise the apparent Ca affinity very little since the pH would be moving away from the highest  $pK_a$  rather than towards it.

These in vitro calibrations show that quin2 responds sensitively to  $[Ca^{2+}]$  near the expected intracellular resting level of  $10^{-7}$  M with little interference from either  $Mg^{2+}$  or  $H^+$ . Thus if our measurements of cytoplasmic free  $Mg^{2+}$  or pH (20) were in

A



B

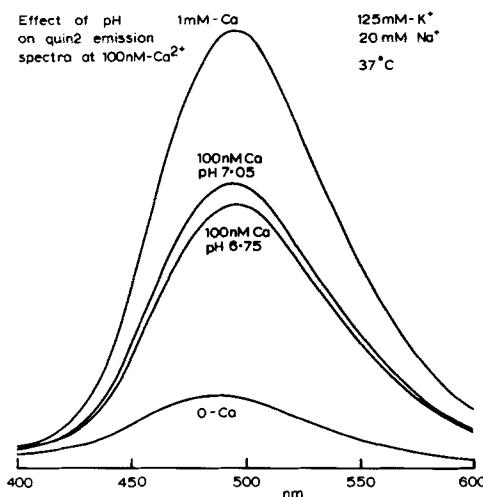


FIGURE 3 Quin2 calibration curves at different  $[Mg^{2+}]$  and pH values. (A) Normalized quin2 fluorescence (ex. 339 nm; em. 492 nm) plotted against free  $[Ca^{2+}]$  at 0 Mg and 1 mM free  $Mg^{2+}$ . The data points are fitted with curves corresponding to one-to-one stoichiometry and the indicated apparent dissociation constants. (B) Shows emission spectra from 20  $\mu M$  quin2 at "0"-Ca ( $<1\text{nM}\ [Ca^{2+}]$ ), 100 nM  $[Ca^{2+}]$ , and 1 mM  $[Ca^{2+}]$ , at both pH 7.05 and 6.75. The medium contained 125 mM  $K^+$  and 20 mM  $Na^+$ . The  $[Ca^{2+}]$  and pH were altered by adding appropriate amounts of Ca-EGTA, CaCl<sub>2</sub>, and K-MOPS while carefully adjusting pH. Changing pH at "0"-Ca and 1 mM  $[Ca^{2+}]$  had no effect on the spectra.

error by  $\pm 30\%$  or  $\pm 0.2$  units respectively, the estimates of  $[Ca^{2+}]_i$  with quin2 would be off by  $\pm 15\%$ .

### Loading of Quin2 into Lymphocytes

Loading of analogous chelators by means of intracellularly hydrolyzable esters has been demonstrated in erythrocytes and mast cells (30). In those experiments, loading was detected by the acid production associated with ester hydrolysis, direct titration of the intracellularly generated chelator, and the temporary suppression of cell responses known to be dependent on rises of  $[Ca^{2+}]_i$ . With the acetoxyethyl ester of quin2, quin2/AM, hydrolysis is most easily monitored by the gradual shift in the emission spectrum from that of the ester, peaking at 430 nm, to that of the final indicator, peaking at 492 nm. Fig. 4 gives an example. Under the conditions chosen,  $10^7$  cells/ml, 20  $\mu M$  initial quin2/AM,  $37^\circ C$ , the spectral shift is practically complete in 75–90 min. Much of the quin2 was

trapped inside the cells as demonstrated by centrifuging them and resuspending them in fresh medium to remove external quin2. The amount trapped can then be quantified by lysing the washed cells, and comparing the resulting fluorescence with the autofluorescence of lysed, unloaded cells and with standards containing known concentrations of quin2 in the same medium. Another way to assay quin2 contents of lysates is to compare the fluorescences with excess  $Ca^{2+}$  and with excess EGTA; the difference signals the quin2 with negligible contribution from cell autofluorescence. The percentage of ester molecules which become hydrolyzed and trapped as indicator molecules inside the cells is defined as the loading efficiency; this is typically 30–40% for cell densities around  $10^7/\text{ml}$ . Since such densities correspond to cytocrits of 0.1% to 0.15%, the final intracellular concentration of quin2 is several hundred times that of the initial concentration of ester in the whole suspension. Somewhat higher loading efficiency (40–60%) is found if the cells are at least initially at  $10^8/\text{ml}$ , possibly because less ester is wasted by precipitation and adsorption to the hydrophobic walls of the container. Loading efficiency varies enough that dye content should be checked empirically whenever it needs to be quantified.

If the ethyl ester rather than the acetoxyethyl ester of quin2 is added to cells, the fluorescence peak stays at 430 nm rather than moving to 492 nm, showing that the ethyl ester is not hydrolyzed.

Loaded and washed cells lose dye very slowly when stored at room temperature (<5%/h). Present data are not sufficient to say how much of the loss is due to occasional cell death and lysis, true permeability through healthy cell membranes, or chemical destruction of the dye.

### Intracellular Location of Quin 2

We find that essentially all the trapped quin2 behaves as if it were free in the cytoplasm, not strongly bound to macromolecules or membranes or sequestered in intracellular organelles. This conclusion is based on the following lines of evidence:

(a) In lymphocytes, the organelles most likely to be able to sequester quin2 or  $Ca^{2+}$  are the mitochondria, which occupy 5–10% of the cell volume. There are very few lysosomes and little smooth endoplasmic reticulum in resting lymphocytes. Quin2 accumulation in mitochondria was assayed by selec-

