[14] Measurement of Cytosolic Free Ca^{2+} with Quin2

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Introduction: General Advantages and Disadvantages

The fluorescent Ca^{2+} indicator dye, quin2 (Fig. 1), is the basis of one of the most popular methods for measuring cytosolic free Ca^{2+} [$(Ca^{2+}]_i$), especially in suspensions of mammalian cells. Briefer reviews of the use of quin2 have appeared previously.[1-3] Its widespread use probably stems from the following advantages that it has over other methods for measuring cytosolic free Ca^{2+}. (1) Quin2 can be loaded into the cytosol of millions of cells simultaneously, without any micromanipulations or any disruption of the plasma membrane.[4] Cells are simply incubated with quin2/AM, the tetra(acetoxymethyl) ester of quin2 (Fig. 1). This uncharged, hydrophobic derivative of quin2 diffuses freely across membranes but is gradually hydrolyzed by cytoplasmic esterases, eventually regenerating quin2 itself, which remains trapped in the cytosol as a relatively impermeant tetranion. All other methods, particularly those using ion-selective microelectrodes, aequorin, and arsenazo III, require breaching of the plasma membrane to enable the sensor molecules to contact the cytoplasm. Because of the enormous electrochemical gradient of Ca^{2+} across the plasma membrane, any threat to membrane integrity is bad for the cells and for the measurement. The nondisruptive method of loading quin2 via permeant hydrolyzable esters permits its use on cells too small or delicate to tolerate puncture or lysis and resealing. Also, the ability to measure an average $[Ca^{2+}]_i$ from large numbers of cells can be very helpful in establishing correlations of $[Ca^{2+}]_i$ with cell responses that must also be measured from whole populations, for example secretion, proliferation, or metabolism of cylic nucleotides, lipids, or proteins. (2) Quin2 is usually monitored with a conventional cuvette spectrofluorimeter. Both the dye and the instrumentation are widely available by purchase or loan from colleagues, whereas other methods tend to require much specialized or custom equipment. (3) Quin2 readings are usually easily calibrated at typical resting levels of $[Ca^{2+}]_i$ ($\sim 10^{-7}$ *M*) and below (to 10^{-8} *M*), whereas all other techniques do their best at detecting activated levels ($\sim 10^{-6}$ *M*)

[1] R. Y. Tsien, *Annu. Rev. Biophys. Bioeng.* **12,** 94 (1983).
[2] R. Y. Tsien, T. Pozzan, and T. J. Rink, *Trends Biochem. Sci.* **9,** 263 (1984).
[3] T. J. Rink and T. Pozzan, *Cell Calcium* **6,** 133 (1985).
[4] R. Y. Tsien, T. Pozzan, and T. J. Rink, *J. Cell. Biol.* **94,** 325 (1982).

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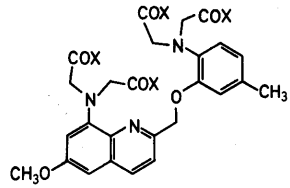

quin2 : X= O^-
quin2/AM : X = OCH_2OCOCH_3

FIG. 1. Structure of Ca^{2+} indicator quin2 and its acetoxymethyl ester quin2/AM. Reproduced from Ref. 4, *The Journal of Cell Biology* (1982) 94:325–334, by copyright permission of the Rockefeller University Press.

and are nearly at their detection limits at resting levels. (4) Quin2 avoids many of the drawbacks particular to other $[Ca^{2+}]_i$ measurement techniques, for example, the irreversible destruction of aequorin by Ca^{2+}, the poor Mg^{2+} rejection and messy Ca^{2+} stoichiometry of arsenazo III and antipyrylazo III, the slow response time and need for voltage referencing of ion-selective electrodes, and the null point method's need to destroy large numbers of cells in low extracellular $[Ca^{2+}]$ for each data point.[1,5]

Of course, quin2 suffers from significant disadvantages, most of which were described in the first full articles[4,6] on the technique and which have been repeatedly rediscovered by subsequent authors. (1) The fluorescence of quin2 is not exceptionally bright, so that intracellular loading of several hundred micromolar is usually required for the quin2 signal to dominate cellular autofluorescence. This much quin2 can partially buffer $[Ca^{2+}]_i$ transients, though it generally does not alter $[Ca^{2+}]_i$ levels set by long-term homeostatic processes. This buffering can actually be extremely useful when examining whether $[Ca^{2+}]_i$ is really essential for a given cell response such as exocytosis or phosphoinositide breakdown.[6–9] $[Ca^{2+}]_i$ rises can often be suppressed to controllable extents by judicious use of varying quin2 loading, extracellular Ca^{2+} deprivation, and ionophore pretreatments. Comparisons of damped $[Ca^{2+}]_i$ signals and cell responses from the very same populations are a powerful tool to show whether $[Ca^{2+}]_i$ rises are really important, often revealing that they are

