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# Photochemically Generated Cytosolic Calcium Pulses and Their Detection by Fluo-3*

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**Joseph P. Y. Kao, Alec T. Harootunian, and Roger Y. Tsien‡**

*From the Department of Physiology-Anatomy, University of California, Berkeley, California 94720*

**Fluo-3, one member of a family of new fluorescent $Ca^{2+}$ indicators excitable at wavelengths in the visible (Minta, A., Kao, J. P. Y., and Tsien, R. Y. (1989) *J. Biol. Chem.* 264, 8171–8178), has been tested in living cells. We demonstrate that fluo-3 can be loaded into fibroblasts and lymphocytes by incubation with the pentaacetoxymethyl ester of the dye and that the ester is hydrolyzed intracellularly to yield genuine fluo-3 capable of indicating changes in $[Ca^{2+}]_i$ induced by agonist stimulation. Fluo-3 can also be microinjected into fibroblasts along with photolabile compounds such as nitr-5 and caged inositol trisphosphate for photorelease experiments. Fluo-3 permits continuous monitoring of $[Ca^{2+}]_i$ without interference with use of UV-sensitive caged compounds. A procedure for combined use of ionophore and heavy metal ions in end-of-experiment calibration of fluo-3 intensities to give $[Ca^{2+}]_i$ is also described.**

With the advent of photolabile compounds which upon photolysis release either $Ca^{2+}$ (nitr-5, nitr-7 (1)) or second messengers which mobilize intracellular $Ca^{2+}$ stores (caged $InsP_3$[1] (2)), or exogenous $Ca^{2+}$ buffer,[2] it has become possible to manipulate or modify the intracellular $Ca^{2+}$ milieu with light. Because light can be easily controlled in intensity, direction, and extent in both space and time, it represents an ideal experimental stimulus in conjunction with intracellularly trapped caged compounds. The ability to generate light flashes of controlled temporal and spatial extent is especially attractive when one wishes to use photorelease techniques for studying phenomena which are spatially heterogeneous (*e.g.* intracellular $Ca^{2+}$ gradients (3, 4)) or rapidly time-varying (*e.g.* $[Ca^{2+}]_i$ oscillations (5–8)). Heretofore, monitoring $[Ca^{2+}]_i$ during a photorelease experiment has been problematical because the commonly used fluorescent $Ca^{2+}$ indicators quin-2, fura-2 and indo-1 all require UV excitation while all known examples of caged compounds are sensitive to UV photolysis. The introduction of fluorescent $Ca^{2+}$ indicators with green excitation wavelengths (see preceding companion paper (9)) in principle provides a solution. We now demonstrate that such an indicator, fluo-3, can easily be loaded into cells either via the pentaacetoxymethyl (AM) ester or by direct microinjection of the free salt and that cells loaded with fluo-3 respond properly to mitogen or peptide hormone stimulation. Fluo-3 permits continuous monitoring of $[Ca^{2+}]_i$ without interfering with UV-triggered release of inositol polyphosphates or $Ca^{2+}$ so that both pre- and post-photolysis $[Ca^{2+}]_i$ levels can be observed. At the end of an experiment, fluo-3 fluorescence intensities are calibrated to yield $[Ca^{2+}]_i$ values by adding a heavy metal in conjunction with ionomycin and then releasing all the dye by permeabilization. Fluo-3, therefore, in addition to being a useful fluorescence $Ca^{2+}$ indicator in its own right, also facilitates quantitative application of photorelease methodology to the study of cellular phenomenon.

## MATERIALS AND METHODS

T-lymphocytes of the tumor line EL4-BU (10) were cultured in RPMI medium 1640 supplemented with 10% (v/v) fetal bovine serum. For AM ester loading, the cells were spun at 1000 rpm for 3–5 min and resuspended at a density of $\sim 10^7$/ml in fresh RPMI containing 20 μM fluo-3/AM. Loading was allowed to proceed for 1 h at 37 °C in a 6% $CO_2$ incubator, with occasional gentle agitation of the cell suspension. To test the completeness of ester hydrolysis, cells were then spun down and resuspended three times in fresh serum-free RPMI 1640 to remove all traces of extracellular fluo-3/AM. The cells were finally resuspended in a $Ca^{2+}$- and $Mg^{2+}$-free supporting electrolyte solution of 100 mM KCl buffered at pH 7.05 with 20 mM MOPS and lysed to liberate the intracellularly trapped fluo-3. Lysis was effected by 3 cycles of rapid freezing in liquid $N_2$ followed by fast thawing in a 37 °C water bath. The lysate was centrifugally filtered through a membrane of 0.45 μm porosity before being used for $Ca^{2+}$/EGTA fluorimetric titration on a spectrofluorometer (Fluorolog 2, Spex Industries, Metuchen, NJ).