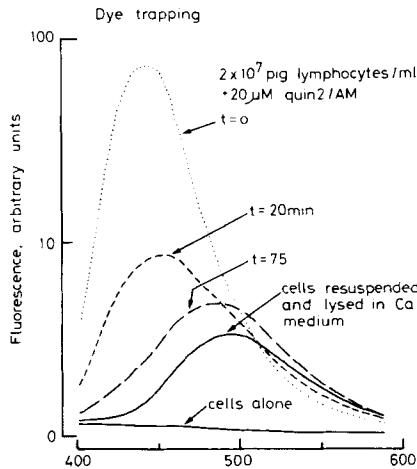


FIGURE 4 Shifts of emission spectra during quin2/AM uptake and hydrolysis. Excitation 339 nm. Dots show the initial fluorescence of external quin2/AM, 20  $\mu M$  in standard, 1 mM Ca solution. Then  $2 \times 10^7$  pig lymphocytes/ml were added. Dashed curves show the suspension spectra at 20 and 75 min. The solid curve was obtained after washing the cells, resuspending in the same volume of fresh medium, then releasing the trapped dye with digitonin. The bottom trace *cells alone* shows the auto-fluorescence measured from unloaded cells at the same density.

tively permeabilizing the plasma membrane with 50–100  $\mu\text{M}$  digitonin, a detergent which leaves mitochondria largely intact (26, 32) so long as  $[\text{Ca}^{2+}]$  is not too high. Mitochondrial integrity was verified here by retention of 80% of the cellular glutamate dehydrogenase and the continued ability to sequester excess  $\text{Ca}^{2+}$ . Nevertheless, digitonin released 98% of the cellular quin2 into the supernatant, showing that the dye was not substantially contained in the mitochondria. (100% release of enzymes and quin2 is defined as that given by solubilization with the nonselective detergent Triton X-100). The probable explanation is that mitochondria have little internal esterase activity, as can be independently demonstrated by the inability of isolated rat liver mitochondria to accumulate quin2 when incubated with quin2/AM.

(b) Since digitonin severely disrupts the plasma membrane and releases 92% of a cytoplasmic soluble protein, lactate dehydrogenase, the release of quin2 by digitonin does not rule out quin2 binding to macromolecules or digitonin-solubilizable membranes. A yet more selective method is required for putting small holes in the plasma membrane. A partial solution was found in the use of high-voltage electric discharges, which are believed to make holes in the plasma membrane by dielectric breakdown (2). Discharge parameters could be found which gave release of 85% of the loaded quin2 but only 28% of lactate dehydrogenase. Considering that the number and pore size of the holes are not perfectly uniform from cell to cell, the differential release suggests no major binding of quin2 to structures of the size of lactate dehydrogenase or larger.

(c) Binding to hydrophobic domains of lipids or proteins would be expected to change the spectral characteristics of quin2 fluorescence, since the chromophore of quin2 is closely analogous to a dansyl moiety, well known to be highly sensitive to the microenvironment. However, the excitation and emission peaks of quin2 have the same wavelengths and shape in cells as in simple saline. Even the fluorescence quantum yield is the same, as shown by the observation that once  $[\text{Ca}^{2+}]_i$  is raised above micromolar levels by large doses of calcium ionophores, lysing the cells and releasing the quin2 does not further alter the fluorescence. Polarization of the emitted fluorescence is another sensitive spectroscopic correlate of binding to membranes or proteins. Only very preliminary experiments have been completed, indicating that quin2 in cells has a small but

nonzero polarization, much less than that observable when the dye is immobilized in artificial viscous media. More experiments will be needed to decide whether the small polarization can be explained by the microviscosity of cytoplasm or whether binding to rotationally immobile sites need be invoked.

(d) Finally it is important to consider the distribution of  $\text{Ca}^{2+}$  and quin2 across the nuclear membrane, since the nucleus occupies 60–70% of resting lymphocyte volume. Nuclear pores pass ions and many macromolecules (17, 27) and are very unlikely to pose a significant diffusion barrier to  $\text{Ca}^{2+}$  or quin2. Cation activities in the nucleus are generally identical to those in cytoplasm (18). When a nuclear pellet, prepared from cells permeabilized with digitonin, is equilibrated with quin2 (the tetraanion, not the ester) and  $^3\text{H}_2\text{O}$ , the ratio of quin2 to  $^3\text{H}_2\text{O}$  is the same in the pellet as in the supernatant, so that the spaces accessible to quin2 and to  $\text{H}_2\text{O}$  are the same. It seems that the nucleus neither binds nor excludes quin2, so that it need not be treated as a compartment separate from cytoplasm.

#### Measurement of $[\text{Ca}^{2+}]_i$

Fig. 5 shows the basic procedure for measuring  $[\text{Ca}^{2+}]_i$ . The fluorescence of loaded and washed cells was measured, giving the first section of trace. Because the dye reports  $[\text{Ca}^{2+}]_i$  by a change in fluorescence intensity, the signal depends not only on  $[\text{Ca}^{2+}]_i$  but on the dye content of the suspension and the sensitivity of the fluorimeter. To calibrate the fluorescence signal it is therefore necessary to expose the dye to known  $[\text{Ca}^{2+}]$  levels while keeping constant the dye content of the suspension and the fluorimeter settings. This is done after the observation period by releasing the dye from the cells with digitonin or Triton X-100. The medium usually contains 1 mM  $\text{Ca}^{2+}$  so  $F_{\max}$  is obtained after dye release.  $F_{\min}$  is obtained by setting  $[\text{Ca}^{2+}]_i$  to 1 nM by adding 2 mM EGTA and enough Tris base to take the pH to >8.3.