[5] R. Y. Tsien and T. J. Rink, *in* "Current Methods in Cellular Neurophysiology" (J. L. Barker and J. McKelvy, eds), pp. 249–312. Wiley, New York, 1983.
[6] T. Pozzan, P. Arslan, R. Y. Tsien, and T. J. Rink, *J. Cell Biol.* **94,** 335 (1982).
[7] F. DiVirgilio, D. P. Lew, and T. Pozzan, *Nature (London)* **310,** 691 (1984).
[8] T. J. Rink, S. W. Smith, and R. Y. Tsien, *FEBS Lett.* **148,** 21 (1982).
[9] L. M. Vicentini, A. Ambrosini, F. DiVirgilio, T. Pozzan, and J. Meldolesi, *J. Cell Biol.* **100,** 1330 (1985).

not as necessary as previously hypothesized. This seems an important and underused application of quin2, especially because other measurement techniques are much less amenable to variation in the extent of $[Ca^{2+}]_i$ buffering. But when rapid $[Ca^{2+}]_i$ transients are to be measured with minimum perturbation, an indicator working at lower loadings than quin2 would be highly desirable. (2) Obviously, quin2 is inappropriate for tissues that are highly opaque due to pigment or scattering or that are particularly sensitive to near-UV-light. (3) Because quin2 signals Ca^{2+} primarily by its fluorescence at a single set of excitation (339 nm) and emission wavelengths (490–500 nm), its signal becomes more difficult to calibrate in terms of absolute $[Ca^{2+}]_i$ values as one switches from suspensions to monolayers to single cells. A dye that shifted its preferred wavelengths rather than just changing its amplitude of fluorescence would be much easier to calibrate, since a mere change in dye content or instrumental sensitivity would not masquerade as a $[Ca^{2+}]_i$ change.[4,10] (4) Quin2 can be bleached by high illumination levels either in a cuvette or on a microscope.[11] (5) Quin2 does have some sensitivity to Mg^{2+}. Mg^{2+} by itself causes little change in the fluorescence with standard wavelengths of 339 nm excitation, but does act as a competitive inhibitor of Ca^{2+}-binding and associated fluorescence enchancement.[4,12] For a given error in estimating or guessing $[Mg^{2+}]_i$, quin2 at resting $[Ca^{2+}]_i$ levels would be much less affected than arsenazo III or an organophosphate-based ion-selective electrode, similarly or slightly less perturbed than aequorin would be, but more affected than an electrode using neutral carriers.[1,5] (6) A few tumor cell types have enough heavy metals in them to perturb the dye.[13] (7) Not all cells can be loaded by the gentle means of hydrolyzing membrane-permanent esters. Plant, bacterial, and a scattering of invertebrate cells load poorly or not at al,[14,15] whereas most vertebrate cells seem to load, with the possible exception of some but not all types of muscle. (8) In cells that do load by ester hydrolysis, the chemical by-products, formaldehyde and protons,[16] could have harmful side effects. (9) Quin2 is maximally sensitive to Ca^{2+} levels near its Ca^{2+} dissociation constant, $\sim 10^{-7}$ M;

[10] G. Grynkiewicz, M. Poenie, and R. Y. Tsien, *J. Biol. Chem.* **260,** 3440 (1985).
[11] B. A. Kruskal, C. H. Keith, and F. R. Maxfield, *J. Cell Biol.* **99,** 1167 (1984).
[12] R. Y. Tsien, *Biochemistry* **19,** 2396 (1980).
[13] P. Arslan, F. DiVirgilio, M. Beltrame, R. Y. Tsien, and T. Pozzan, *J. Biol. Chem.* **260,** 2719 (1985).
[14] R. J. Cork, *Plant Cell Environ.* **9,** 157 (1986).
[15] J. I. Korenbrot, D. L. Ochs, J. A. Williams, D. L. Miller, and J. E. Brown, *in* "Optical Methods in Cell Physiology" (P. DeWeer and B. M. Salzberg, eds), pp. 347–363. Wiley, New York, 1986.
[16] R. Y. Tsien, *Nature (London)* **290,** 527 (1981).

much higher levels ($>10^{-6}$ *M*) nearly completely saturate the dye and cannot be distinguished from each other.[4] Moreover, if the dye resides in several compartments with different $[Ca^{2+}]_i$, a measurement that ignores the compartmentation will underestimate the deviations from the mean.[6]

Many of the above problems can be alleviated by more recently introduced relatives of quin2. These newer fluorescent indicators,[10] especially fura-2 and indo-1, are much brighter in fluorescence than quin2. Because so much less dye needs to be introduced, both the Ca^{2+} buffering and the potential for toxicity from hydrolysis products are greatly reduced. The new dyes shift wavelengths not just intensity upon binding Ca^{2+}, so calibration of signals from monolayers and single cells is greatly eased. Fura-2 is much more resistant to photobleaching[17] than quin2 and has better selectivity for Ca^{2+} over Mg^{2+} and heavy metals. Because fura-2 and indo-1 are sensitive enough to give large calibratable signals from single cells or regions of cells,[18–20] they allow direct measurement of compartmentalization and heterogeneity. For those reasons, we expect quin2 eventually to be replaced in most applications by fura-2, indo-1, or even newer relatives under development. This article, written in 1985–1986, concentrates mainly on quin2, the longer established indicator, whereas the newer dyes are discussed in more recent reviews.[21–25] Many of the protocols and cautions described below are similar for all the dyes. Moreover, to take full advantage of the advanced properties of the new dyes does require more elaborate equipment, so many newcomers to the field of $[Ca^{2+}]_i$ measurement will still want to get started using quin2 in a conventional cuvette fluorometer.