The quality of dye loading and extent of dye compartmentation in the EL4-BU cells were assessed by comparing the amount of acridine orange from intracellular organelles (11), lactic dehydrogenase (a cytosolic enzyme), and fluo-3 released by 20 μM digitonin to the total amount of each liberated by 1% (w/w) Triton X-100. EL4-BU lymphocytes were loaded in a 37 °C incubator, at a cell density of 5–10 × $10^6$/ml in RPMI 1640, either for 30 min with 10 μM acridine orange or for 1 h with 20 μM fluo-3/AM. A control suspension with matched cell density was always treated identically except for the absence of dye. The cells were then spun down and resuspended twice in fresh RPMI 1640, and twice in low $Ca^{2+}$ lysis buffer containing 125 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1.1 mM EGTA and 10 mM HEPES, at pH 7.35. The cells were lysed by exposure to either 20 μM digitonin or 1% Triton X-100 in lysis buffer for 5 min at room temperature before the cell suspensions were centrifuged to yield clear supernatant lysates which were used for lactic dehydrogenase assays and dye quantitation. Acridine orange in the lysate was measured by integrating the 500–600 nm fluorescence emission intensity while exciting the solution at 480 nm. To assay the lactic dehydrogenase activity, an aliquot (0.2–0.7 ml) of lysate was rapidly mixed into a fluorescence cuvet containing a solution of NAD and sodium DL-lactate in lysis

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‡ To whom correspondence should be addressed.

[1] The abbreviations used are: caged $InsP_3$, *myo*-inositol 1,4,5-trisphosphate, 4 (or 5)-1-(2-nitrophenyl)ethyl ester; DME, Dulbecco's modified Eagle's medium; AM, acetoxymethyl ester; HEPES, *N'*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, [ethylene bis (oxyethylenenitrilo] tetraacetic acid.

[2] S. R. Adams, J. P. Y. Kao, and R. Y. Tsien, *J. Am. Chem. Soc.*, submitted for publication.

buffer (final concentrations were 1 mM NAD and 10 mM lactate) and measuring the rate of NADH fluorescence emission increase at 440 nm while exciting at 340 nm. To quantitate fluo-3, 1.0 M $CaCl_2$ was added to the sample and control lysates to yield 2 mM free $Ca^{2+}$. The control lysate was titrated with fluo-3 until the amplitude of the emission spectrum matched that of the sample lysate. EL4-BU cell volumes were estimated by measuring cell dimensions under a microscope.

Fisher rat embryo fibroblasts of the cell line REF52 (12; a gift of J. Feramisco) were seeded onto 25-mm diameter glass coverslips and cultured in DME supplemented with 10% (v/v) fetal bovine serum for 3–4 days before use, at which time the cells were effectively serum-starved. During the experiments, which were conducted in air, the fibroblasts were bathed in bicarbonate-free DME buffered only with 20 mM HEPES. NIH 3T3 fibroblasts were cultured in the same way as the REF52 cells but were not serum-starved before use.

For the mitogen-stimulation studies, the REF52 cells were loaded with fluo-3 by incubation at 23 °C for 1 h with 10–20 μM fluo-3/AM in serum-free DME. For photorelease experiments with nitr-5, the REF52 cells were either incubated for 1 h at 23 °C with 10–20 μM nitr-5/AM and 10 μM fluo-3/AM in serum-free DME or directly pressure injected with a microinjection solution containing 110 mM nitr-5 tetrapotassium salt and 10 mM fluo-3 pentapotassium salt in $H_2O$. In the caged $InsP_3$ photorelease experiments, the REF52 cells were injected with a solution consisting of 1 mM caged $InsP_3$ trisodium salt, 10 mM fluo-3 pentapotassium salt, 27 mM $K_2HPO_4$, 8 mM $NaH_2PO_4$, and 26 mM $KH_2PO_4$, pH 7.3. The cells were always allowed 30–40 min to recover from impalement injury before measurements were begun.