Part of the signal comes from cell autofluorescence. If this were constant throughout, it would not affect the calculation of  $[\text{Ca}^{2+}]_i$  from equation 1 since it would contribute equally to  $F$ ,  $F_{\min}$ , and  $F_{\max}$ . The fluorescence of unloaded cells is not affected by elevation of  $[\text{Ca}^{2+}]$  with A23187, nor is the signal from lysed cells detectably changed by altering  $[\text{Ca}^{2+}]$  over the range 1 nM to 1 mM. Autofluorescence does however show a

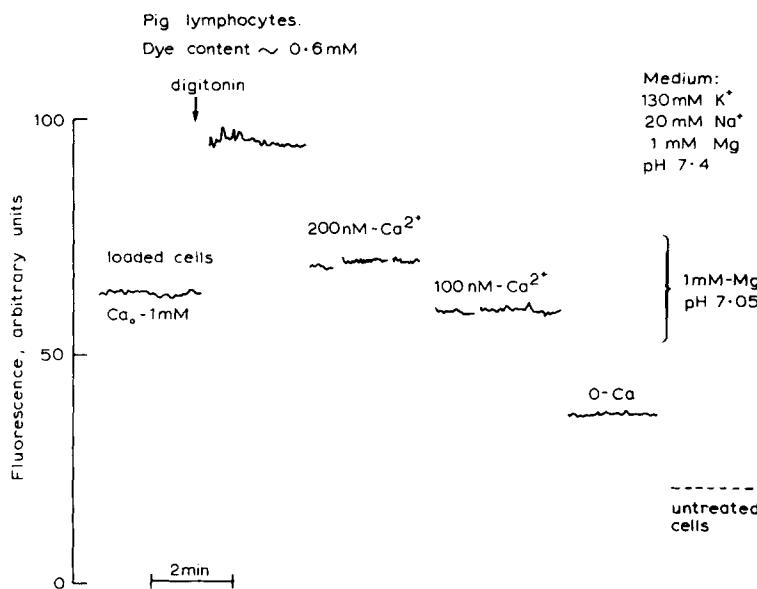


FIGURE 5 Measurement of  $[\text{Ca}^{2+}]_i$ . Fluorescence was recorded from pig lymphocytes containing ~0.5 mM quin2, suspended at  $5 \times 10^6$  cells/ml in medium containing 130 mM KCl, 20 mM NaCl, 1 mM  $\text{MgCl}_2$ , 5 mM glucose, 10 mM MOPS, pH 7.4. 50  $\mu\text{M}$  digitonin was added to lyse the cells as indicated.  $[\text{Ca}^{2+}]$  was then set to 200 and 100 nM by appropriate additions of EGTA, with  $\text{MgCl}_2$  and KOH added to maintain free  $[\text{Mg}^{2+}]$  at 1 mM and pH at 7.05. The  $[\text{Ca}^{2+}]$  was set to <1 nM by titration to pH 8.4. The dotted line shows the autofluorescence signal from the same density of unloaded cells. The time bar applies only until the Ca titrations of the medium. Then the periods for addition of EGTA,  $\text{MgCl}_2$ , and KOH are deleted.

small (~25%) enhancement when the cells are disrupted with digitonin or Triton X-100, which necessitates an appropriate correction to the measured  $F_{\max}$  and  $F_{\min}$ . This correction is equivalent to the fluorescence from ~30  $\mu\text{M}$  trapped quin2-Ca complex, and so with the higher loadings is almost negligible. With this correction, the initial fluorescence in Fig. 5 is found to correspond to 150 nM.

The fluorescence levels in the lysate at 100 and 200 nM  $[\text{Ca}^{2+}]_i$  conform to Eq. 1 with the same dissociation constant, 115 nM, as was obtained in Fig. 3 with pure quin2 which had not been esterified and de-esterified. This shows that the cells had fully regenerated quin2 with the correct  $\text{Ca}^{2+}$ -affinity. Therefore it becomes sufficient as a routine to note the fluorescences in saturating and near-zero calcium,  $F_{\max}$  and  $F_{\min}$ , in the lysate at the end of an experiment. Eq. 1, then, calibrates all the fluorescence levels from the intact cells. Our calibration also assumes that the quin2 has the same indicator properties at millimolar concentrations in the cells as in micromolar levels in physiological saline. One can eliminate major sources of error from likely interfering substances but never absolutely rule out possible effects from untested components of cytoplasm. One potential problem is that traces of heavy metals such as  $\text{Mn}^{2+}$  in the medium can depress the fluorescence of released quin2 and give a falsely low  $F_{\max}$ . This can be overcome by addition of 10  $\mu\text{M}$  diethylenetriaminepentaacetic acid, a chelator more specific than EGTA for heavy metals, conveniently added as a Ca complex. Such interference with the intracellular signal is unlikely to be a problem since the traces of contaminants which can influence micromolar quin2 after release could barely perturb the signal from millimolar quin2 inside the cells.

#### *Quin2 Loading in Normal Medium Does Not Alter Resting $[\text{Ca}^{2+}]_i$*

The mean value for basal  $[\text{Ca}^{2+}]_i$  from measurements of pig mesenteric node lymphocytes in the standard 145 mM-Na<sup>+</sup>, 1 mM-Ca was 136 nM ( $\pm 10$  nM, SE,  $n=13$ ). For mouse thymocytes the mean was  $118 \pm 9$  nM ( $n=14$ ). However, quin2 is inherently a high-affinity chelator of  $\text{Ca}^{2+}$  and is generated in a Ca-free form inside the cells. One might expect the dye to strip  $\text{Ca}^{2+}$  from internal sites and to perturb the  $[\text{Ca}^{2+}]_i$  level which it was intended to measure. If so, higher dye loadings ought to give cells with lower indicated  $[\text{Ca}^{2+}]_i$ . In fact, this is not observed, as shown by Fig. 6 which is a "scattergram" of  $[\text{Ca}^{2+}]_i$  readings vs. dye loadings. The regression line shows that  $[\text{Ca}^{2+}]_i$  if anything increases with quin2 content, though the slope is not statistically different from zero. Nor was any correlation seen when one batch of cells was subdivided into aliquots and loaded to different final dye contents.

The explanation seems to be that during the quin2 loading, the plasma membrane maintains homeostasis of  $[\text{Ca}^{2+}]_i$  by allowing net entry of  $\text{Ca}^{2+}$ . Thus in loaded cells the total calcium, as measured by atomic absorption spectrophotometry, increases from the normal value of ~1 mmol/liter cells by an amount approximating half of the quin2 loading. The maintenance of the quin2 at half-saturation is an independent though imprecise confirmation that  $[\text{Ca}^{2+}]_i$  approximately equals the effective  $K_d$  for intracellular dye.