Dye Loading

The protocol for loading quin2 into intact cells was originally described in lymphocytes[4,26] and has been successfully applied with minor modifications to a variety of cell types, mostly mammalian. Basically,

[17] D. A. Williams, K. E. Fogarty, R. Y. Tsien, and F. S. Fay, *J. Gen. Physiol.* **86,** 37a (1985).
[18] R. Y. Tsien, T. J. Rink, and M. Poenie, *Cell Calcium* **6,** 145 (1985).
[19] D. A. Williams, K. E. Fogarty, R. Y. Tsien, and F. S. Fay, *Nature (London)* **318,** 558 (1985).
[20] M. Poenie, J. Alderton, R. Steinhardt, and R. Y. Tsien. *Science* **233,** 886 (1986).
[21] R. Y. Tsien and M. Poenie, *Trends Biochem. Sci.* **11,** 450 (1986).
[22] P. H. Cobbold and T. J. Rink, *Biochem. J.* **248,** 313 (1987).
[23] R. Y. Tsien, *Trends Neurosci.* **11,** 419–424.
[24] R. Y. Tsien, *Methods Cell Biol.* **30,** 127 (1989).
[25] R. Y. Tsien, *Annu. Rev. Neurosci.* **12,** in press.
[26] R. Y. Tsien, T. Pozzan, and T. J. Rink, *Nature (London)* **295,** 68 (1982).

quin2/AM from a stock solution in dimethyl sulfoxide (DMSO) is added to cell suspensions or monolayers. After some time the cells are washed, resuspended in fresh medium, and recording of fluorescence is initiated. Major variables in this protocol are (1) source of quin2/AM, (2) quin2/AM concentration and cell density, (3) incubation time and temperature, and (4) addition of serum or albumin.

Source

Quin2 and quin2/AM are now available from several commercial sources: Lancaster Synthesis, Amersham, Calbiochem-Behring, Sigma, Aldrich, and Dojindo Laboratories. The first five firms have branches both in the United States and Europe, while Dojindo is at 2861 Kengun-machi, Kumamotoshi (862), Japan. [It is our understanding that Amersham nonradioactive material and Sigma are merely repackagings of the Dojindo material.] We have not seen any consistent difference in quality between products from different companies, though there have been several isolated incidents of bad batches. Quin2 free acid is a free-flowing powder; its color is white or yellow, depending on purity and degree of protonation. Quin2/AM is a yellow gum or resin that should be fairly hard at refrigerator temperature but usually becomes sticky at room temperature. Routinely we keep both compounds desiccated over silica gel at −20° for long-term storage, since the protonated free acid can gradually decarboxylate and the AM ester can hydrolyze and/or oxidize. (The only derivative known to be crystallizable and indefinitely stable at room temperature is the tetraethyl ester, which is still the best source for critical studies on quin2 tetranion.)

Tests for the quality of quin2, in order of increasing laboriousness and stringency, are determination of extinction coefficient ($\varepsilon = 5000\ M^{-1}\ cm^{-1}$ at 354 nm in Ca^{2+}- and Mg^{2+}-free medium above pH 7.0 with millimolar EDTA or EGTA to chelate contaminating Ca^{2+}), determination of fluorescence excitation spectra before and after addition of excess Ca^{2+}, and determination of K_D for Ca^{2+}. Measurement of the extinction coefficient tests for gross inert contamination. Fluorescence excitation spectra at micromolar dye concentrations in high Ca^{2+} should show a peak near 339 nm excitation, whose amplitude above blank background is five- to seven-fold greater than the amplitude above background of the same dye concentration at the same wavelength in zero Ca^{2+} with EDTA or EGTA. Examples of such excitation spectra at saturating and zero Ca^{2+} are shown in Fig. 2 as the two extremes of the series of spectra. If adequate signal amplitude or stability cannot be attained, either the dye or fluorometer could be deficient; if an adequate enhancement due to Ca^{2+} cannot be

