Fura-2 Measurement of Cytosolic Free Ca^{2+} in Monolayers and Suspensions of Various Types of Animal Cells

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Abstract. The fluorescent indicator fura-2 has been applied to a variety of cell types in order to set up appropriate conditions for measurements of the cytosolic concentration of free ionized Ca^{2+} ($[Ca^{2+}]_i$) in both cell suspensions and single cells analyzed in a conventional fluorimeter or in a fluorescence microscope equipped for quantitative analyses (with or without computerized image analyses), respectively. When the usual procedure for fluorescence dye loading (i.e., incubation at 37°C with fura-2 acetoxy-methyl ester) was used, cells often exhibited a nonhomogeneous distribution of the dye, with marked concentration in multiple small spots located preferentially in the perinuclear area. These spots (studied in detail in human skin fibroblasts), were much more frequent in attached than in suspended cells, and were due to the accumulation (most probably by endocytosis) of the dye within acidic organelles after hydrolysis by lysosomal enzyme(s). When loading with fura-2 was performed at low (15°C) temperature, no spots appeared, and cells remained diffusely labeled even after subsequent incubation at 32–37°C for up to 2 h. Homogeneous distribution of the dye is a prerequisite for appropriate $[Ca^{2+}]_i$ measurement. In fact, comparison of the results obtained in human skin fibroblasts labeled at either 37 or 15°C demonstrated in spotty cells a marked apparent blunting of Ca^{2+} transients evoked by application of bradykinin. Additional problems were encountered when using fura-2. Leakage of the dye from loaded cells to the extracellular medium markedly affected the measurements in cell suspensions. This phenomenon was found to depend on the cell type, and to markedly decrease when temperature was lowered, suggesting the involvement of a facilitated transport. Calibration of fluorescence signals in terms of absolute $[Ca^{2+}]_i$ was complicated by the increased fluorescence of fura-2 in the intracellular environment. To solve this problem we propose an in situ calibration procedure based on measurements carried out on cells in which $[Ca^{2+}]_i$ was massively lowered (by loading the probe in a Ca^{2+}-free medium) or increased (by treatment with the Ca^{2+} ionophore ionomycin, applied in a medium containing 3 mM Ca^{2+}). These results provide explanations and, at least partial, solutions to the major problems encountered when using fura-2, and should thus be of help in clarifying the proper usage of the dye in $[Ca^{2+}]_i$ measurements.

THE fine control of the cytosolic concentration of free ionized Ca^{2+} ($[Ca^{2+}]_i$)[1] has long been recognized as a fundamental mechanism of cell activation (2–4, 29, 37). A direct knowledge of $[Ca^{2+}]_i$ is now possible in virtually all cell types, due to the development of intracellularly trapped fluorescent indicators: first quin2 (34) and, more recently, fura-2 (14, 33). The remarkable advantages of the latter dye with respect to its older congener, discussed in detail by Tsien and his associates (14, 33, 35–37), include higher fluorescence and quantum yield, lower affinity for Ca^{2+}, and favorable spectral properties, including shift of the excitation spectrum induced by Ca^{2+} binding, with reciprocal changes of fluorescence across the isosbestic point. Using fura-2 $[Ca^{2+}]_i$ can thus be adequately measured ratiowise, i.e., by computing the ratio of the fluorescence at two wavelengths. Such a procedure, because it is independent of the geometry and (above certain limits) of the dye concentration in the preparation under study, can be used not only in cell suspensions, but also in single cells analyzed individually in a fluorescent microscope (13, 20, 32). The investigation with fura-2 can be further pursued at the subcellular level (6, 18, 25, 26, 27, 39) through the use of imaging techniques.

In spite of the advantages of fura-2, the use of the dye has not become general because several problems have been encountered. The first, and most annoying, problem concerns

1. *Abbreviations used in this paper:* AM, acetoxy-methyl ester; AO, acridine orange; $[Ca^{2+}]_i$, free ionized Ca^{2+}; HSF, human skin fibroblasts; KRH, Krebs-Ringer medium buffered with Hepes.

the cytosolic loading of the dye. In some cells the classical procedure used with quin2 (administration to the cells of hydrophobic acetoxy-methyl ester (AM), destined to generate the Ca^{2+}-binding probe after cleavage by cytosolic esterase[s] [14, 34, 35]) has been found to cause the accumulation of fura-2 not only in the cytosol, but also (and especially) within multiple, discrete subcellular compartments (1, 7, 12, 15, 17, 21). A second problem concerns calibration of fura-2 signals in terms of absolute $[Ca^{2+}]_i$ (24). Finally, many cell types investigated were found to release appreciable amounts of fura-2 to the medium during incubation.

In our laboratories fura-2 has been applied to a variety of cell types. The three problems mentioned above have been systematically reinvestigated, and the observations made have led to the development of protocols for cell loading and incubation as well as for signal calibration that might be of widespread applicability.

Materials and Methods

Cell Cultures

Both primary cultures and cell lines were used. Human skin fibroblasts (HSF) were prepared and cultured as described in reference 11. Information about neurons of the superior cervical ganglion, bovine chromaffin cells, PC12, 3T3, RINm5F, HL60, and A431 cells is given in references 8, 23, and 24. Vero and HeLa cells were cultured by conventional procedures. Rat pituitary mammotroph cells were dissociated, purified, and cultured as previously described (31).

Cell Loading with Fluorescent Probes

Before being loaded with fura-2, the cells were either detached and put in suspension, or attached to glass coverslips. Some cell types (HSF, 3T3, and HeLa) attached directly to the coverslips, others (neurons, PC12, mammotrophs, and RINm5F) required coating of the glass with polyornithine with or without collagen. Unless otherwise indicated, loading was started by adding a small volume (usually 1/1,000) of fura-2/acetoxy-methyl ester (fura-2/AM) dissolved in DMSO to either the culture media or a physiological medium (KRH) containing, in mmol/liter: NaCl, 125; KCl, 5; $MgSO_4$ and KH_2PO_4, 1.2; $CaCl_2$, 2; glucose, 6; Hepes-NaOH buffer 25, pH 7.4. Final concentration of fura-2 in the incubation media ranged between 1 and 10 μM; temperature and length of incubation between 0 and 37°C and 10–120 min, respectively. Rhodamine 123 and acridine orange (AO) were added to KRH (final concentrations of 0.5 and 2 μg/ml, respectively) and incubated at 37°C in the dark for 30 min. AO-loaded cells were then rinsed with fresh KRH and reincubated at 37°C for 10 min before fluorescence microscopy (19, 27).

Fluorescence Microscopy

Labeled cells attached to coverslips were studied in a Zeiss photomicroscope III using excitation lights centered at the following wavelengths (nm): 360 (fura-2), 540 (rhodamine 123), and 485 (AO). At the emission site cutoff filters (450, 580, and 520 nm, respectively) were used.

Electron Microscopy

Monolayers of HSF before and after loading with fura-2 (15 and 37°C) were fixed with a glutaraldehyde-formaldehyde mixture (2.5 and 1.5%, in 125 mM phosphate buffer), postfixed in 2% OsO_4 in the same buffer, pH 7.4, and embedded in Epon. Ultrathin sections were cut and observed in a Philips CM 10 electron microscope after uranyl-lead staining.