Very different behavior of  $[\text{Ca}^{2+}]_i$  can be observed when calcium homeostasis is blocked by carrying out the loading in low- $\text{Ca}^{2+}$  medium. Fig. 7 shows the signal from cells which were incubated with quin2/AM in RPMI medium to which EGTA had been added to set  $[\text{Ca}^{2+}]_0$  at 100 nM, then washed

Does quin2 loading lower basal  $[\text{Ca}^{2+}]_i$ ?

- pig node lymphocytes
- mouse thymocytes

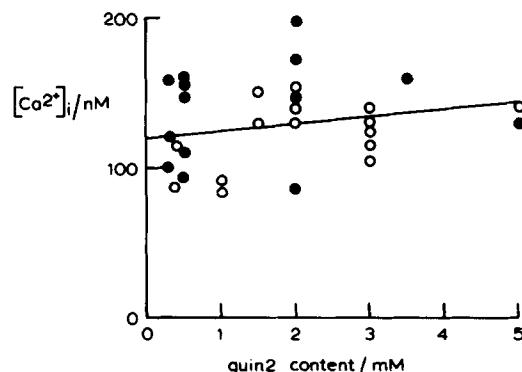


FIGURE 6 Effect of different quin2 loadings on  $[\text{Ca}^{2+}]_i$ . Each symbol represents one measurement of  $[\text{Ca}^{2+}]_i$  in mouse thymocytes (open circles) or pig node lymphocytes (closed circles). The line shows the linear regression through the points, but the slope is not significantly ( $P > 0.2$ ) different from zero.

and resuspended in  $\text{Ca}^{2+}$ -free simplified medium. The indicated  $[\text{Ca}^{2+}]_i$  was now below 20 nM, because the loaded quin2 had chelated internal  $\text{Ca}^{2+}$  which could not be resupplied from outside. Adding back external  $\text{Ca}^{2+}$  either all at once (upper trace) or in steps (lower trace) enabled the cells to take up  $\text{Ca}^{2+}$  and within a few minutes to stabilize at  $[\text{Ca}^{2+}]_i$  slightly higher than normal. (The final supranormal level may reflect slight membrane damage due to the prolonged exposure to low  $\text{Ca}^{2+}$ ). It is therefore not surprising that when the cells have access to normal external  $\text{Ca}^{2+}$  throughout the >1-h loading period, the resulting  $[\text{Ca}^{2+}]_i$  is not depressed by the dye.

#### *Nontoxicity of Quin2*

The finding that resting  $[\text{Ca}^{2+}]_i$  is in the range normally found in other types of cells at rest and is independent of quin2 loading argues against gross toxicity. Further evidence was that (a) loaded cells maintained high (>90%) eosin exclusion; (b) ATP levels remained within 20% of normal at quin2 loadings of up to 2 mM in mouse thymocytes and 3 mM in splenocytes, as shown in Fig. 8. 5 mM quin2 more seriously lowered ATP particularly in thymocytes, which may contribute to the slightly positive slope in Fig. 6. Whether the ATP drop is due to the chelator properties of quin2 or to the other hydrolysis products of the ester is being investigated with ester loading of an analogous but low-affinity  $\text{Ca}^{2+}$ -chelator. (c) Capping of anti-immunoglobulin on B-cells occurs normally with up to 5 mM intracellular quin2 (19); (d) membrane potentials of quin2-loaded mouse thymocytes were similar to those of normal cells and gave a similar hyperpolarization in response to concanavalin A (31); (e) thymocytes and spleen cells loaded with quin2 then treated with concanavalin A at normal optimal doses can undergo mitogenic transformation as assessed by thymidine uptake (T. R. Hesketh, personal communication); (f) human platelets loaded with 1–2 mM quin2 showed virtually normal shape change and aggregation in response to thrombin (25). However, cells loaded in HEPES media with several millimolar quin2 seem to have an internal pH 0.1 U more acid than cells without quin2, as assessed either by a trapped pH-indicating

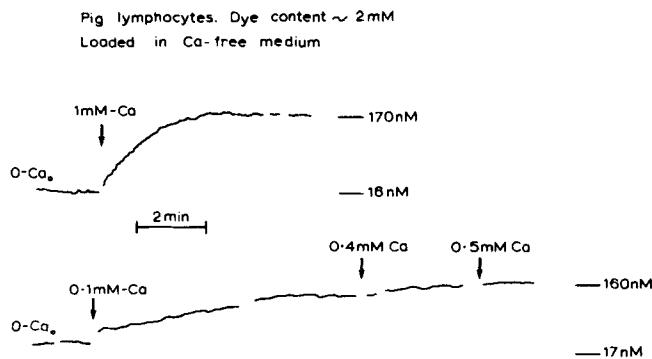


FIGURE 7 Records from cells loaded in low  $[Ca^{2+}]_o$  medium. EGTA was added to the loading medium to reduce  $[Ca^{2+}]_o$  below 100 nM. The cells were then washed and resuspended in simplified medium, without added  $Ca^{2+}$ .  $Ca^{2+}$  was then added back as indicated. The gaps in the traces here, and in subsequent figures, show the times for stirring or addition of reagents.

fluorescein derivative, or by a null-point method previously described (21). This small shift may be a residual effect of the acid generated by the ester hydrolysis, half of which can diffuse out as acetic acid but half of which is nondiffusible (quin2). Loading in bicarbonate-buffered media might well reduce the pH shift.