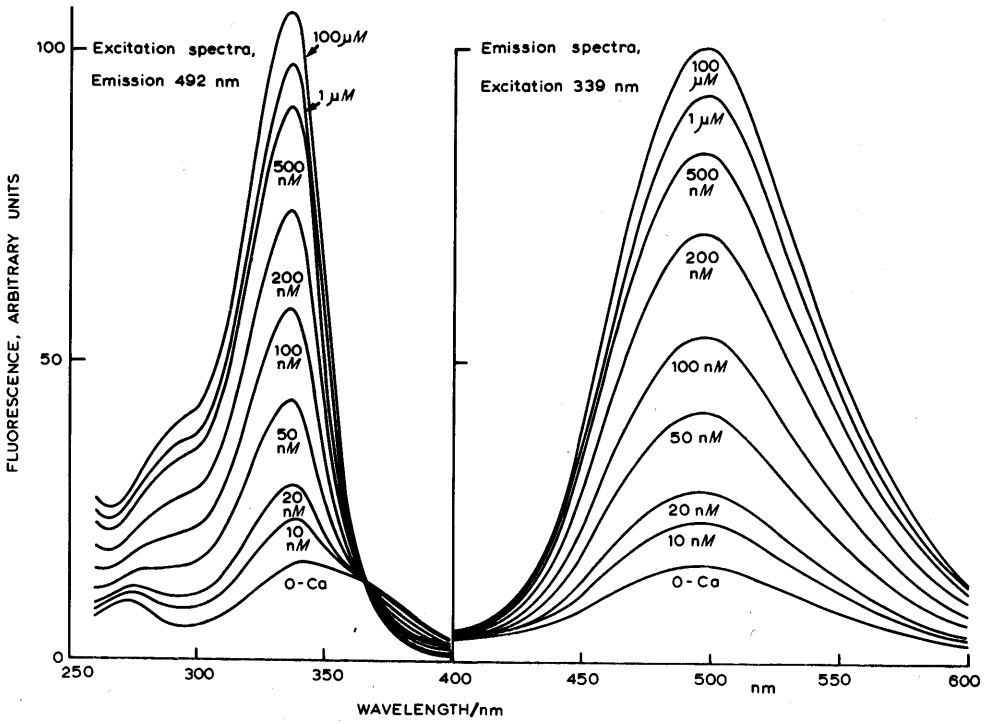

FIG. 2. Excitation and emission spectra of 20 μM quin2, with varying [Ca^{2+}] as shown against an ionic background of 120–135 mM K^+, 20 mM Na^+, 1 mM free Mg^{2+}, and pH 7.05 at 37°. Reproduced from Ref. 4, *The Journal of Cell Biology* (1982) 94:325–334, by copyright permission of the Rockefeller University Press. Further experimental details may be found in that article.

found with a small excitation bandpass (<5 nm), then bad dye should be suspected. Measurement of the K_d for Ca^{2+} using intermediate buffered Ca^{2+} values (as in Fig. 2 and the section Calibration Procedure: Quin2 Affinity for Ca^{2+}, below) tests that all the molecules have the same high-affinity binding site; if some molecules lack one or more carboxylates, they would have lower affinities, yet would show the same spectra at zero and very high Ca^{2+}.

Unlike quin2, the ester quin2/AM is much more soluble in organic solvents than in water and has no Ca^{2+}-binding properties. When one has access to a cell system known to hydrolyze quin2/AM, the latter is usually tested merely by its ability to load quin2 into the cells. If other tests are required, thin-layer chromatography on silica gel is convenient. A solution of the dye in chloroform or dichloromethane is spotted on a

Merck 5554 silica gel TLC plate, 10 cm long, developed with hexane–ethyl acetate (1 : 1, v/v), and visualized under 254 or 365 nm illumination. Quin2/AM runs with R_f = 0.27 in this system. Unfortunately DMSO perturbs this R_f. Minor fluorescent contaminants are often present and usually tolerable. Yet another test of quin2/AM is to hydrolyze the dye chemically. Thus a small aliquot of a 1–10 m*M*, quin2/AM solution in DMSO is mixed with an equal volume of 0.1–1 M aqueous NaOH or KOH. After waiting 5–10 min, the mixture should be a stock solution of quin2 tetranion which can be tested as described above. Of course, base hydrolysis does not test the completeness or correctness of esterification.

Quin2/AM stock solutions of 1–10 m*M* concentration are usually made in DMSO because of its ready miscibility with water, biological inertness in most systems, nonvolatility, and ease of microliter pipetting. The DMSO should be anhydrous, and stock solutions of quin2/AM should be kept frozen and desiccated. Because the melting point of DMSO (18°) is sharply depressed by water content, moisture pickup is easily seen as rapid melting of the solution upon removal from the refrigerator.

Quin2/AM Concentration and Cell Density

Typically, the 1–10 m*M* stock solution of quin2/AM in DMSO is diluted 100- to 1000-fold into the cell suspension while stirring or shaking. Depending on cell type and other variables discussed below, typically 5–40% of the total quin2/AM added will become trapped as quin2 inside the cells. Since the cells usually occupy 0.1–1% of the volume of the suspension, intracellular quin2 concentrations reach 0.1 to 5 m*M*, substantially higher than the 1–100 μM initial concentration of quin2/AM in the suspension.

Quin2/AM before hydrolysis is actually quite hydrophobic and poorly soluble in simple salines. Light-scattering measurements (T. Pozzan, unpublished observations) suggest that above a few micromolar, the ester forms colloidal suspensions or precipitates. Therefore loading efficiency, or the percentage of quin2/AM usefully trapped, is improved by squirting the dye solution directly into a dense and stirred suspension of cells.[4] High concentrations of dye ester without sufficient cells or protective proteins simply precipitate dye wastefully, some of which may then be endocytosed and generate undesired intracellular background fluorescence.

Though loading efficiency is improved by high initial cytocrits, nearer 1% than 0.1%, the cells soon acidify the medium. Also, the unavoidable death of a fraction of the cells may release enzymes that can harm other

cells and hydrolyze extracellular quin2/AM. Therefore, dense suspensions should be diluted up to 10-fold a few minutes after the ester is added, since that interval seems sufficient to complete the initial association of the ester with cells, though not the full hydrolysis.