Spectro- and Microspectrofluorometry

Suspensions and monolayers of cells loaded with fura-2 were analyzed in a Perkin Elmer LS5 fluorometer. Cell monolayers grown on a glass slide were positioned at appropriate angles in the cuvette by a custom-made adapter. Microspectrofluorometry was carried out on single cells attached to glass coverslips that were placed, facing downwards, on a small (1 ml) thermostated glass perspex chamber equipped for medium stirring and replacement. Temperature, controlled by an electronic digital thermometer, ranged between 30 and 37°C. A Zeiss photomicroscope III equipped for photometry (Zeiss PMT system) was used. The light from a xenon lamp (XBO75w/2; Osram) passed through either one of two narrowband interference filters that were alternated in the light pathway by an air-pressured system. The filters used in most experiments (bandwidth 10 nM; Oriel, Stratford, CT) were centered at 340 and 380 nm. Although most of the light passed through the 340-nm filter was not transmitted through the glass optics of the microscope, this filter proved to be more appropriate than its homologue centered at 350 nm, a wavelength too close to the isosbestic point to yield measurable fluorescence decreases upon Ca^{2+} binding to the dye. The objective used was a Zeiss Plan Neofluar (40×, water immersion, NA 0.9). The emitted fluorescence, before feeding into the photomultiplier, was filtered through a cutoff filter (490 nm). To delimit the field of analysis, an aperture was placed in the image plane in front of the photomultiplier. Analyzed fields ranged between 5 and 20 μm in diameter. The photomultiplier was coupled to a Zeiss A-D converter interfaced to a personal computer (SX 64; Commodore) that also controlled filter alternation. Averaged light intensities over excitation periods (0.5–2 s) at each of the two wavelengths were used by the computer to calculate 340/380 ratios after background subtraction. Data were stored in sequential files. At the end of each experiment they were plotted and calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkyewicz et al. (14) based on the equation

$$[Ca] = K_d \times \frac{(R - Rmin)}{(Rmax - R)} \times \frac{Sf2}{Sb2}$$

and assuming the K_d of the Ca^{2+}-fura-2 interaction to be 225 nM in the cytosolic environment (14). Rmin, Rmax, and Sf2/Sb2 were measured in the same conditions using fura-2 standards in high (10^{-3} M) and low (10^{-8} M) $[Ca^{2+}]$.

Image Analysis

Analysis of the images of fura-2-loaded HSF was carried out at excitation wavelengths ranging from 340 to 400 nM by using a Zeiss photomicroscope equipped with a monochromator attached to a television camera (high sensitivity) (TYK; Bosch, Federal Republic of Germany) connected to a computerized system (Kontron-IBAS, FRG).

Controlled Cell Lysis with Digitonin

HSF monolayers loaded with fura-2 at either 15 or 37°C were exposed to increasing concentrations of digitonin in KRH at 37°C for 1 min, after which the supernatants were collected, the cells stained with Trypan blue (0.5% in KRH) for 1 min, rinsed, and finally aldehyde fixed. The supernatants were analyzed for the released fura-2, and the cells for trypan blue positivity.

Isolation of Subcellular Fractions from Rat Liver and Fura-2 Hydrolysis Assays

High speed supernatant and lysosome fractions, isolated from rat liver homogenates by Borgese and Pietrini (5), were used after freezing and thawing and treatment with Triton X-100 (0.2%).

Assays were carried out by mixing, in the fluorometer cuvette (37°C), 0.2 mg of enzyme protein and fura-2/AM with 2 ml of either Hepes or acetate buffers, pH 7 or 5, respectively, containing 2 mM $CaCl_2$ or devoid of added Ca^{2+} but supplemented with 1 mM EGTA. The conversion of the ester to fura-2 free acid was estimated by following the increase of fluorescence at 340 nm and the decrease at 390 nm. Blank samples were incubated similarly, but with no added enzyme.

Bradykinin-induced $[Ca^{2+}]_i$ Transients in HSF

Single HSF of monolayers loaded with fura-2 at either 15 or 37°C were incubated in Ca^{2+}-free KRH containing 1 mM EGTA, and exposed to arg-bradykinin (0.1 μM).

Other Assays

Lactic dehydrogenase was assayed spectrophotometrically at 340 nM; protein was measured by the Brandford procedure; ethidium bromide nuclear staining was measured either in the LS5 spectrofluorometer, or by direct counting of stained cells in the fluorescence microscope.

Materials

Media and sera for cell cultures were obtained from Flow Laboratories, Milan, Italy. Fura-2 and fura-2/AM were from Molecular Probes, Junction City, OR. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Results

A number of different cell types were investigated during the present studies: fibroblasts (HSF and Swiss 3T3 mouse fibroblasts); tumor cell lines (HeLa, Vero, A431, and HL60); various neurosecretory cells, both in a continuous line (PC12 rat pheochromocytoma and RINm5F insulinoma) and in primary culture (bovine chromaffin and rat pituitary mammotroph cells); neurons of the rat sympathetic superior cervical ganglion; and blood cells (human neutrophils and various types of lymphocytes).

Fura-2 Studies in Adherent Cells

Punctate and Diffuse Distribution of Fura-2. The conditions used for loading fura-2 into adherent cells were initially similar to those routinely used to load cell suspensions with quin2, except that lower dye concentrations were used (e.g., 1–10 μM fura-2/AM compared with 10–100 μM quin2/AM). Cells were attached (either directly or through a polyornithine coat) to glass coverslips that were placed at the bottom of a petri dish filled with either the culture medium or KRH. Fura-2/AM was then added from a concentrated (at least 10^3-fold) solution in DMSO, and incubations carried out for 30–60 min at 37°C. Examples of the fluorescence patterns obtained with this loading protocol are given in Fig. 1, *A–D*. Many cells were not labeled uniformly, but showed bright discrete spots glowing over a diffuse background. The number of these spots varied greatly in different cell types. They were rare or absent in neurons and neurosecretory cells (Fig. 1 *D*), and extremely numerous in fibroblasts. Particularly in fibroblasts, the spots appeared to be located preferentially in the perinuclear area, from which they tended to depart radially in ordered rows (Fig. 1, *A* and *B*).

Attempts to eliminate the punctate distribution of the dye were made by systematically modifying the loading protocol. No improvement was observed when the length of the loading incubation was changed (10–120 min). Changes of the pH (6.5–8) or introduction of proteins (BSA, 1–10 mg/ml; FCS, 1–10%) into the medium induced only marginal differences. Centrifugation (10,000 *g*, 1 min) of the fura-2–containing medium resulted in the precipitation of the dye, so that the cells loaded by incubation with the supernatant were virtually unlabeled. In contrast, when the temperature of the loading incubation was lowered, the spots decreased or disappeared, and the cells looked uniformly, although often rather faintly, labeled (Fig. 1, *E–H*). Optimal conditions of loading were established empirically for each type of cell by independently varying the dye concentration and the temperature of incubation. When diffusely labeled cells, after being washed with fresh medium, were warmed to 32 or 37°C, the fluorescence remained diffuse (i.e., no appearance of spots occurred) even after prolonged (1–2 h) incubations (not shown). To get information on the cellular mechanisms responsible for the distribution of fura-2, we focused our at-