Studies on the effects of intracellular pH changes were done using NIH 3T3 fibroblasts loaded with fluo-3/AM or, for comparison, fura-2/AM. The intracellular pH was clamped to well defined values (13, 14) by equilibrating the cells with solutions containing 5 μM nigericin, 140 mM potassium gluconate, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and buffered to known pH values in the range of 6.6–7.4 with either MOPS or HEPES at 20 mM.

In all AM ester loadings, a non-ionic surfactant, Pluronic F-127 (BASF Wyandotte Corp., Wyandotte, MI), was used to aid solubilization of the AM esters into aqueous medium (15). 0.5–1 μl of a 25% (w/w) stock solution of Pluronic F-127 in dry dimethyl sulfoxide was mixed with every 10 nmol of AM ester before thoroughly dispersing the mixture into aqueous solution.

Fluo-3 and its AM ester were initially synthesized as described in the preceding companion paper (9) and were subsequently supplied by Molecular Probes, Inc. Thrombin and Arg-vasopressin were purchased from Sigma and used as aqueous stock solutions. The trisodium salt of caged $InsP_3$ and nitr-5/AM were from Calbiochem. All AM esters were dissolved in dry dimethyl sulfoxide for use. For fluo-3 intensity calibrations, 1.0 M aqueous stock solutions of $MnSO_4$ and $ZnCl_2$ were used. All media and sera for cell culture were obtained from GIBCO.

The fluorescence imaging apparatus was essentially as described previously (14, 15). One 150-watt xenon lamp was used to deliver 490-nm excitation light through a grating monochromator to cells loaded with fluo-3. Flash protolysis was effected by moving a chopper mirror so that a 75-watt xenon lamp, filtered only by a broad-band UV filter (UG-1) illuminated the same cells through the same objective. A custom dichroic mirror (DR505LP/Extended 405DCLP, Omega Optical, Inc., Brattleboro, VT) was placed in the epifluorescence filter cube to reflect both UV and 490 nm light efficiently but transmit the fluorescence emission at >510 nm.

## RESULTS AND DISCUSSION

*Dye Loading and Response*—One of the most useful features of tetracarboxylate indicators and chelators is the possibility of loading them into large populations of cells by hydrolysis of membrane-permeant esters, such as AM esters, without disruption of the cells' plasma membranes. However, experience with previous chelators suggests that increasing molecular weight and decreasing aqueous solubility of AM esters tend to prevent effective loading and complete hydrolysis. Fortunately, fluo-3 does seem to accumulate in lymphocytes and fibroblasts incubated with fluo-3/AM, the pentaacetoxymethyl ester of fluo-3. We have tested loading of fluo-3 into T-lymphocytes of the tumor line EL4-BU by incubation of a cell suspension with 20 μM fluo-3/AM. Fluorescence spectra taken on the loaded cells resuspended after three washes revealed that the fluorescence intensity arising from intracellularly trapped fluo-3 was more than 10 times brighter than the autofluorescence intensity measured for unloaded control cells (data not shown). The trapped dye appeared to be real fluo-3, since upon cell lysis and titration with $Ca^{2+}$ its fluorescence properties matched never-esterified fluo-3. Such titration is illustrated by Fig. 1, which shows a comparison of Scatchard-type plots of $Ca^{2+}$ titration data for a sample of genuine fluo-3 free acid and for the cell lysate from fluo-3/AM-loaded EL4 lymphocytes. Both the 40-fold enhancement of fluorescence upon binding $Ca^{2+}$ and the ~400 nM $Ca^{2+}$ dissociation constant were reproduced, the latter being an important criterion for complete hydrolysis of all the ester groups.

We have checked the intracellular concentration and location of the trapped fluo-3 in AM ester-loaded EL4-BU cells. 20 μM digitonin was sufficient to release essentially all (101 ± 6%) of the cellular lactic dehydrogenase, a standard cytosolic marker enzyme, while liberating only 21% of the acridine orange trapped in subcellular organelles. The same concentration of digitonin released >98% of the intracellular fluo-3. We thus infer that in the EL4-BU lymphocytes the fluo-3 resides predominantly in the cytosol. Using an estimate of EL4-BU cell volume (4.6 $\mu l/10^7$ cells) and the concentration of the fluo-3 in cell lysates, we calculate the average intracellular fluo-3 concentration to be 900 μM after the cells have been loaded with 20 μM fluo-3/AM for 1 h at 37 °C.