#### Effects of Quin2 on $[Ca^{2+}]_i$ Transients

Although intracellular quin2 seems not to perturb steady-state  $[Ca^{2+}]_i$ , it is expected to buffer  $[Ca^{2+}]_i$  transients. Fig. 9 shows the effect of very small, submitogenic and nontoxic doses of the calcium ionophore A23187, in cells with low or high quin2 content. In both cases the ionophore produces a rise in  $[Ca^{2+}]_i$  to about 1  $\mu M$ , enough to nearly saturate the dye, but the rise is substantially slower in the presence of the higher dye content. The ratios of the half times for responses to the same dose of A23187 in this and other similar experiments were roughly in the ratios of dye contents. Closely similar results were seen with another  $Ca^{2+}$ -ionophore, ionomycin (9).

#### Effects of Small Ions and Membrane Potential on $[Ca^{2+}]_i$

Numerous maneuvers have been demonstrated or postulated to influence  $Ca^{2+}$  homeostasis in various cell types. We have briefly examined the effect of some of them on the quin2 signal from lymphocytes.

In several tests reduction of  $[Na^+]_o$  to a few millimolar by replacement of NaCl by KCl or choline chloride had no obvious effect on  $[Ca^{2+}]_i$  observed over many minutes. In both of these low Na solutions  $[Ca^{2+}]_i$  was slightly higher than the mean but not outside the normal range, and there was no sign of a progressive increase. In isotonic choline solution, 20 nM A23187 produced the usual rise to  $\geq 1 \mu M$   $[Ca^{2+}]_i$  which was then followed by a slow trend towards the resting value, similar to that in the upper trace of Fig. 9. This may indicate that  $Ca^{2+}$ -extrusion does not require the normal  $Na^+$  gradient.

Choline substitution slightly hyperpolarizes the membranes of lymphocytes, whereas isotonic potassium strongly depolarizes them (24) so the lack of effect of these solutions suggests that alterations in membrane potential have little effect on  $[Ca^{2+}]_i$ . The antibiotic gramicidin D creates a large permeability to  $Na^+$  and  $K^+$ ; in Na medium it both depolarizes the cells

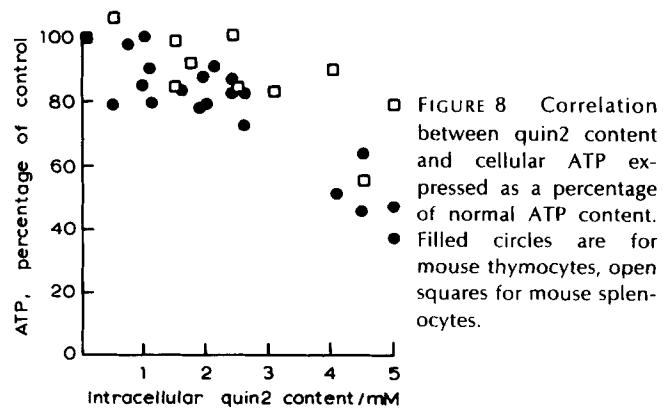


FIGURE 8 Correlation between quin2 content and cellular ATP expressed as a percentage of normal ATP content. Filled circles are for mouse thymocytes, open squares for mouse splenocytes.

and allows rapid exchange of intracellular  $K^+$  for  $Na^+$ . A fully effective dose of gramicidin, 20 nM, had no effect on the quin2 signal over several minutes, further supporting the view that changes in neither membrane potential or  $Na^+$  gradient have any measurable short-term influence on  $[Ca^{2+}]_i$  in lymphocytes.

Increasing the extracellular  $Ca^{2+}$  concentration,  $[Ca^{2+}]_o$ , from the normal 1 mM had only small effects, as shown in Fig. 10, where an increase to 5 mM caused an apparent increase of  $[Ca^{2+}]_i$  from 125 nM to 200 nM. This relative insensitivity to  $[Ca^{2+}]_o$  was a general finding, though occasionally there were quite marked step changes in signal on addition of the concentrated  $CaCl_2$  stock. These seem to come from cells damaged during the addition and mixing; any dye released into the medium sees a higher  $[Ca^{2+}]_i$  and gives an increased signal. Leakage of dye can then be confirmed by abruptly lowering  $[Ca^{2+}]_o$  by addition of EGTA; a step decline in signal probably represents external dye while a slow decline is probably a genuine reduction in  $[Ca^{2+}]_i$ . Such a test in Fig. 10 reveals slight dye leakage, so that the apparent increase in  $[Ca^{2+}]_i$  probably overestimates the true sensitivity.

Intracellular acidification produced by an increase in  $pCO_2$  is known to increase  $[Ca^{2+}]_i$  in some tissues and decrease it in others (1). However, adding 25 mM  $HCO_3^-$  at pH 7.5 (increasing  $pCO_2$  from near zero to  $\sim 40$  mm Hg) gave no detectable change in quin2 signal, although intracellular pH monitored with a trapped fluorescein derivative showed the expected transient acidification (Rink, Pozzan and Tsien, manuscript in preparation). In one trial half of a batch of cells was kept in bicarbonate-buffered medium during dye loading and  $[Ca^{2+}]_i$  measurement, and the other half was kept in HEPES-buffered medium. The two  $[Ca^{2+}]_i$  readings did not differ measurably.

#### Effects of Mitochondrial Inhibitors on $[Ca^{2+}]_i$

It is quite widely held that mitochondria may constitute an important labile store of  $Ca^{2+}$  and exert a strong influence on  $[Ca^{2+}]_i$ . Mitochondrial inhibitors applied to quin2-loaded lymphocytes gave somewhat variable results, but in all cases the changes in signal were small, corresponding to at most a twofold increase. Fig. 11 shows some records of responses to various inhibitors. In pig lymphocytes, the uncoupling agent FCCP, which is expected to rapidly release mitochondrial stores, produces a transient elevation of  $[Ca^{2+}]_i$ . The elevation was much diminished in  $Ca^{2+}$ -free medium. This may be due to a general depletion of cellular  $Ca^{2+}$ , since  $[Ca^{2+}]_i$  was 85 nM under those conditions. The return to resting  $[Ca^{2+}]_i$  is probably due to extrusion of the released  $Ca^{2+}$  via the plasma membrane  $Ca^{2+}$ -pump, since pig cells, unlike mouse thymocytes, can maintain reasonable levels of cellular ATP by glycolytic activity.