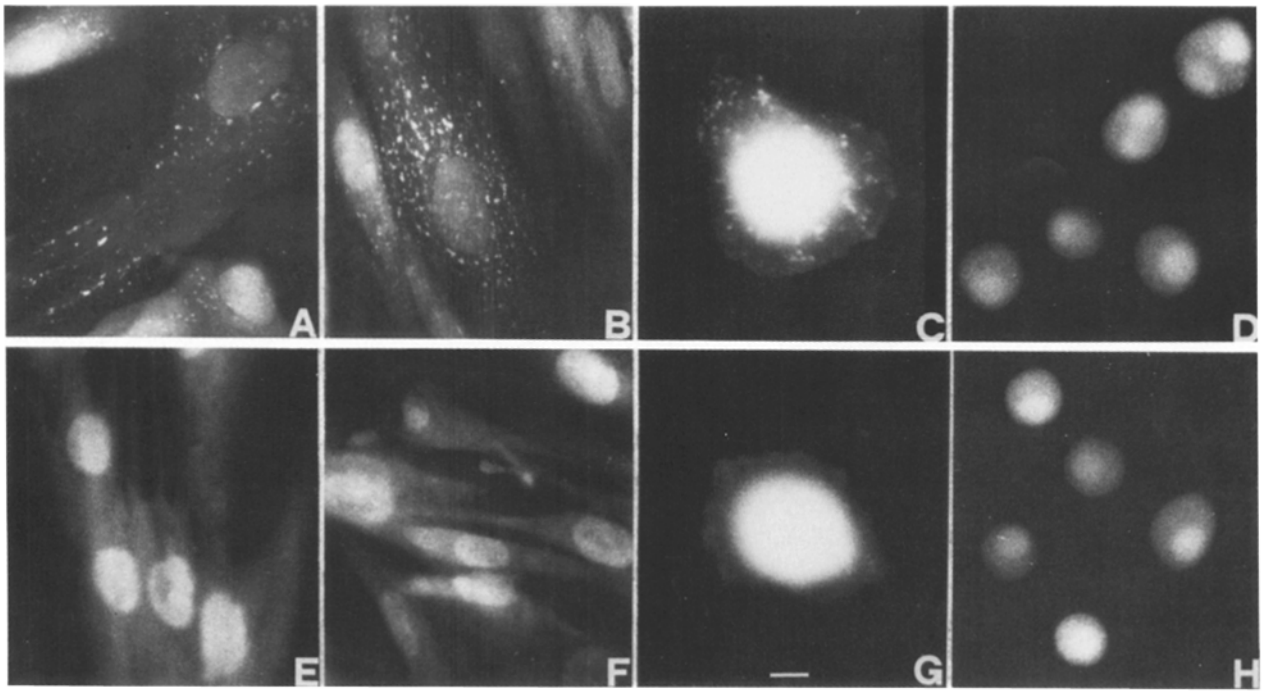

Figure 1. Fura-2 fluorescence microscopy of various types of cell monolayers loaded with the probe at either 37°C for 30 min (*A–D*) or 15°C for 60 min (*E–H*). In the cells loaded at 37°C (concentration of fura-2/AM was 2 μM) highly fluorescent spots are seen, localized primarily in the perinuclear area. These spots, particularly prominent in HSF (*A*) and 3T3 fibroblasts (*B*), but also evident in A431 cells (*C*), are often arranged in linear rows, departing radially from the center of the cell. In primary cultures of rat mammotroph cells (*D*), on the other hand, spots were only occasionally seen, even with this loading protocol. In the same cell types loaded at 15°C (*E*, HSF; *F*, 3T3; *G*, A431; *H*, mammotrophs; concentration of fura-2/AM was 10 μM) the homogeneous distribution of fura-2 fluorescence is illustrated. Bar, 10 μm.

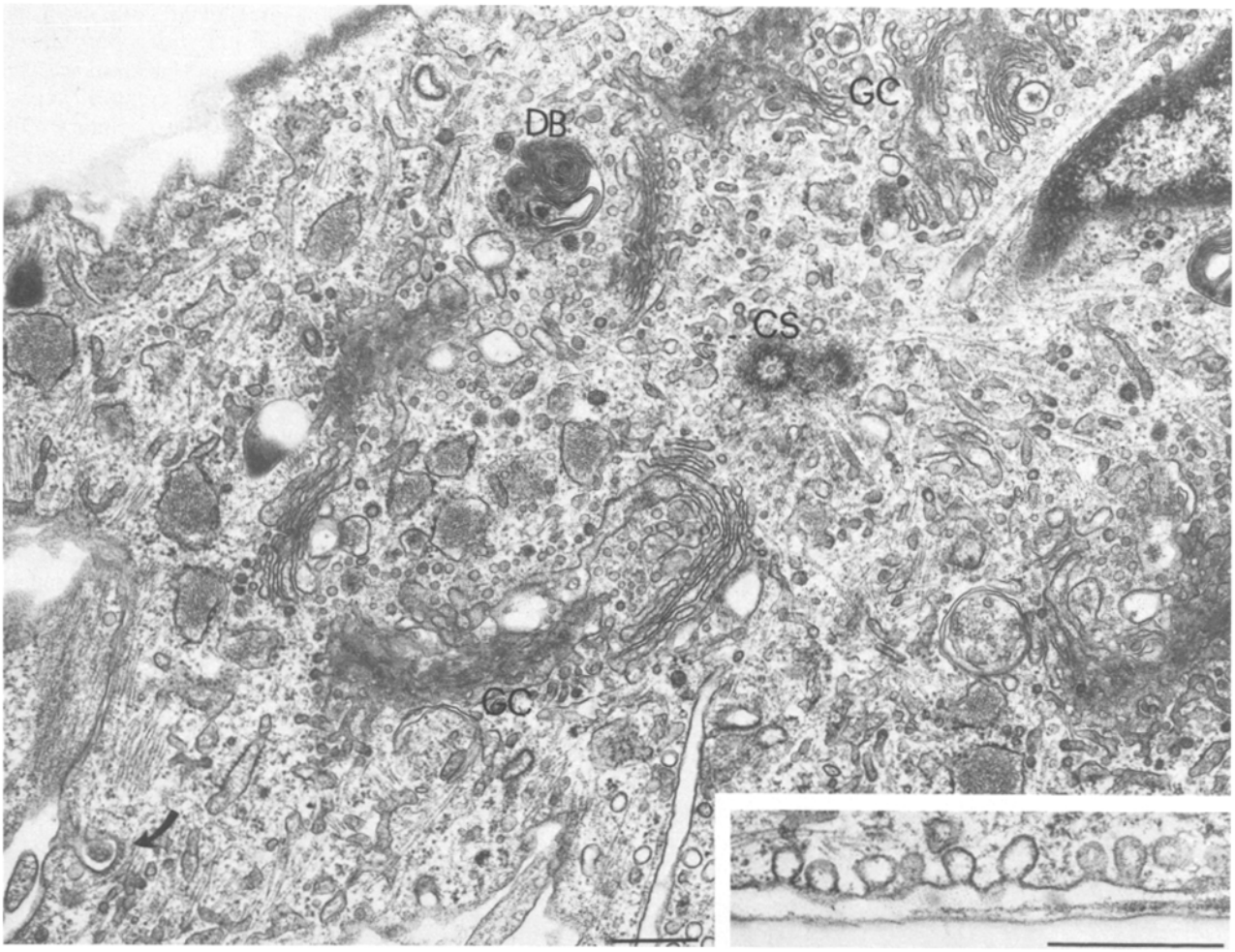

Figure 2. Thin-section electron microscopy of the centrosomal region in an HSF cell. *M*, mitochondrion; *DB*, dense body; *CS*, centrosome; *GC*, Golgi complex; the arrow points to a large coated pit. (*Inset*) Various vesicles in continuity with the plasma membrane (probably endocytic vesicles). Bar, 0.5 μM.

tention on HSF, a cell type also suited for these studies because of the large difference in labeling patterns at 37 and 15°C. When studied by conventional thin-section electron microscopy, HSF appear as flat and irregularly shaped cells. Their perinuclear region (i.e., the region where fluorescent spots accumulate at 37°C) is occupied by the centrosome, a well-organized Golgi complex, some endoplasmic reticulum cisternae, and by populations of vesicles, anastomosing tubules, vacuoles, and dense bodies. An example of those structures is shown in Fig. 2. After loading with fura-2 at either 15 or 37°C no obvious difference could be detected (not shown).