Fluo-3/AM loading in REF52 fibroblasts was also effective, although in these cells some compartmentation into internal organelles could be observed. Fig. 2 compares fibroblasts loaded via fluo-3/AM hydrolysis with cells directly microinjected with fluo-3 free acid. In the ester-loaded cells (Fig. 2*a*), some inhomogeneity of fluorescence was visible as a corona around the nucleus, presumably due to some dye uptake into organelles in the cytoplasm. By contrast, in cells injected with free acid (Fig. 2*c*) the cytoplasmic dye fluorescence appeared

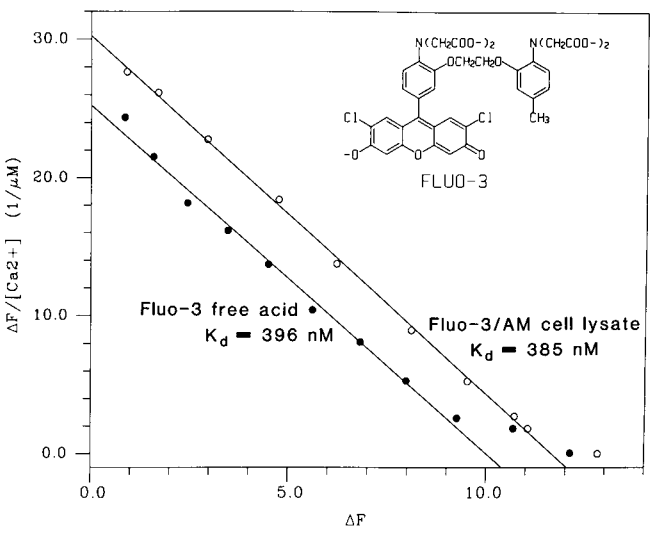


FIG. 1. **Fluo-3/AM is hydrolyzed by cells to yield genuine fluo-3.** Scatchard analysis of the $Ca^{2+}$ titration data for a lysate from EL4-BU T-lymphocytes incubated for 1 h at 37 °C with 20 μM fluo-3/AM (○) and for a genuine, never-esterified sample of fluo-3 free acid (●). The lines shown are linear least squares fits of the experimental data. Data at high $\Delta F$ values with significant deviations from linearity (1 point in the set (○) and 2 points in the set (●)) were excluded from the least squares fits. The chemical structure of the tetracarboxylate form of fluo-3 is also shown.

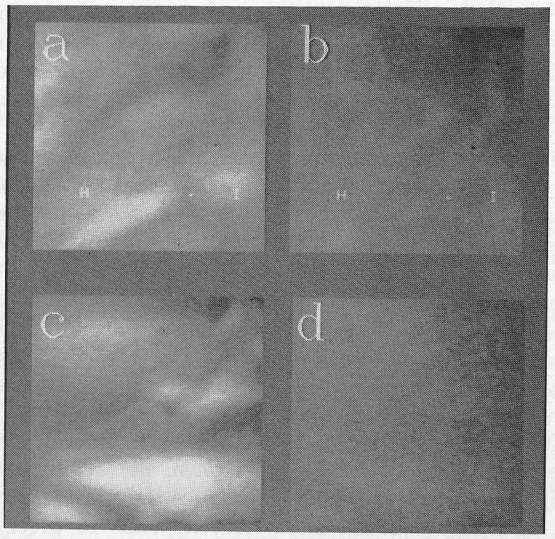


FIG. 2. **Fluorescence video microscopic images of REF52 fibroblasts loaded with fluo-3 via the AM ester (*a, b*) or by direct injection (*c, d*).** *a*, cells incubated for 1 h at 23 °C with 10 μM fluo-3/AM; *b*, the same cells after lysis with 40 μM digitonin. *c*, cells pressure-injected with the pentapotassium salt of fluo-3 (10 mM in micropipette); *d*, the same cells after 40 μM digitonin lysis. The labels (the letters *H* and *I*) appearing in *a* and *b* are remnants inadvertently left by image analysis software and are not germane to the present illustration.