Incubation Time and Temperature

Quin2/AM is transformed to quin2 tetranion by four separate hydrolysis steps, one for each acetoxymethyl group. It is essential that this process be completed before measurements are begun. Any dye molecule with incompletely hydrolyzed ester groups contributes fluorescence yet is crippled in its Ca^{2+}-binding capability, leading to an underestimate of $[Ca^{2+}]_i$. If hydrolysis continues during the observation of fluorescence, the gradual increase in Ca^{2+} affinity will cause a rising curve that will simulate a progressive rise in $[Ca^{2+}]_i$. To aid completion of hydrolysis, it is important that the free concentration of the starting tetraester be reduced practically to zero well before observation begins. Otherwise the cells never get a chance to clear the metabolic pipeline of partially hydrolyzed intermediates. In dense suspensions of cells with high esterase activity, as in our initial studies with blood-forming cells, this exhaustion of quin2/AM is automatic as the cells take up and hydrolyze the ester. In some cases precipitation can help reduce the quin2/AM concentration. But if cell density or esterase activity is low, the experimenter should help by washing the cells with ester-free media after the initial contact period of a few minutes to 30 min. The cells should then be incubated in dye-free media until complete hydrolysis of the trapped intermediates which still bear one, two, or three ester groups. A quick and crude indication[4] of hydrolysis is a shift in the emission peak from 430 to 490–500 nm, matching that of the dye tetranion with or without Ca^{2+}. However, this widely used test is not fully rigorous, because an acetoxymethyl group lingering on the benzene ring portion of quin2 would not affect the fluorescence properties of the quinoline portion. The most rigorous test for full hydrolysis is to release the dye by lysing the cells, then titrate the dye with known buffered Ca^{2+} levels and quantitatively verify its responsiveness.[4] Obviously, such titration does not have to be done routinely, but should be checked during the establishment of a loading protocol for the particular tissue being studied.

We and most others have allowed about 1 hr incubation for full hydrolysis of the four ester groups. Some cells apparently hydrolyze the ester faster (for example, hepatocytes load well in just 2.5 min[27]), some more

[27] A. Binet, B. Berthon, and M. Claret, *Biochem. J.* **228,** 565 (1985).

slowly (i.e., some cultured cell lines), some not at all. In most cases the loading temperature has been 37°. There are a few reports, mainly in monolayers, where the loading was performed at room temperature. One of us (TP) has observed in brain synaptosomes, neutrophils, and the insulinoma cell line RINm5F, that loading at temperatures below 37° seems to result in trapping of quin2/AM in cell compartments different from the cytoplasm. In these cases, the emission spectrum retains a significant shoulder at 430 nm, indicating noncomplete hydrolysis. This shoulder surprisingly cannot be abolished either by prolonging the incubation time or by rewarming the cell to 37°. Another sign of such compartmentation is that digitonin treatment, which normally releases most cellular quin2, releases a smaller fraction (70–80%) of the dye; the residue requires Triton X-100 for release.

Albumin and Serum during Loading

Inclusion of either bovine serum albumin (0.5–1%) or fetal calf serum (1–5%) in the loading medium generally tends to increase the loading efficiency, probably because the proteins reduce the precipitation of quin2/AM and act as buffers for it. Moreover they increase the viability of most cell types. Very high concentrations of protein, as in platelets in their own plasma, may somewhat reduce loading efficiency by excessive competition for the dye, but the minimization of cell manipulation more than outweighs the waste of dye (T. J. Rink, personal communication). BSA and FCS can have some fluorescence of their own, and definitely reduce the potency of calcium ionophores,[28] so they are omitted or minimized in the final medium during actual measurements.

Recording the Fluorescence

Once dye loading is complete, the cells are typically washed once or twice to remove leaked or excess dye and hydrolysis by-products. Resuspension for fluorescence measurements should be in a medium containing as little as possible of such absorbing and fluorescing substances as phenol red, fetal calf serum, tryptophan, riboflavin, and pyridoxine. Cells that are not to be used immediately are resuspended in whatever medium at whatever temperature will best preserve their viability and dye content; often, room temperature is suitable. If the cells have been kept for a considerable time, a final wash just before use is helpful again to remove leaked dye and fluorescent additives from the medium.

[28] L. O. Simonsen, *J. Physiol. (London)* **318,** 34P (1981).

A wide variety of commercially available spectrofluorometers have been used successfully with quin2 in cell suspensions in cuvettes, so instrumental requirements are not stringent. However, it is important to scan the excitation and emission spectra to verify the quality and rate of dye loading. Therefore, filter fluorometers working at fixed wavelengths are not adequate unless the user is very experienced with quin2 and the tissue particularly tractable. Probably the most important characteristics of a good spectrofluorometer for quin2 studies are stability of output signal over the time scale of an experiment, and ability of the sample compartment to maintain whatever thermostatting, gassing, and/or stirring are needed for the particular cell type. Instrumental stability is easily tested by placing an unstirred solution of quin2 free acid in buffer of known high or low $[Ca^{2+}]$ and looking for an unchanging signal amplitude. Assuming temperature equilibrium has been reached, any progressive decline in signal suggests a failing instrument, or more likely, dye bleaching due to excessive illumination intensity. Bleaching is confirmed if the signal stops declining or even partially recovers either upon stirring the solution or temporarily blocking the illumination. Bleaching can be minimized by reducing the monochromator slit settings or inserting filters, screens, or apertures in the excitation beam. Most commercial instruments have enough sensitivity if in good repair to reach a stable and sufficiently quiet signal with excitation intensity low enough to avoid significant bleaching over the 0.5–1 hr time scale. Very high sensitivity is not required, since intrinsic autofluorescence from the cells is usually readily detectable at moderate gain and one generally has to load enough dye (usually several hundred micromolar) to overcome autofluorescence by a factor of two or more.