Fluorescence Spectral Analysis of Punctate and Diffusely Labeled HSF

To learn about the state of the probe in HSF loaded at different temperatures, spectra were recorded from both monolayers and single cells. As illustrated in Fig. 3 *A*, no difference was observed in the shape of the excitation spectra of cell monolayers loaded with fura-2 at either 37 or 15°C, although in the first case (punctate pattern) the amount of loaded fura-2 was greater than in the second (diffuse pattern). Spectra had a peak at ~350 nm, with a visible shoulder at ~390 nm. The shoulder coincides with the excitation peak of fura-2/AM (Fig. 3 *B*), and might thus be due to uncleaved dye associated with the monolayers. In monolayers loaded at 4°C the fluorescence attributable to fura-2/AM appeared to predominate (not shown).

To investigate the intra- or extracellular location of the two forms of fura-2 (cleaved and uncleaved), monolayers were rinsed after loading with incubation media supplemented with BSA (10 mg/ml), which is known to bind hydrophobic molecules such as fura-2/AM. When this was done the 390-nm shoulder decreased considerably (Fig. 3 *A*). Parallel microscopic examinations were carried out on coverslips bearing low cell density monolayers. After BSA washing, fluorescence spots originally present on the empty regions of coverslips were no longer seen, whereas the cellular fluorescence patterns (both diffuse, 15°C, or punctated, 37°C) were unaffected (not shown). Taken together these results indicate that most of the spots visible in the cells loaded at 37°C are located intracellularly and are due to the accumulation not of the AM ester, but of fura-2 that has been at least partially deesterified (14, 30, 33, 36). Further support for this conclu-

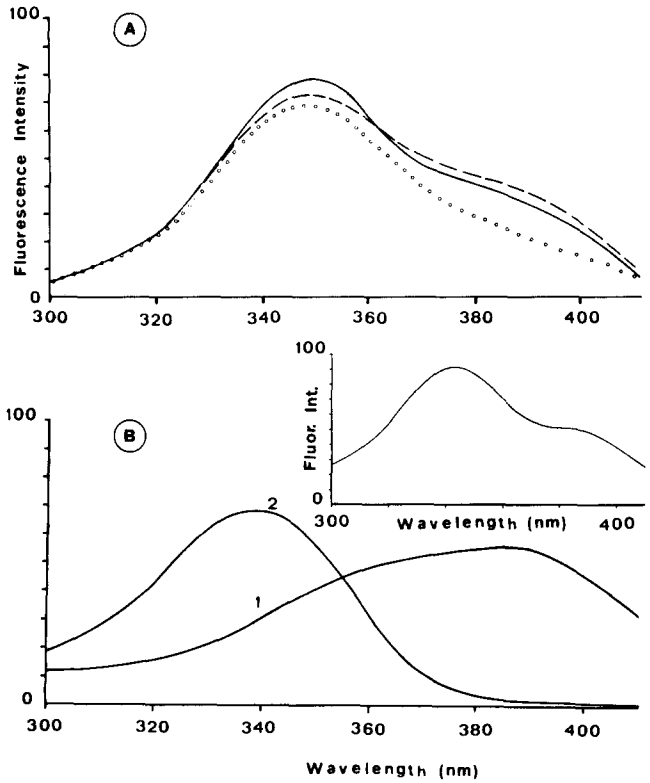

Figure 3. Fura-2 fluorescence spectra recorded from either (*A*) monolayers of HSF on glass coverslips loaded at 37°C (*continuous line*) and 15°C (*broken line*), or (*B*) solutions of fura-2/AM (*1*) and fura-2 free acid (*2*) (0.5 μM, in KRH containing 2mM Ca^{2+}). The dotted line in *A* is the spectrum recorded from a monolayer washed with 1% BSA in KRH after loading at 15°C. Similar results were obtained with monolayers loaded at 37°. Concentrations of fura-2/AM and length of loading incubations were as in Fig. 1. Spectra of monolayers were subtracted from the contribution of glass coverslips, which is considerable below 320 nm. (*Inset*) Spectrum recorded from a sample (in KRH) containing a mixture of fura-2/AM and free acid, each at 0.5 μM concentration. Fluorescence intensity is expressed in arbitrary units.

sion comes from results obtained with image analysis in a single HSF. Fig. 4 (*A–D*) shows the fluorescence emitted by a spotty cell excited at four different wavelengths; 340, 360, 380, and 390 nm. The brightness of the cells was clearly nonhomogeneous: higher in the nucleus and spots than in the cytoplasm. In addition, it is worth noting that in all compartments, and especially in the spots, fluorescence at 360 was higher than at 390 nm (cf. *B* and *D* in Fig. 4). When the 360:390 ratio was computed, the resulting image was still moderately inhomogeneous, with higher values in the spots (Fig. 4 *E*).

Subcellular Distribution of Fura-2 in Spotty and Diffusely Loaded HSF

To identify the nature of intracellular fluorescent spots, we made use of fluorescent markers specifically addressed to individual cytoplasmic organelles. Fig. 4 *F* shows that the distribution of the mitochondrial fluorescent marker, rhodamine 123 (19), is completely different from that of the fura-2 spots. In contrast, the distribution of AO, a marker of intracellular acidic compartments (27), appears to mimic closely the pattern observed after fura-2 loading at 37°C (cf. Fig. 4 *G* with Figs. 1 *A* and 4 *H*). To confirm the identification of fura-2 spots as acidic organelles, double label experiments were carried out. Fig. 4, *H* and *I* illustrate the coincidence of the discrete labeling in HSF first loaded with fura-2 at 37°C (Fig. 4 *H*) and then with AO (Fig. 4 *I*). When doubly labeled cells, such as those of Fig. 4 *I*, were excited at 340 nm (the fura-2 wavelength), no spotted fluorescence was revealed, suggesting that the fura-2 signal within the acidic vesicles (but not that in the cytoplasm) was being quenched by the colocalization with AO. Evidence along the same line was obtained by the use of drugs known to affect acidic compartments. Both chloroquine (200 μM, a weak base that accumulates within acidic organelles, Fig. 4 *J*) and monensin (1 μM, an ionophore that causes Na^+/H^+ exchange, and thus the collapse of proton gradients, not shown), when added to HSF together with fura-2, prevented the formation of spots. Results of this type were obtained not only in HSF, but also in other cell types (3T3 and PC12). The involvement of acidic organelles seems therefore widespread, if not general.

A final series of experiments aimed to characterize the nature of the spots was carried out by the use of the cholesterol-binding detergent, digitonin. As can be seen in Fig. 5 an almost complete release of fura-2 from cells loaded at 15°C occurred over a narrow range of digitonin concentrations, and was paralleled by the death of the cells, revealed by trypan blue staining. In contrast, the curve of fura-2 release from HSF loaded at 37°C was biphasic, with 65% of the probe released in parallel to the appearance of trypan blue positivity, and the rest occurring at higher digitonin concentrations. The 37°C-loaded HSF that were trypan blue–positive and had released a large part of their fura-2 by a low concentration of digitonin exhibited fluorescent spots glowing over a dark background (not shown).

Cytosolic trapping of fluorescent indicators is due to the cleavage of their hydrophobic AM precursors, to yield the hydrophilic free acids. If indeed the fura-2 free acid accumulated not only in the cytoplasm, but also within acidic organelles (presumably lysosomes), one would expect a dual localization of the cleaving enzyme(s) as well. Fig. 6 demonstrates that this is indeed the case, at least in rat hepatocytes, from which well-characterized subcellular fractions are available. The study of excitation spectra revealed that the lysosomal lysate is able to cleave fura-2/AM, however, with a specific activity lower than that of the high speed supernatant (the cytosolic fraction). In the two compartments cleavage might be due to different enzymes, as the cytosolic activity was greater at neutral, the lysosomal at acidic pH.