Attempts to detect a similar small mitochondrial store of  $\text{Ca}^{2+}$  in mouse thymocytes were unsuccessful. FCCP produced no measurable immediate rise in  $[\text{Ca}^{2+}]_i$ , suggesting that there is no  $\text{Ca}^{2+}$  quickly releasable from mitochondria. Identical results were obtained with valinomycin, another powerful uncoupler and metabolic poison in lymphocytes (12). The slow rise in  $[\text{Ca}^{2+}]_i$  to some higher steady level seen in the first two traces in Fig. 11 B looks like a new balance between plasma membrane  $\text{Ca}^{2+}$ -influx and efflux. The absence of any effect in  $\text{Ca}^{2+}$ -free medium, shown here with valinomycin, also argues for an effect at the plasma membrane. It seems likely that the profound fall in cellular ATP produced by uncoupling agents may have increased  $\text{Ca}^{2+}$  permeability or depressed the activity of the  $\text{Ca}^{2+}$ -pump. In accord with this explanation, oligomycin, which does not release stored mitochondrial  $\text{Ca}^{2+}$  but produces a moderate fall in ATP levels (20), produces a similar but somewhat slower rise in  $[\text{Ca}^{2+}]_i$ . A method which should reveal  $\text{Ca}^{2+}$  stored in any membrane-bounded organelle, not just mitochondria, is to add a  $\text{Ca}^{2+}$ -ionophore in  $\text{Ca}^{2+}$ -free medium. With pig cells, 10 or 20 nM A23187 quite rapidly produced about a 50% increase in  $[\text{Ca}^{2+}]_i$  which then slowly returned to, or even below, the initial level, whereas with mouse thymocytes there was no detectable change. By contrast, in mouse spleen cells there are intracellular  $\text{Ca}^{2+}$  stores releasable by A23187; see the following paper (19).

## DISCUSSION

These results show that quin2 can be rather easily loaded into intact lymphocytes via uptake and cytoplasmic hydrolysis of the acetoxymethyl ester. Loadings of up to several mM intracellular quin2 have little untoward effect. The in vitro properties of quin2 should make it a suitable fluorescent indicator of  $[\text{Ca}^{2+}]_i$ , and the results show that it does work inside cells. The indicated resting  $[\text{Ca}^{2+}]_i$  is in the range expected from studies on other cells and the known  $\text{Ca}^{2+}$ -sensitivities of cellular processes. The responses to  $\text{Ca}^{2+}$ -ionophores are those anticipated. The available evidence indicates that the dye stays in the cytoplasm and does not report intraorganelle  $[\text{Ca}^{2+}]$ .

Quin2 has distinct advantages as a  $[\text{Ca}^{2+}]_i$  indicator over previous techniques and the unique feature of a nondisruptive loading procedure. Quin2 seems to be as good as, or better than, other detectors at quantifying  $[\text{Ca}^{2+}]_i$  below  $10^{-7}$  M. The 1:1 stoichiometry of complexation makes the quin2 signals much simpler to calibrate than those from photoproteins or bisazo dyes. Fluorescence minimizes problems due to light-scattering by the tissue. Ester-loaded quin2 should make  $[\text{Ca}^{2+}]_i$  measurement possible in many preparations hitherto inaccessible.

However quin2 still has aspects which could be improved: (a) Any indicator dye will be most sensitive around its  $K_d$ . While quin2 is fine for resting levels, it is hard to quantitate levels above 1  $\mu\text{M}$ . Other dyes with  $K_d$  values near 1–5  $\mu\text{M}$  are needed to quantitate stimulated levels of  $[\text{Ca}^{2+}]_i$ . (b) Quin2 loadings of at least several tenths of millimolar are generally needed for stable well-calibrated recordings. This requirement is set not by the absolute sensitivity of a conventional spectrofluorimeter, but rather by the autofluorescence of the cells at the wavelengths used for quin2. Much of the autofluorescence may be due to NADPH. Lower loadings would suffice if the dye worked at longer wavelengths exciting less autofluorescence, and if it had a higher extinction coefficient and quantum efficiency. For comparison a fluorescein derivative used for measuring pH has ample fluorescence at micromolar intracellular loadings (Rink, Tsien and Pozzan, In press. *J. Cell Biol.*), and there is no inherent reason why a calcium indicator could not do as well. (c) Quin2 signals  $[\text{Ca}^{2+}]$  only as a change in fluorescence intensity, so that any loss of quin2 from the illumination zone undermines the end-of-experiment calibration. This would present a problem if one wished to calibrate  $[\text{Ca}^{2+}]_i$  readings from single cells under a microscope. Simple lysis could not be used for calibration since it would rapidly disperse the dye from the field of view. An indicator whose Ca-free and Ca-bound forms both fluoresced but at different wavelengths would be greatly preferable, since the ratio of

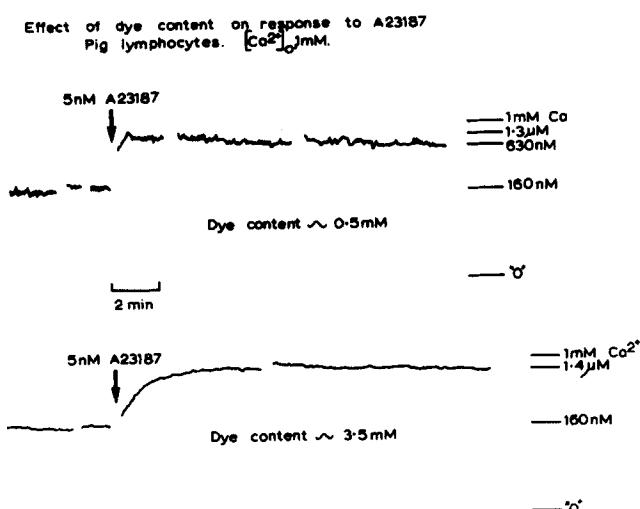


FIGURE 9 Effect of 5 nM A23187 on pig lymphocytes with two different quin2 loadings, suspended in 1 mM  $\text{Ca}^{2+}$  solution.