Thermostatting and gassing of the cuvette are obvious requirements for many cell preparations. Continual stirring is often unnecessary with small and well-dispersed cells, which settle sufficiently slowly so that occasional manual stirring with a Pasteur pipette is adequate. However, larger cell or clumps of cells need continual stirring. Magnetic drive is better than an overhead paddle because addition of solutions is unobstructed.

Traditional 1 cm square quartz cuvettes are expensive, fragile, and very wasteful of solution volume and cells. We find that cheap round shell vials or test tubes made of borosilicate glass are usually adequate[3] except for the most precise physicochemical measurements. The glass walls are thin enough to absorb a negligible amount (a few percent) of the incident UV beam. The cylindrical shape reduces wasted volume in the corners, improves stirring, yet seems not to cause optical problems. Depending on precise dimensions, sample volumes one-fourth to one-half that of 1 cm^2

cuvettes are sufficient, reducing usage of tissue and dyes correspondingly. The vials are rugged and cheap enough to be centrifugable, a help when assessing leakage. The main disadvantage of the vials is the need to build a metal adapter to center the vial in a conventional square sample holder.

Quin2 has also been used in cell monolayers grown on glass coverslips.[29] The coverslip is clamped by spring tension in a holder that fits in a square cuvette and maintains the coverslip at a near-diagonal orientation to the fluorimeter beams. Coverslips obviously are advantageous for anchorage-dependent cells; also they permit complete *in situ* replacement of the solution, whereas one can add to but not subtract from a cuvette of suspended cells. However, stability of the fluorescence amplitude is more difficult with coverslips, since minute motions of the coverslips, bleaching in the fixed zone of illumination, and detachment of cells can cause severe artifacts. Some success has been reported[30] in growing anchorage-dependent cells on microcarrier beads and suspending them in cuvettes, but this variant has not achieved popularity, perhaps because the considerable light scattering from the beads tends to contaminate or obscure the fluorescence signal, and the vigorous stirring needed to keep the beads in suspension tends to scrape the cells off.

Calibration Procedure

The fluorescence signal recorded from loaded cells reflects not only some sort of mean cytosolic free Ca^{2+} concentrations, $[Ca^{2+}]_i$, but also many other factors. These necessarily include the effective affinity of quin2 for Ca^{2+}, the concentration of quin2 within the cells, the number of cells in the effective sample volume, and the sensitivity of the fluorometer. Furthermore, if any significant quantity of dye is present in noncytoplasmic compartments such as extracellular space or intracellular organelles, such dye will contaminate the overall fluorescence signal. The purpose of calibration is to give a quantitative estimate of $[Ca^{2+}]_i$ that is as independent of the other variables as possible. We first discuss the determination of the effective affinity of quin2 for Ca^{2+}, then the several methods available for handling uncertainties in dye content, location, and instrumental sensitivity. Operationally, calibration is a much simpler procedure than one might think from the length of the following discussion, which attempts to summarize the major alternative procedures and their implicit assumptions.

[29] W. H. Moolenaar, L. G. J. Tertoolen, and S. W. deLaat, *J. Biol. Chem.* **259,** 8066 (1984).
[30] J. D. R. Morris, J. C. Metcalfe, G. A. Smith, T. R. Hesketh, and M. V. Taylor, *FEBS Lett.* **169,** 189 (1984).

Quin2 Affinity for Ca^{2+}

Ideally, one would determine the dissociation constant (K_D) of the quin2-Ca^{2+} complex in actual cytoplasm. However, this would require a highly accurate method for setting or reading $[Ca^{2+}]_i$ independently of quin2 itself. Such methods are rarely available in the cell systems to which quin2 is most suited. Instead one has to determine the K_D *in vitro* in buffers believed to mimic the main ionic constituents of cytoplasm, as described below. Are these *in vitro* K_D values applicable to cytoplasm? Probably yes, because quin2 estimates of $[Ca^{2+}]_i$ are rather close to those obtained by quite independent techniques. For example, estimates of resting $[Ca^{2+}]$ in toad stomach smooth muscle[19] cells are 129 ± 4 n*M*, 137 ± 13 n*M*, and 148 ± 20 n*M* by quin 2, fura-2, and ion-sensitive microelectrodes, respectively. In hepatocytes, quin2 values of 200 n*M*[31] and 160 n*M*[27,32] compare well with 211 ± 9 n*M* and 190 ± 10 n*M* using aequorin[33] and null points,[34] respectively.