$[Ca^{2+}]_i$ Measurement in Single Fura-2–loaded Cells

Fig. 7 compares representative transients of apparent $[Ca^{2+}]_i$ obtained in single HSF loaded with fura-2 at either 15 or 37°C and then exposed to arg-bradykinin while suspended in a Ca^{2+}-free medium. It can be assumed by analogy with results for other types of cells (16) that these transients are due to bradykinin-induced generation of inositol-1,4,5-trisphosphate and consequent release to the cytosol of Ca^{2+} segregated within a "microsomal" store. The rise of apparent $[Ca^{2+}]_i$ measured in the spotty cells was consistently and considerably lower than in diffusely labeled cells (on the av-

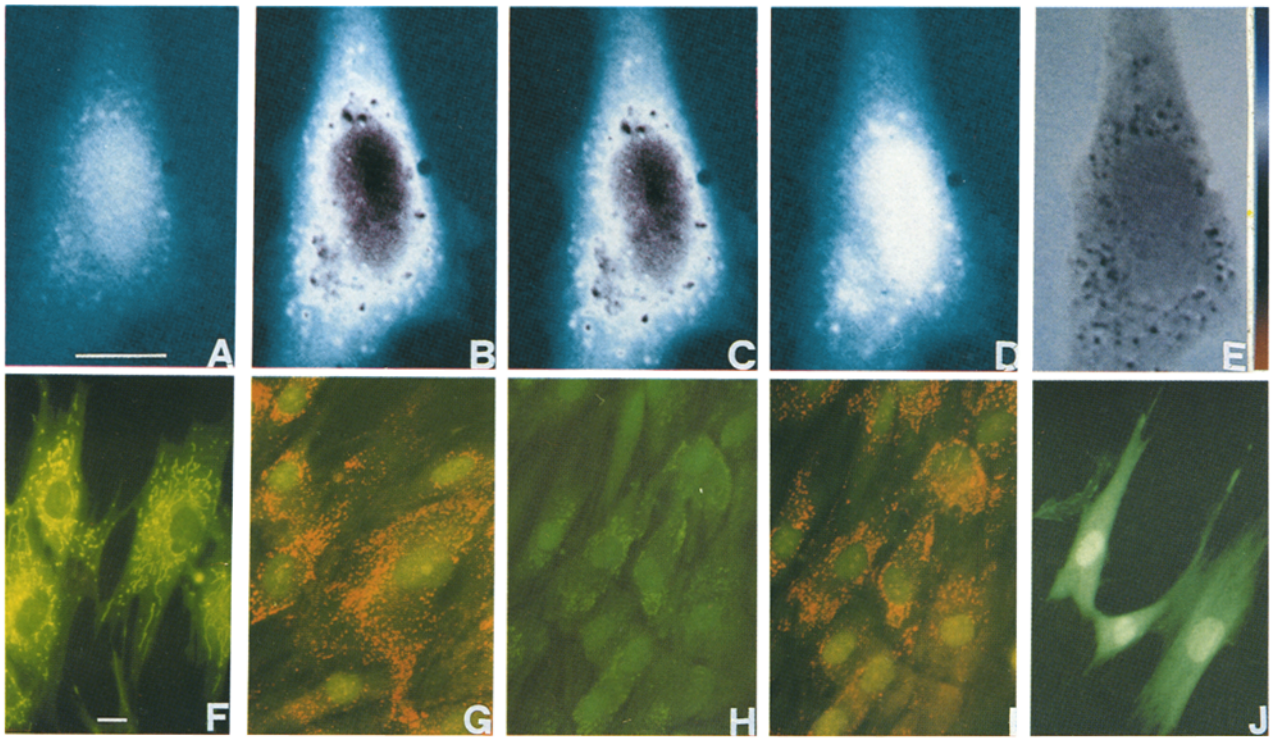

Figure 4. Computerized images taken directly from the video display (*A–E*), and fluorescence microscopy (*F–J*) of HSF loaded with either fura-2 or other fluorescent markers. For computerized images, cells were loaded at 37°C with 4 μM Fura-2/AM and then analyzed. The excitation beam passed through a monochromator (slit, 10 nm), while the emitted light passed through a cutoff filter (450 nm). Each image represents the average of 20 images subtracted from the background. *A–D* were recorded from the same cell at 340, 360, 380, and 390 nm excitation, respectively. Notice that fluorescence at 340 nm was considerably cut down by the glass optics used in the microscope. *E* is a ratio image resulting from the computerized division of images *B* and *D*, normalized to a 0–255 gray scale level. The false color scale for *A–E*, shown to the right of *E*, was obtained by conversion of the gray values. Distribution of fluorescent markers (*F–J*) was investigated in HSF loaded at 37°C with either rhodamine 123 (*F*), AO (*G*), fura-2 (*H*) followed by AO (*I*), or fura-2 administered together with chloroquine (*J*). Notice the coincidence of most fluorescent spots in the cell loaded first with fura-2, then washed, photographed (*H*), and finally loaded on the stage of the microscope with AO before the second picture (*I*) was taken. Bar, 10 μm.

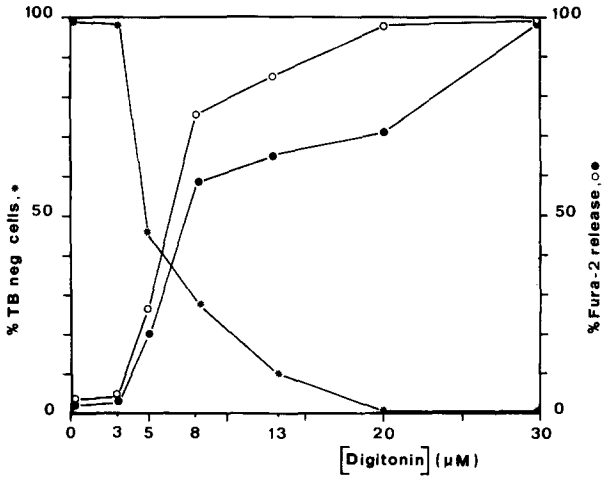

Figure 5. Release of fura-2 and cytotoxicity in HSF treated with digitonin. Cell monolayers were loaded with fura-2/AM (concentration during the loading incubation was 10 μM) at either 37 or 15°C for 60 min, washed and treated with KRH containing digitonin (concentrations specified in abscissa) for 1 min, and then were stained with trypan blue (0.5%, 60 s), washed with KRH, and finally fixed with 4% paraformaldehyde in 125 mM phosphate buffer. (*Solid and open circles*) The percent release of fura-2 to the media containing digitonin from the cells labeled at 37 and 15°C, respectively; (*asterisks*) cytotoxicity (trypan blue positivity) of cells loaded at both 15 and 37°C.