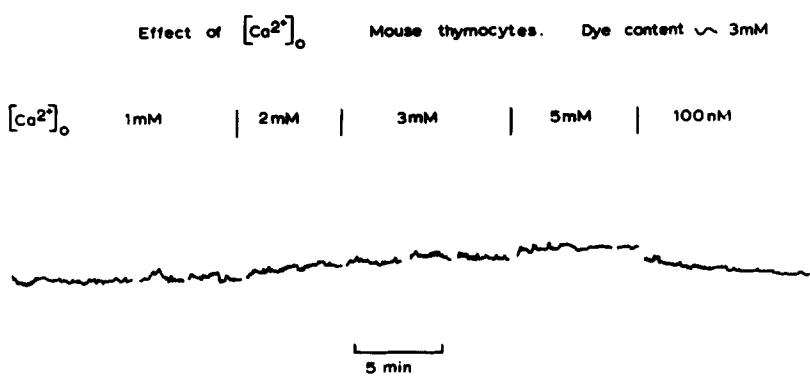


FIGURE 10 Effects of changing external  $[\text{Ca}^{2+}]_o$  on  $[\text{Ca}^{2+}]_i$  in mouse thymocytes. Quin2 loading  $\sim 3$  mM.  $[\text{Ca}^{2+}]_o$  was changed by successive small additions of 0.2 M  $\text{CaCl}_2$ , then finally by addition of EGTA with Tris to maintain external pH. The gaps in the trace indicate additions and/or stirring with a pasteur pipette.

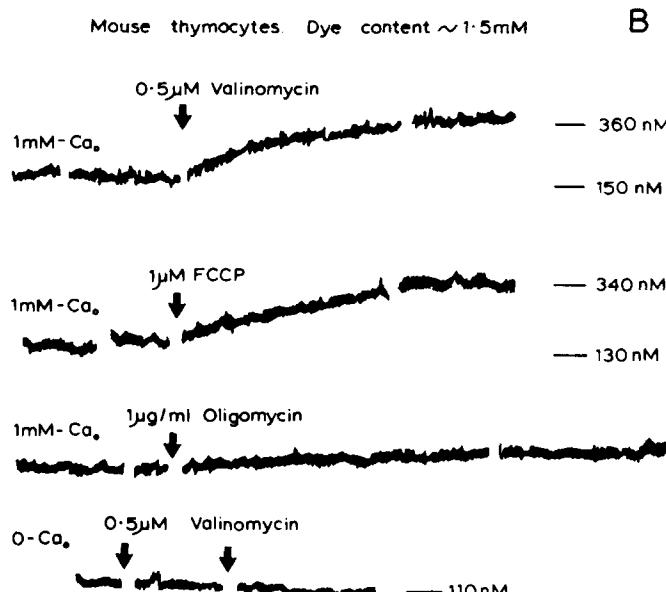
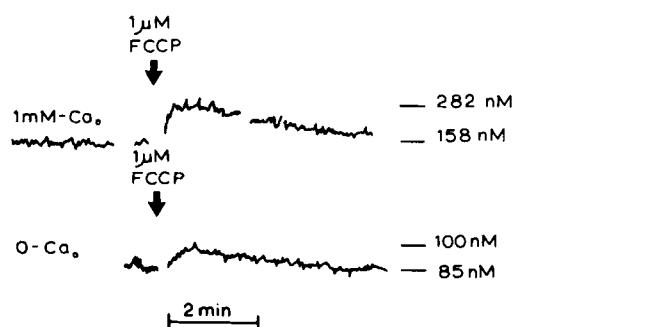


FIGURE 11 Effects of mitochondrial inhibitors. (A) Pig lymphocytes, quin2 loading ~0.3 mM. (B) Mouse thymocytes, quin2 loading ~1.5 mM. O-Ca denotes solution with no added  $\text{Ca}^{2+}$ . The traces in B appear noisier because the amplifier damping was set to a lower value than usual.

fluorescences at the two wavelengths would signal  $[\text{Ca}^{2+}]_i$  independently of dye concentration, making unnecessary any final lysis step. Judicious molecular design and some luck should allow better dyes to be synthesized. It may also be possible to replace the acetoxyethyl groups in the ester with alternatives allowing faster loading and releasing products of lesser potential toxicity.

Meanwhile, quin2/AM has allowed us to obtain the first measurements of  $[\text{Ca}^{2+}]_i$  in lymphocytes. Because millimolar contents are needed for decent signals, the buffering effect of the intracellular chelator had to be considered. The experimental results show that in the steady state, additional  $\text{Ca}^{2+}$ -buffering has no effect on  $[\text{Ca}^{2+}]_i$ . This is just what is expected, as previously discussed (23); steady-state  $[\text{Ca}^{2+}]_i$  is set exclusively by pump/leak balance at the plasma membrane, while the total Ca content of the cells is set by the amount of  $\text{Ca}^{2+}$  sequestered or held in buffers at that  $[\text{Ca}^{2+}]_i$ . Adding chelator should (and did) increase only Ca content, not  $[\text{Ca}^{2+}]_i$ . Loading cells with quin2 in the absence of external  $\text{Ca}^{2+}$  prevented the normal maintenance of  $[\text{Ca}^{2+}]_i$  by influx from the medium. Quin2 then stripped  $\text{Ca}^{2+}$  from intracellular sites and markedly lowered  $[\text{Ca}^{2+}]_i$ , as shown in Fig. 7.