The *in vitro* affinity of quin 2 for Ca^{2+} is determined by setting free Ca^{2+} to a series of accurately known values, measuring the dye fluorescence at each value, and fitting the data to the theoretical equation for one-to-one binding.

$$F = F_{min} + (F_{max} - F_{min}) \left(\frac{[Ca^{2+}]}{[Ca^{2+}] + K_D} \right)$$

Examples of the fluorescence data and the fit are shown in Figs. 2 and 3. This titration was done against a constant background of 120 m*M* K^+, 20 m*M* Na^+, 1 m*M* free Mg^{2+}, pH 7.05, at 37°, an ionic milieu believed to correspond in major cation composition to lymphocyte cytoplasm.[4,35] Nearly all other investigators using quin2 have continued to use the K_D, 115 n*M*, obtained from Fig. 3. However, the effective K_D is definitely sensitive to ionic strength, temperature, free Mg^{2+}, and pH values if they fall below about 6.5–6.8. In addition, any deterioration of the dye sample or incompleteness of ester hydrolysis will generate chemical heterogeneity that can jeopardize the quantitative calibration. Therefore it is highly desirable, though admittedly tedious, to generate the equivalent of Figs. 2 and 3 on lysates of loaded cells from each new tissue system being studied, with the correct temperatures and ionic background for that system.

[31] R. Charest, P. F. Blackmore, B. Berthon, and J. H. Exton, *J. Biol. Chem.* **258,** 8769 (1983).

[32] A. P. Thomas, J. Alexander, and J. R. Williamson, *J. Biol. Chem.* **259,** 5574 (1984).

[33] N. M. Woods, K. S. R. Cuthbertson, and P. H. Cobbold, *Nature (London)* **319,** 600 (1986).

[34] E. Murphy, K. Coll, T. L. Rich, and J. R. Williamson, *J. Biol. Chem.* **255,** 6600 (1980).

[35] T. J. Rink, R. Y. Tsien, and T. Pozzan, *J. Cell Biol.* **95,** 189 (1982).

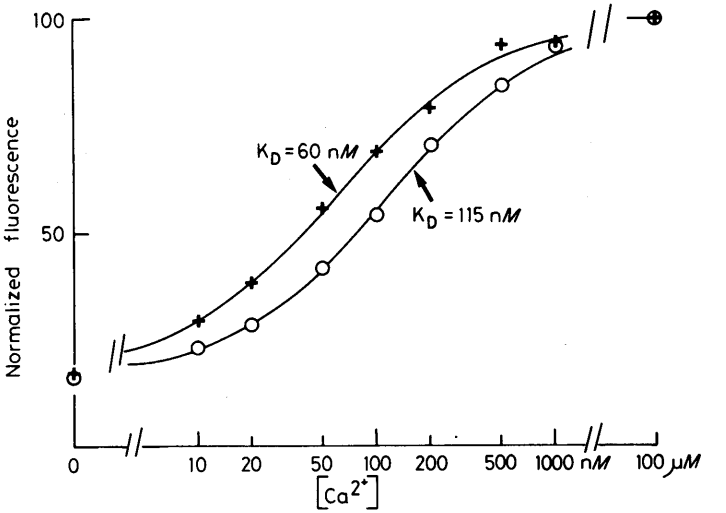

FIG. 3. Quin2 calibration curves in 120–135 m*M* K^+, 20 m*M* Na^+, pH 7.05, at 37°, and two different $[Mg^{2+}]$ values. Normalized quin2 fluorescence (excitation 339 nm; emission 492 nm) plotted against free $[Ca^{2+}]$ at 0 Mg (crosses) and 1 m*M* free Mg^{2+} (circles). The data points are fitted with curves corresponding to one-to-one stoichiometry and the indicated apparent dissociation constants. Reproduced from Ref. 4, *The Journal of Cell Biology* (1982) 94:325–334, by copyright permission of the Rockefeller University Press.

Ionic Strength and Monovalent Cation Concentration. So far, quin2 has been mostly used in mammalian cells, though a few reports of its application to other eukaryotic cells have also been published. In mammalian cells it is likely that the ionic strength and total monovalent cation concentration are normally rather constant. However, a change in ionic strength from that typical of a mammalian cell to that typical of marine invertebrate axoplasm increases the Ca^{2+} dissociation constant of related tetracarboxylate chelators by three- to fourfold.[10] Though we have not measured the quin2 K_D at high ionic strength, we would expect the same increase to apply.

Temperature. Increasing the temperature moderately increases the dissociation constant for Ca^{2+}, but a more significant effect is to decrease the fluorescence intensity of quin2 at fixed $[Ca^{2+}]$. A change from 20° to 37° decreases the fluorescence intensity of quin2 by a factor of approximately 2. This effect explains the common experience that cold solutions that are put in a warm thermostatted cuvette often decrease in fluorescence for the first few minutes before reaching a steady baseline level.

pH. Quin2 and other tetracarboxylate Ca^{2+} indicators are much less sensitive to pH changes around 7 than their parent EGTA is. This is because the highest pK_a of quin2 is ≤ 6.5 compared to 9.58 for EGTA. The effective K_D of quin2 for Ca^{2+} is increased by acidification of the medium below pH 6.8, while above this value pH changes are practically ineffective. In lymphocytes we measured[35] a resting pH_i of 7.05 for $pH_o = 7.4$, hence the choice of pH for Fig. 2. Since in most mammalian cells pH_i has been shown to range between 6.9 and 7.4, pH_i is probably not a major source of error in the calibration of $[Ca^{2+}]_i$. There are, however, some manipulations or drugs which can acidify pH_i considerably, which would complicate their effects on quin2 signals. Examples are mitochondrial uncouplers, anoxia, and permeant weak acids.