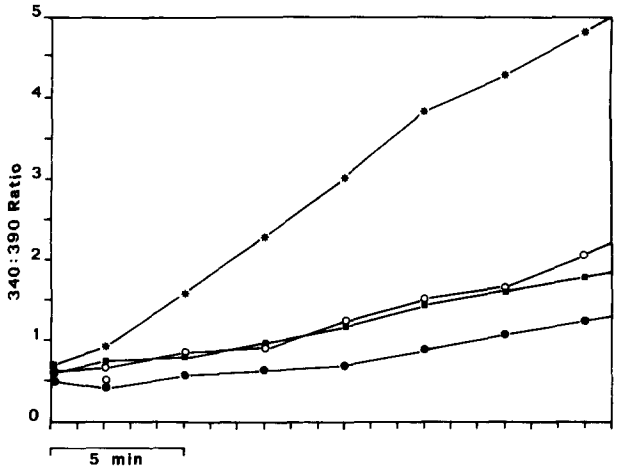

Figure 6. Hydrolysis of fura-2/AM incubated in vitro with liver subcellular fractions. Aliquots of high speed (110,000 *g* for 1 h) supernatant and lysosome fractions were treated with Triton X-100 (0.2%), mixed with fura-2/AM (2 μM) in either Hepes-NaOH (pH 7) or acetate (pH 5) buffers, supplemented with $CaCl_2$, 2 mM, and incubated at 37°C in a fluorimeter cuvette. The conversion of fura-2/AM to the hydrolyzed forms was followed by monitoring the fluorescence excitation ratio 340:390. (*Asterisks and solid squares*) High speed supernatant; (*solid and open circles*) lysosomal lysate, pH 7 and 5, respectively. Concentration of protein in the incubation mixtures was 0.1 mg/ml.

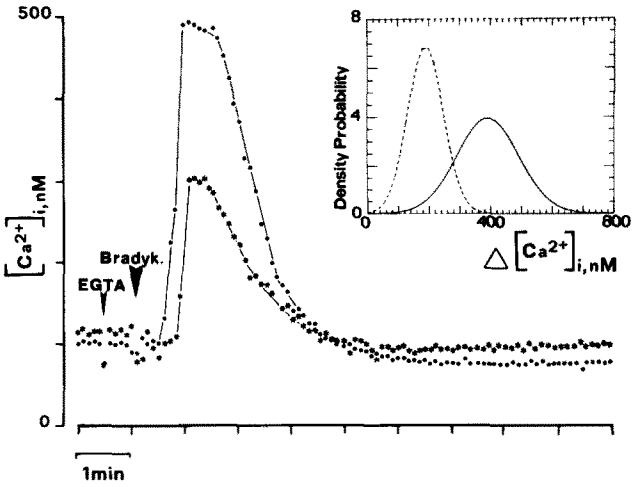

Figure 7. Apparent Ca^{2+} transients induced by bradykinin (100 nM) in single HSF loaded with fura-2 at either 37 or 15°C (conditions as in Fig. 1). Changes in fluorescence in two representative HSF loaded at either temperature (37°, *asterisks*; 15°, *solid circles*) and then treated with bradykinin (Bradyk.) at 32°C in a KRH medium without Ca^{2+} (EGTA, 1 mM). (*Inset*) The density probability of Ca^{2+} rise maxima in the population of analyzed HSF; (*continuous and broken lines*) loading at 15 and 37°C, respectively.

erage 189 ± 58 and 391 ± 101 nM, $n = 7$, respectively, over a resting level of 105 ± 63 nM). Under the experimental conditions used, the concentration of fura-2 was approximately the same in the cells loaded at either temperature. Thus, the result obtained cannot be due to different cytosolic Ca^{2+} buffering. In contrast, this is the result expected in two cells, one (diffuse labeling) with fura-2 entirely available, the other (spotty labeling) only partially available for $[Ca^{2+}]_i$ sensing. In other cell types the influence of spotty and diffuse labeling on apparent $[Ca^{2+}]_i$, although investigated in less detail, yielded results similar to those for HSF (not shown).

Fura-2 Loading of Cell Suspensions

The application of fura-2 to cell suspensions should not be viewed only as a fashionable alternative to the widely used quin2. There are a number of situations in which fura-2 is the first choice Ca^{2+} indicator, e.g., when buffering and/or side effects of quin2 loading are a problem (see, for example, references 9, 14, 22, 24, 36). Intracellular sequestration of fura-2 into acidic organelles, discussed above for cell monolayers, was found to be definitely less dramatic in the suspensions of cells that we have tested so far: normal cells (such as human neutrophils, mouse, and rat thymocytes; cytotoxic mouse and human T lymphocytes; and bovine adrenal chromaffin cells) and cell lines (such as the leukemic HL60, the neurosecretory PC12, and several sublines of the insulin secreting-cell line RINm5F). In all these cells loaded at either 15 or 37°C fura-2 fluorescence was usually diffuse. Spotty fluorescence was observed only occasionally, especially in RINm5F.

Leakage of Fura-2 to the Extracellular Medium

Fura-2 has been noticed to leak out from loaded cells during incubation. Such a phenomenon is particularly annoying with cell suspensions, because it yields a progressive increase of the extracellular background fluorescence. Although all cell types we have investigated were found to leak out fura-2 appreciably, the rate of the process was quite variable, and depended considerably on the temperature of incubation. At room temperature leakage never exceeded 10%/h, but above 30°C it rose considerably. For example, in PC12 cells incubated at 37°C the loss of fura-2 was linear with time and represented ~45% of the total cellular dye in 30 min. In human neutrophils the leakage rate was lower (~15% in 30 min), while in J774 macrophages, a mouse cell line (Di Virgilio, F., T. H. Steinberg, J. A. Swanson, and S. C. Silverstein, manuscript submitted for publication), the loss of trapped dye can be almost complete (~80%) in 30 min. Leakage of fura-2 clearly occurs from intact cells. During the same period of time the release from PC12 cells of a cytoplasmic marker, lactic dehydrogenase, was found to be <10%, and a cytotoxicity index, the staining of nuclei with ethidium bromide (a dye with a molecular weight lower than fura-2), to increase only negligibly (not shown).

Calibration of the Fura-2 Signal in Terms of $[Ca^{2+}]_i$

As already mentioned in the introduction, the spectral properties of fura-2 appear to be particularly suited for a dual wavelength ratio estimation of $[Ca^{2+}]_i$. In the experiments reported so far, the 340:380 nm ratio values measured experimentally were calibrated as recommended by Grynkyewicz et al. (14), i.e., by making reference to solutions of fura-2 free acid in buffers mimicking the ionic composition of the cytosol. Studies by Tsien and associates (26, 36) indicated, however, that the $[Ca^{2+}]_i$ values measured by this procedure are markedly lower with respect to those measured by the classical single wavelength–lysis procedure. As this discrepancy has been suggested to be due to the greater fluorescence of fura-2 in the cytosolic environment, we developed a protocol of fura-2 signal calibration in which reference standards are not saline solutions of known $[Ca^{2+}]_i$, but suspensions of intact cells in which $[Ca^{2+}]_i$ is modified to either very low or very high levels. Fig. 8 illustrates four spectra recorded in sequence from the same batch of suspended PC12 cells, before and after lysis. Spectrum *a* was recorded first, immediately after the cells were loaded with fura-2 in a Ca^{2+}-free medium with EGTA, washed, and resuspended in the same medium. ($[Ca^{2+}]_i$ was measured in parallel with quin2 $\leq$5 μM). $CaCl_2$ (3 mM) was then added to the cell suspension together with the Ca^{2+} ionophore, ionomycin (1 μM), and spectrum *b* was recorded 5 min later ($[Ca^{2+}]_i \geq 3$ μM, measured as above); cells were then lysed with 1% Triton X-100 and spectra *c* and *d* were recorded before and after addition of Tris and EGTA to bring $[Ca^{2+}]$ to ~1 nM. From the four spectra the values of Rmin, Rmax (ratios of fluorescence intensities at the selected excitation wavelengths with very low and very high $[Ca^{2+}]$, respectively) and Sf2/Sb2 (ratio of fluorescence intensities at the longer wavelength used, with very low and very high $[Ca^{2+}]$) can be calculated for intact cells and solutions. By comparing the spectra recorded in low $[Ca^{2+}]$ before and after lysis (Fig. 8, traces *a* and *d*) it is immediately evident that disruption of cell integrity causes a decrease of total fura-2 fluorescence, not just a shift, as one would expect if the dye was simply released into a medium with a different $[Ca^{2+}]$. Furthermore, no isosbestic point between these two spectra is observed. The