Another interesting feature of Fig. 7 is the rapid return to normal or supranormal  $[\text{Ca}^{2+}]_i$  on readmission of external  $\text{Ca}^{2+}$ . This contrasted with the small effects of removing and readmitting external  $\text{Ca}^{2+}$  when  $[\text{Ca}^{2+}]_i$  started from normal. This suggests that low  $[\text{Ca}^{2+}]_i$  may greatly increase  $\text{Ca}^{2+}$ -permeabil-

ity which is restored to near normal values as  $[\text{Ca}^{2+}]_i$  reaches its normal resting level. It is even conceivable that part of the  $\text{Ca}^{2+}$  influx with very low  $[\text{Ca}^{2+}]_i$  goes by reversal of the  $\text{Ca}^{2+}$ -pump (6).

The presence of intracellular  $\text{Ca}^{2+}$ -chelators does of course buffer  $\text{Ca}^{2+}$ -transients. If the perturbation is a maintained increase in  $\text{Ca}^{2+}$ -influx, as is presumably the case with  $\text{Ca}^{2+}$ -ionophores and apparently with mitogenic plant lectins (31), then the intracellular chelator should slow the  $[\text{Ca}^{2+}]_i$  rise, as seen in Fig. 9, but not influence the new steady level. If however there is a pulse input of  $\text{Ca}^{2+}$ , either via a short-lived increase in plasma membrane  $\text{Ca}^{2+}$ -permeability, or by release from limited internal stores, then intracellular chelator will slow and truncate the subsequent  $[\text{Ca}^{2+}]_i$  spike. The following paper (19) discusses the effect of quin2 loading on the response to  $\text{Ca}^{2+}$ -release from internal stores in B cells. It is unfortunate that quin2 cannot be used at concentrations low enough to be sure of not blunting such transients, but this awaits improvement of the dye properties. At least however the actual  $[\text{Ca}^{2+}]_i$  changes in the chelator-loaded cells can be monitored, and the relation between the observed  $[\text{Ca}^{2+}]_i$  changes and the cell function gives valid information about the  $\text{Ca}^{2+}$ -sensitivity of that function. Often it is positively useful to load a known amount of  $\text{Ca}^{2+}$  chelator into a cell specifically to examine the effects of buffering. A chelator which simultaneously detects  $[\text{Ca}^{2+}]_i$  is much better than one that does not.

The present results have given some information on the

mechanism of  $\text{Ca}^{2+}$  homeostasis in lymphocytes, though much further work is needed, particularly studies combining monitoring of  $[\text{Ca}^{2+}]_i$  with  $^{45}\text{Ca}$  flux measurements. The absence of any effects of  $\text{Na}^+$  substitution argues against the presence of a  $\text{Na}^+:\text{Ca}^{2+}$  exchange process of the type reported in invertebrate nerve and vertebrate heart. We assume that  $\text{Ca}^{2+}$ -extrusion in lymphocytes is achieved by an ATP-fueled  $\text{Ca}^{2+}$  pump. A  $\text{Ca}^{2+}$ -sensitive ATPase of appropriate properties has recently been partially purified from lymphocyte plasma membranes (8). The lack of response to depolarization or hyperpolarization likewise argues against any voltage-dependent  $\text{Ca}^{2+}$ -channels, although a rapidly inactivating channel might give only a minute  $\text{Ca}^{2+}$ -influx with the slow maintained depolarizations imposed by  $\text{K}^+$ -rich solutions or gramicidin.

The small response to alterations of  $[\text{Ca}^{2+}]_i$  suggests either that at resting  $[\text{Ca}^{2+}]_i$ ,  $\text{Ca}^{2+}$ -permeability is very low, or that the pump has a steep activation curve and gives large alterations in efflux to stabilize  $[\text{Ca}^{2+}]_i$  over a small range. These questions should be resolvable by combined quin2 and  $^{45}\text{Ca}$  studies. However the existence of substantial Ca tracer exchange in resting lymphocytes is inconclusive since much could be tightly coupled Ca:Ca exchange which could make no contribution to net movements (22).

The lack of effect of internal acidification with  $\text{CO}_2$  presumably shows that the relevant  $\text{Ca}^{2+}$ -binding sites do not have proton dissociations near pH 7. It was important to see that basal  $[\text{Ca}^{2+}]_i$  was the same in HEPES-buffered and bicarbonate-buffered medium, since most of our experiments (and those of many others) are done in  $\text{CO}_2$ -free media, whereas living pigs and mice use bicarbonate-buffered fluids. The effect on  $[\text{Ca}^{2+}]_i$  of raising  $\text{pCO}_2$  has been examined in several cell types. Small falls or elevations have been reported in different tissues. (For a brief discussion see reference 1).

There is still contention over the question of whether mitochondria constitute an important Ca store in healthy cells, and whether  $[\text{Ca}^{2+}]_i$  regulates or is regulated by mitochondria (5, 14). Our data suggest that little, if any, of the  $\text{Ca}^{2+}$  in mouse lymphocytes is sequestered in mitochondria, and that mitochondrial poisons act mostly through ATP depletion and alteration of plasma membrane function. A small amount of Ca does seem to be releasable from pig lymphocyte mitochondria, judging from data such as these in Fig. 11A, not more than 10% of the total cell Ca. On the other hand, there may be genuine differences between cell types. Other cells in which mitochondrial poisons have minimal effects on basal  $[\text{Ca}^{2+}]_i$  include freshly dissected squid axons (3), snail ganglion cells (28), mouse spleen lymphocytes (19), and human platelets (S. Smith and T. J. Rink, unpublished observations). The lack of  $\text{Ca}^{2+}$  in the mitochondria of such cells at rest correlates with the inability of mitochondria to accumulate  $\text{Ca}^{2+}$  from solutions containing 100–200 nM  $[\text{Ca}^{2+}]$  and intracellular pH and free  $\text{Mg}^{2+}$  (16).

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