$[Mg^{2+}]_i$. There are many fewer direct measurements of free $[Mg^{2+}]_i$ than of pH_i. Our measurement of about 1 m*M* in lymphocytes[35] was the basis for the choice of $[Mg^{2+}]$ in Fig. 2. The affinity of quin2 for Mg^{2+} is in the 1–2 m*M* range, so an increase of Mg^{2+} can significantly increase the effective K_D of quin 2 for Ca^{2+}. Thus a change of Mg^{2+} from 0 to 1 m*M* results in a increase of the apparent K_D of quin 2 for Ca^{2+} from 60 to 115 m*M*, respectively.[4] Fortunately, the standard excitation wavelength of 339 nm not only maximizes the effect of Ca^{2+} on the fluorescence but also minimizes the direct effect of Mg^{2+} on the fluorescence, which is why 339 mn was chosen. At other wavelengths Mg^{2+} directly affects the spectrum as well as competing for Ca^{2+} (Fig. 4). There is little evidence up to now for changes of $[Mg^{2+}]_i$ upon cell stimulation, but the possibility of variability of the $[Mg^{2+}]_i$ between cell types has to be remembered.

Quin2 Localization in Cells

One of the most attractive features of the methodology is that most if not all of the trapped quin2 seems to reside in the cytoplasm or nucleus as opposed to other organelles. This generalization seems valid for the cells we have investigated, i.e., lymphocytes, platelets, neutrophils, Ehrlich and Yoshida carcinomas, and the cell lines PC12, RINm5F, and HL60. However, few workers have investigated in detail the localization of quin2 within other cell types, which may act differently.

Intracellular localization has been determined in various ways: (1) Direct morphological observation by fluorescence microscopy[11] reveals mainly diffuse rather than punctuate fluorescence, suggesting a cytoplasmic localization. (2) Detergents like digitonin, which preferentially permeabilize membranes with high-cholesterol content (like the plasma membrane), release practically all trapped quin2 in parallel to cytoplasmic

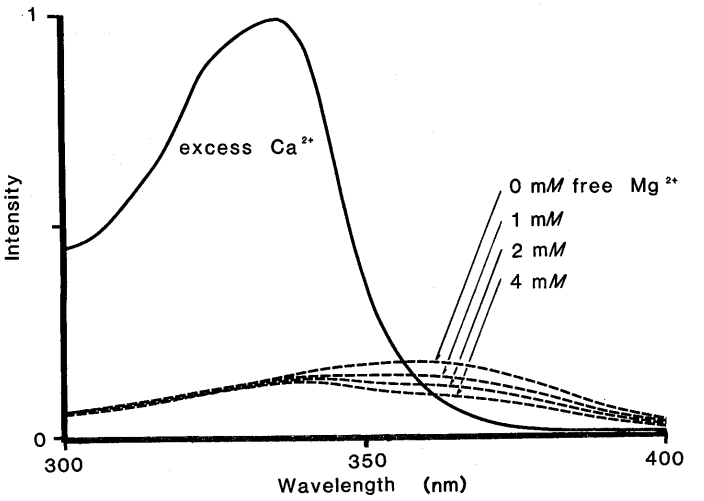

FIG. 4. Effect of Mg^{2+} on the excitation spectrum of quin2. Though Mg^{2+} has little effect at 335–340 nm, it significantly depresses the excitation amplitude at 350–370 nm, so that the ratio of the excitations at these two bands can be perturbed by physiological $[Mg^{2+}]$. The solution contained 10 μM quin2 in 132 mM KCl, 1 mM K_2H_2EGTA, 10 mM K-MOPS, pH 7.18, at 20°. $MgCl_2$ was added to reach 1, 2, and 4 mM free $[Mg^{2+}]$, then 4 mM $CaCl_2$ was added to saturate the dye with Ca^{2+}. Excitation bandpass was 1.8 nm; emission was collected at 500 nm with 9.25 nm bandpass. Excitation spectra were corrected by a rhodamine B quantum counter.

markers, with minor release of enzymes from mitochondria, lysosomes, and endoplasmic reticulum.[4,13,36,37] (3) High voltage discharge, which makes small holes in the plasma membrane, releases most intracellular quin2.[4] (4) Incubation of quin2/AM with partially purified cellular fractions reveals that the relevant esterase activity is localized in the soluble cytosolic fraction.[4] (5) Measurement of extracellular quin2 after strong stimulation of secretion indicates that, in platelets[8] and neutrophils,[36] quin2 is not appreciably contained in secretory granules. However, it appears that mast cell granules can pick up substantial amounts of fluorescence.[38]

In lymphocytes we also found[4] that the nuclear membrane does not constitute a permeability barrier for quin2, so that the dye is neither concentrated nor excluded from the nucleus. Since most evidence had suggested that ionic activities are the same in the nucleus as in the cyto-

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