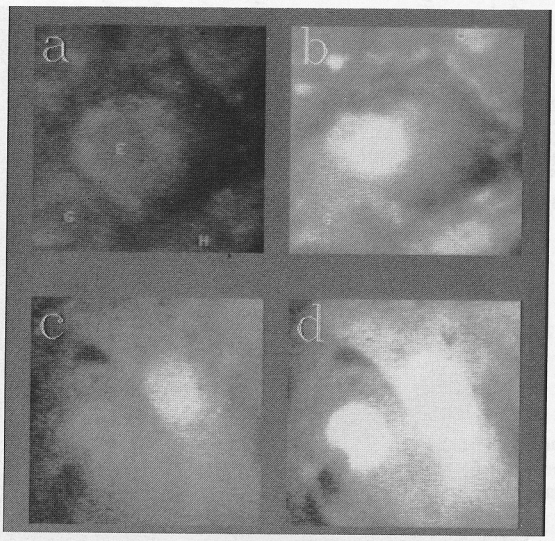


FIG. 3. **REF52 fibroblasts loaded with fluo-3 can respond to stimulation by Arg-vasopressin.** *a*, cells loaded with fluo-3 by incubation with AM ester; *b*, the same cells immediately after stimulation with 30 nM Arg-vasopressin. *c*, cells injected with fluo-3; *d*, same cells immediately following stimulation with 30 nM Arg-vasopressin. The labels (the letters *E, G,* and *H*) in *a* and *b* are remnants inadvertently left by image analysis software and are not germane to the present illustration.

homogeneous and diffuse. The nucleus was brighter than the surrounding cytoplasm, indicating greater cell thickness or possibly some binding in the nuclear region. Further evidence for partial compartmentation of ester-loaded fluo-3 was seen upon lysis of the cells with the plasma membrane-selective detergent digitonin. Ester-loaded cells (Fig. 2*b*) retained a small but noticeable fraction of their original fluorescence as an irregular halo around the nucleus, whereas lysis of injected cells (Fig. 2*d*) left proportionally less fluorescence, which also was more diffuse.

Upon stimulation of the fibroblasts with mitogens or peptide hormones, the intracellular fluo-3 fluorescence intensity increased markedly as expected for a sizable increase in cytosolic $[Ca^{2+}]_i$, suggesting that the normal signal transduction mechanism was not impaired by the presence of fluo-3. Immediately after stimulation, both ester-loaded and directly injected cells (Fig. 3, *b* and *d*) showed a bright nucleus surrounded by diffuse cytoplasmic fluorescence. In the ester-loaded cells, the compartmentalized fluorescence surrounding the nucleus was no longer visible although it is not clear whether those compartments actually lost fluorescence or merely became swamped by the increase in diffuse fluorescence. Even in the continued presence of the agonist, most of the diffuse fluorescence increase (and presumably $[Ca^{2+}]_i$ elevation) was transient (Fig. 4, *b* and *c*), with a decay time of ~1 min. This decline was not due to any failure of the dye's ability to respond to $[Ca^{2+}]_i$, since subsequent addition of 2.5 μM of the $Ca^{2+}$ ionophore ionomycin raised the fluorescence again to even higher levels (Fig. 4*d*). Lysis with 40 μM digitonin induced rapid loss of fluo-3 fluorescence, although once again, in these AM ester-loaded cells, a small amount of fluorescence not immediately releasable by digitonin remained with the cell remnants (Fig. 4*f*).