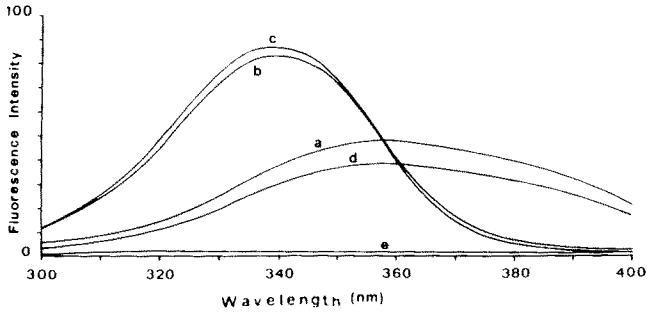

Figure 8. Excitation spectra of fura-2–loaded PC12 cell suspensions at different $[Ca^{2+}]_i$ levels. Effect of cell lysis on the spectral properties of fura-2. Cells were washed and resuspended (3×10^6/ml) in a Ca^{2+} free KRB medium (EGTA, 1 mM) containing 3% FCS. Fura-2/AM (4 μM) was added together with MAPTA/AM (an intracellularly trappable, nonfluorescent Ca^{2+} chelator, 11 μM), and the cell suspension incubated at 37°C under continuous stirring. A parallel batch of cells was incubated in the same medium but in the presence of quin2/AM. After 30 min the cells were centrifuged (500 *g* for 5 min), washed with KRB-EGTA containing 10% FCS, resuspended in KRB-EGTA, and finally analyzed in the spectrofluorometer as specified in the text. Spectrum *a* was recorded from cells with a $[Ca^{2+}]_i$ <5 nM; spectrum *b* from cells with $[Ca^{2+}]_i$ >3 μM; spectra *c* and *d* are from loaded cells after lysis by treatment with Triton X-100, 1% at $[Ca^{2+}]$ of 2 mM and <1 nM, respectively.

spectra taken at high $[Ca^{2+}]$ (*b* and *c*) are more similar, but not identical. In addition, the isosbestic point obtained in intact cells (*a* and *b*) is ~357–358 nm, i.e., ~3 nm blue-shifted compared with that of fura-2 free acid in normal solution. The spectra after cellular hydrolysis and cell lysis (*c* and *d*) are indistinguishable from those of fura-2 solutions. These results confirm and expand the observations of Tsien and associates (26, 36) that the spectral properties of fura-2 within cells are different from those in solution. An important consequence of the higher fluorescence of the dye in the cytoplasm compared with saline media, especially at low $[Ca^{2+}]_i$, is the overestimation of $[Ca^{2+}]_i$ that is expected when calibration is carried out by the lysis procedure.

The deviation of ratio in intact cells versus free solutions depending on the wavelength pairs used was investigated next. Fig. 9 (*a* and *b*) shows the values of ratio calculated as a function of $[Ca^{2+}]$ at either 340:380 or 340:361 nm. It is clear that, compared with the 340–361 nm pair, the 340–380 pair gives a 10-fold larger change of ratio, which, however, is primarily due to a mathematical expansion of the scale (26). Fig. 9 (*c* and *d*) represents the theoretical curves of ratio versus $[Ca^{2+}]_i$ calculated by using the values of Rmax, Rmin, and Sf2/Sb2 obtained by the internal calibration procedure discussed above (because of the shift in isosbestic point within cells the 340:357 R was used). While Rmin is similar for both wavelength pairs, whether obtained in saline or intact cells, Rmax is distinctly lower in the latter than it is in the first condition (but see Discussion).

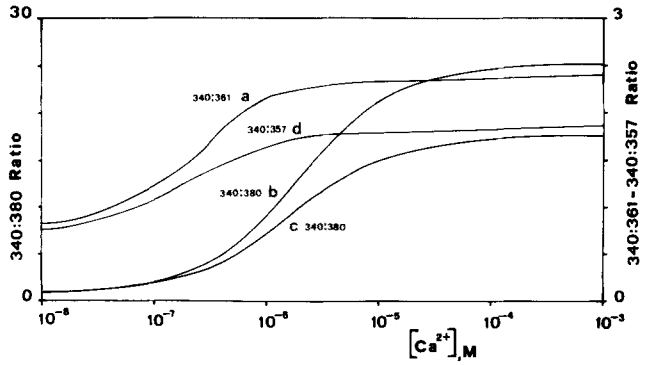

Figure 9. Changes of calculated fluorescence ratios (*R*) at various wavelength pairs as a function of Ca^{2+} concentrations. Values of Rmin, Rmax, and Sf2/Sb2 were measured in either fura-2 free acid solutions (*a* and *b*) or intact PC12 cells as illustrated in Fig. 8 (spectra *c* and *d*). The selected wavelength pairs were 340:380 for spectra *b* and *c*; 340:361 for spectrum *a*; and 340/357 for spectrum *d*. R was calculated based on Eq. 5 of Grynkyewicz et al. (14) reported in Materials and Methods.

Discussion

Subcellular Distribution of Fura-2

A uniform and selective distribution of the fluorescent probe in the cytosol of analyzed cells represents a prerequisite to most $[Ca^{2+}]_i$ measurements. If the dye distribution is heterogeneous, with accumulation into discrete spots, reliable values of $[Ca^{2+}]_i$ can be obtained only by imaging techniques, such as those used for the data of Fig. 4 (*A–E*). These techniques, however, not only require very expensive equipment, but are also so slow and laborious that they are not used in most laboratories. Up to now the problem of spotty distribution of fura-2 has not been dealt with in detail, although several reports have appeared showing that, when the distribution of the dye is compartmentalized, accurate measurement of $[Ca^{2+}]_i$ is unfeasible (1, 7, 12, 19). These results cast doubt as to the applicability to fura-2 of the loading procedure with AM esters proposed by Grynkyewicz et al. (10). If confirmed, this conclusion would represent a big limitation because the present popularity of fluorescent trappable dyes greatly depends on the convenience of their loading procedure.

The results that we have now obtained in a large number of animal cells of different type and origin confirm that fura-2 administered to the cells at 37°C as the AM ester has the tendency to become sequestered in high concentrations within discrete intracellular spots that were found to correspond to membrane-bounded organelles. These organelles contain cholesterol in their limiting membrane (as they are sensitive to digitonin) and possess an acidic content. This latter property is revealed by the overlapping distribution of fura-2 and AO (27), and by the effect of other drugs (chloroquine and monensin). Because of their preferential localization in the perinuclear area and their higher frequency in fibroblasts with respect to bona fide secretory cells such as neutrophils, chromaffin, and PC12 cells, we believe that these acidic organelles are lysosomes. Within the organelles fura-2 was found to be present not as the AM ester, but as a hydrolyzed form(s). The spectrofluorometric technique used, however, cannot discriminate precisely among the fully and the various partially hydrolyzed forms of fura-2 (14, 30). Thus, although the segregated dye might be, at least in part, in the Ca^{2+} binding form, the uncertainties about both the degree of hydrolysis and the pH and ionic environment within the organelles prevented a calculation of absolute $[Ca^{2+}]$ in that segregated compartment. The inaccessibility

to cytosolic Ca^{2+} of the fraction of fura-2 sequestered within the organelles appeared to be responsible for the blunting of $[Ca^{2+}]_i$ transients observed after activation with bradykinin in spottily versus diffusely labeled HSF.