We have examined the response of cells loaded with fluo-3 to changes in intracellular pH. NIH 3T3 fibroblasts loaded with fluo-3 became marginally brighter as the intracellular pH was lowered from 7.4 to 6.6. This implied that the resting $[Ca^{2+}]_i$ in these cells rose very slightly upon acidification of the cytoplasm. *In situ* $Mn^{2+}$ calibration (see below) of the fluo-3 intensity data from 15 NIH 3T3 cells showed that the average $[Ca^{2+}]_i$ changed from 51 nM at $pH_i = 7.4$ to 93 nM at $pH_i = 6.6$. The pH dependence is very weak, as a drop of 0.8 pH unit resulted in an apparent change in mean $[Ca^{2+}]_i$ of only 42 nM. The dependence of resting $[Ca^{2+}]_i$ on $pH_i$ is probably a real cell biological phenomenon and not an artifact arising from an intrinsic pH-dependence of the fluo-3 indicator. First of all, from *in vitro* measurements, we know that acidification has no effect on fluo-3 fluorescence until the pH drops to ~6.4 (see preceding paper, Ref. 9), whereas the pH effects we observed in the NIH 3T3 cells were evident in the physiological pH range 6.6–7.4. Second, we have performed the $[Ca^{2+}]_i$-$pH_i$ experiment using the well established fluorescent $Ca^{2+}$ indicator fura-2. The fura-2 measurements parallel the fluo-3 results: for 11 cells loaded with fura-2, the mean $[Ca^{2+}]_i$ changed from 72 nM at $pH_i = 7.4$ to 144 nM at $pH_i = 6.6$

*Calibration*—Because fluo-3 responds to $Ca^{2+}$ with only an intensity increase rather than a useful change in excitation or emission ratio, we chose to calibrate fluo-3 using ionomycin and a heavy metal, a method modified from that introduced by Hesketh *et al.* (16) for calibrating quin-2 in single cell microfluorometry. Saturation with $Mn^{2+}$ or $Zn^{2+}$ brings fluo-3 to fluorescences about 20 and 61%, respectively, of that of the $Ca^{2+}$-saturated dye, simulating 100 and 500 nM $Ca^{2+}$, respectively. At the end of an experiment, we usually added micromolar levels of ionomycin, which produced a large $Ca^{2+}$ rise on its own. Enough of a 1 M stock solution of divalent heavy metal ($MnSO_4$ or $ZnCl_2$) was then added to yield a final heavy metal cation concentration in the experimental medium of 2 mM. Once the fluorescence intensity had stabilized under heavy metal treatment, a process requiring normally 10–15 min in REF52 cells, lysis of the cells with digitonin released the dye and permitted recording of the background signal composed of camera dark current and residual autofluorescence from the optics, cell remnants, and media. Control experiments showed that the signal from unloaded cells at similar camera gain settings was unaffected by lysis. Fig. 4,

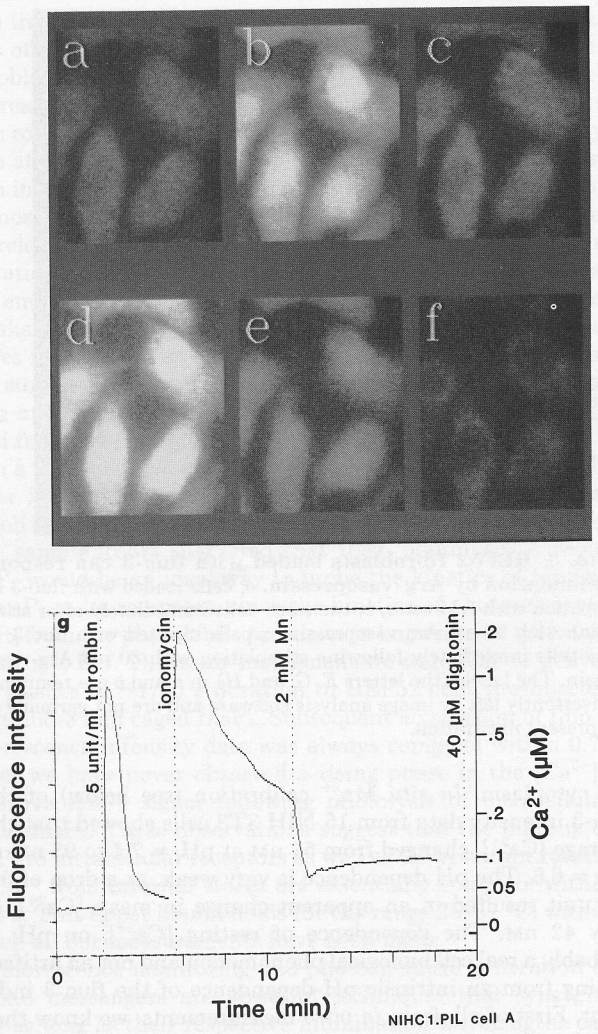


FIG. 4. **Thrombin response of NIH 3T3 fibroblasts loaded with fluo-3/AM.** Fluorescence video images of cells loaded with fluo-3 before stimulation (*a*); 20 s after addition of 5 units/ml thrombin (*b*); 2 min after thrombin addition (*c*); 19 s after adding 2.5 μM ionomycin (*d*); 8 min after adding 2 mM $MnSO_4$ in the continued presence of 2.5 μM ionomycin (*e*); and 34 s after lysis with 40 μM digitonin (*f*). *g* is a time plot of the fluo-3 intensity data from a single cell similar to those shown in *a–f*. Ionomycin-$Mn^{2+}$-digitonin treatment permitted the construction of the $[Ca^{2+}]$ calibration scale shown on the *right axis*. The calibration procedure is described in detail under "Results."

*a–f*, shows selected images of a field of cells, whereas Fig. 4*g* shows the complete time course of response of a single cell loaded with fluo-3/AM to treatment with thrombin followed by ionomycin. A $Mn^{2+}$ calibration was performed before digitonin lysis. From the intensities recorded with either heavy metal before and after lysis, a $Ca^{2+}$ calibration curve could be constructed. For example, if $F_{Mn}$ and $F_{bkg}$ represent the camera signals with ionomycin-$Mn^{2+}$ before and after lysis, then the predicted fluorescence $F_{max}$ from $Ca^{2+}$-saturated cellular dye would be $(F_{Mn} - F_{bkg})/0.2 + F_{bkg}$. Since metal-free fluo-3 has 1/40 the fluorescence of the $Ca^{2+}$ complex, the camera signal $F_{min}$ from the cellular dye should be $(F_{max} - F_{bkg})/40 + F_{bkg}$. Cytosolic $Ca^{2+}$ for any previous signal $F$ can then be estimated by the well known equation $[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$, where $K_d$ is 400 nM at vertebrate ionic strength. The $[Ca^{2+}]_i$ values on the right hand axis of Fig. 4*g* were calculated using the procedure described above. Like all calibration methods in which cytosolic $Ca^{2+}$ is not actually clamped to known levels *in situ*, this procedure assumes that dye in cells behaves similarly to dye *in vitro*, particularly with regard to the $K_d$ values for $Ca^{2+}$ and the relative fluorescence efficiencies for the $Ca^{2+}$ and heavy metal complexes with respect to the uncomplexed species.

A consistency check for this procedure was that the fluorescence obtained from ionomycin in $Ca^{2+}$ medium before heavy metal addition should approach the calculated $F_{max}$, as if $[Ca^{2+}]_i$ levels of many micromolar were attained. This test was generally successful, though we preferred not to rely on the signal in ionomycin-$Ca^{2+}$ as the primary measure of $F_{max}$, because the rise in $[Ca^{2+}]_i$ was often poorly sustained. Because fluo-3 has a higher $K_d$ for $Ca^{2+}$ than does quin-2, it is correspondingly more difficult to saturate fluo-3 with $Ca^{2+}$, especially to reach a well-defined steady state without causing toxic cell injury and dye loss.

Efforts to use both $Mn^{2+}$ and $Zn^{2+}$ consecutively on the same cells were not successful. Indeed, $Zn^{2+}$ calibration is much more difficult to apply in practice than $Mn^{2+}$. Because $Zn^{2+}$ is a highly acidic metal ion, it tends to precipitate hydroxides at physiological pH. This drawback makes $Zn^{2+}$ less attractive for *in situ* calibration than $Mn^{2+}$.

*Intracellular $Ca^{2+}$ Release by Nitr-5 Flash Photolysis*—Once fluo-3 was shown to work as a $Ca^{2+}$ indicator in intact cells, it was appropriate to use it to calibrate the effectiveness of photosensitive $Ca^{2+}$ chelators as well as caged $InsP_3$. Fig. 5*a* is a record of the fluorescence intensity of a fibroblast injected