Fura-2 could end up within acidic organelles by at least three different processes: (*a*) diffusion of the hydrophobic AM into the organelles, followed by cleavage and trapping; (*b*) active transport of the cytosolic free acid across the organelle membrane; and (*c*) endocytosis followed by hydrolysis. Various reasons lead us to believe that endocytosis is the responsible process. Spottiness was in fact found to be greater in cells (HSF and 3T3) with high endocytic activity, and to be inhibited at low temperature, as endocytosis does (11). In contrast, no dependence was found on the concentration of fura-2/AM in the loading fluid, and no correlation with the activity of cleaving enzymes, which was higher in the cytosol than in the segregating organelles, the lysosomes. Both these results are the opposite of what is expected if diffusion is involved. Finally it should be emphasized that formation of spots occurred only during, and not after, loading. In other words, cells showing diffuse labeling at the end of the loading incubation did not become spotty when further incubated at 32 or 37°C (see also reference 12). In plant cells and in a macrophagic cell line, however, transport of the cytosolic fura-2 to vacuoles and endosomes, respectively, has been noticed (Hepler, P., M. Poenie, and R. Y. Tsien; and Di Virgilio, F., T. H. Steinberg, A. S. Newman, J. A. Swanson, and S. C. Silverstein, manuscripts submitted for publication). Endocytosis of fura-2/AM during loading (already reported by Giligorski et al. [12]) might be a consequence of the hydrophobic properties of the dye. When fura-2 is added to the medium, it tends to partition into small droplets, removable by filtration or short centrifugation, which fluorescence microscopy shows to be attached to the cell surface. From the cell surface they might be trapped into endocytic vesicles.

In contrast to spotty cells, the diffusely labeled cells were found to be perfectly suited for $[Ca^{2+}]_i$ assays. The results were always satisfactory for the various cell types investigated in terms of reproducibility, sensitivity, and resolution. It should be emphasized, however, that optimal loading conditions were not identical for various cell types. A proper adjustment of those conditions to the experimental system used therefore appears to be necessary. In addition, we recommend a quick check in the fluorescence microscope before any cell batch loaded with fura-2 is used.

Leakage of Fura-2

Leakage to the extracellular medium of the dye already hydrolyzed and trapped into the cytosolic compartment was found to have different consequences in cell suspensions and monolayers. In the fluorometer cuvette, the dye that leaked out of the cells can account for a considerable and increasing proportion of the total fluorescence signal, which must be subtracted before calibration (by whichever procedure) is performed. In contrast, in the microscopic experiments, leakage only results in a decrease of the total signal because the dye that leaks out rapidly diffuses away from the focal plane of the microscope. The ratio calibration then automatically compensates for the loss of the dye (14).

In our experiments leakage was shown to occur from viable cells, as it could be dissociated from two classical signs of cytotoxicity, release to the medium of a cytosolic enzyme and nuclear staining by a nonpermeant dye. Leakage was found to vary markedly among cell types, and to greatly decrease at temperature below 37°C (for example 30–34°C). These temperatures, however, might not be advisable in various kinds of experiments. Leakage of fura-2 (a molecule endowed with five carboxyl residues that in the ionic conditions of the cytosol are expected to be almost all charged) cannot occur by simple diffusion. Rather, the characteristics of the process suggest involvement of a transporter. Such a hypothesis appears to be supported by recent observations in a macrophage line, where leakage of fura-2 has been found to be inhibitable by blockers of anion transport, such as probenecid (10). These drugs appear to work in other cell types as well (Di Virgilio, F., personal communication) and might therefore be destined to widespread use.

Calibration of Fura-2 Signals in Terms of $[Ca^{2+}]_i$

There are essentially two ways of calibrating fura-2 fluorescence in terms of $[Ca^{2+}]_i$: (*a*) by using one single wavelength, e.g., 340 nm, as with quin2, and applying eq. 1 of reference 29 ($[Ca^{2+}]_i = K_d\,[F - Fmin]/[Fmax - F]$; values of Fmin and Fmax can be obtained by either the lysis or the Mn^{2+}-ionomycin procedures [34, 35]); or (*b*) by the ratio calibration mode of Grynkyewicz et al. (14). This is the procedure of choice in microfluorometry, which can also be used in cell suspensions. In the latter condition, however, the procedure is advantageous only when using a fluorometer equipped for rapidly alternation of the excitation wavelengths, otherwise the moment to moment $[Ca^{2+}]_i$ calibration becomes unfeasible. Discrepancies in the results obtained by the two calibration procedures have already been noticed by Tsien and associates (26, 36). An in vitro calibration using a medium with a viscosity similar to that of intact cells was therefore suggested (26). However, it is assumed, but not demonstrated, that in the cytoplasm viscosity is the only parameter affecting fura-2 fluorescence; moreover, cytoplasmic viscosity might not be constant, but might vary as a function of $[Ca^{2+}]_i$. If this was the case, the mimicking of viscosity would become problematic. Our approach to the problem has been different, i.e., we have developed an in situ calibration. Our method is far simpler technically and requires no assumptions as to the factor(s) affecting fura-2 fluorescence within the cytoplasm, but only assumes the K_d of the fura-2–Ca^{2+} interaction to be the same in the intra- and extracellular environments (see also references 14, 26, 28, 32, 34–39). Our method, however, has its own drawbacks. First, reference $[Ca^{2+}]_i$ values are questionable because they are measured by the quin2 technique. This is particularly true for the high $[Ca^{2+}]_i$, because sensitivity to quin2 is very low above 1–2 μM (34). Second, application of the procedure to adherent cells in a microscope is difficult, although not impossible.

The choice of the wavelength pair is another critical point. Tsien and co-workers recommended usage of the 340–380-nm pair in order to make full use of the wavelength shift of fura-2 as a function of calcium concentration. As an alternative, they suggested usage of the 340–361-nm pair (the latter is the isosbestic point of fura-2 in free solution) (14). It is immediately evident from Fig. 9 that even at internal Ca^{2+} as high as 3 μM the value of the ratio is only 62% of Rmax when using the 340–380-nm wavelength pair, while it is ~95% of

Rmax with the 340–361-nm pair. Since it is doubtful that cells with $[Ca^{2+}]_i$ higher than 10 μM will remain healthy for the period of time needed to record a spectrum, it is clear that calibration in intact cells is unsuited for determination of Rmax using the 340–380-nm pair. In addition, a small error in the autofluorescence, which, at high $[Ca^{2+}]_i$ might be comparable to the dye fluorescence at 380 nm, or the presence of a small amount of uncleaved fura-2/AM, which peaks at 390 nm, could cause large errors in the absolute calibration of $[Ca^{2+}]_i$. For all these reasons, 340–361 nm should be considered the pair of choice whenever calibration in intact cells is attempted. Since, however, the intracellular isosbestic point of fura-2 is blue-shifted compared with that in free solution, the wavelength pair to be used is 340–357 nm.

The choice of the wavelength pair can also be limited by the equipment available. Most commercially available microscopes use glass objectives, which drastically cut off light below 350 nm. With such instruments a wavelength pair of 340–357 nm would be impractical to use, as the fluorescence ratio would change only marginally as a function of $[Ca^{2+}]_i$. Most investigators, ourselves included, therefore use a 340–380-nm pair for microfluorometry experiments. Chances of large (and variable) experimental errors are limited by the fact that with a given experimental setting the above values tend to remain constant, and need, therefore, to be checked only occasionally. Moreover, based on our present experience, they seem to be quite similar in different types of cells.

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