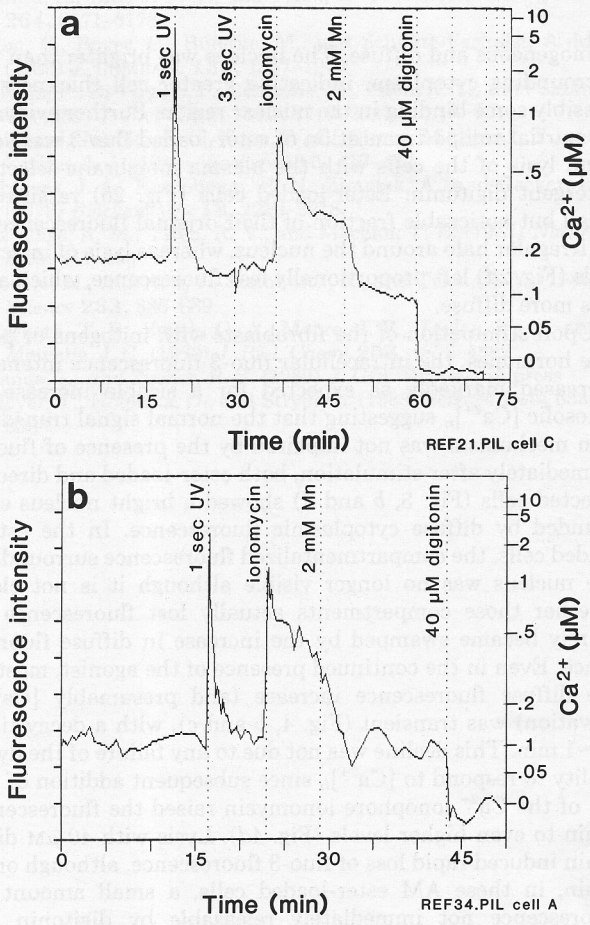


FIG. 5. **Fluo-3 monitoring of $Ca^{2+}$ photoreleased from intracellular nitr-5 in cells coinjected with nitr-5 and fluo-3 (*a*) and cells incubated for 1 h at 23 °C with 20 μM nitr-5/AM and 10 μM fluo-3/AM (*b*).** 2.5 μM ionomycin was added at the times indicated. The signal-to-noise is better for *trace a* than for *trace b* because more fluo-3 and nitr-5 were loaded by microinjection into the cell used to obtain *trace a*.

with a mixture of 100 mM of the photolabile chelator nitr-5 and 10 mM fluo-3. Although no $Ca^{2+}$ was deliberately added to the pipette filling solution, the fluorescence of the cells just after injection (not shown) was high, indicating that enough $Ca^{2+}$ entered due to impalement damage so as not to need any supplementation from the pipette contents. (Experiments with fura-2 and $Na^+$ indicators confirm that the act of pressure microinjection in normal medium is sufficient to raise fibroblast $[Ca^{2+}]_i$ and $[Na^+]_i$ drastically.)[3] However, the elevated $[Ca^{2+}]$ soon declined as the impalement wound healed and $Ca^{2+}$ pumping and sequestration proceeded.

Illumination for 1 s with 340–380 nm light from a xenon arc focused through the objective caused a massive rise in $[Ca^{2+}]_i$ (Fig. 5*a*). This elevation was surprisingly transient, as shown in Fig. 7, which is an expansion of the time interval around the photolysis. The recovery of $[Ca^{2+}]_i$ seemed quite non-exponential, consisting of at least two phases, a fast drop to micromolar levels followed by a much slower final adjustment to pre-flash levels. Subsequent re-exposure to UV light (Fig. 5*a*) even for the longer duration of 3 s had no effect, showing that the first 1 s of illumination had completely photolyzed the nitr-5 and that UV did not bleach the fluo-3 or detectably damage the cell. Addition of ionomycin elevated $[Ca^{2+}]_i$ again, although not even as high as the first nitr-5 photolysis had reached. However, the ionomycin effect was at least partly sustained, presumably by continued influx of $Ca^{2+}$ across the plasma membrane. The pre-illumination resting value of $[Ca^{2+}]_i$ was about 170 nM, which would be sufficient for the nitr-5 ($K_d \approx 150$ nM) to have been about half-saturated with $Ca^{2+}$. Upon complete photolysis of the nitr-5, the $[Ca^{2+}]_i$ should therefore have jumped to ~6 μM, the $K_d$ of photolyzed nitr-5, as indeed the calibration indicates.

Nitr-5 can also be co-loaded with fluo-3 into cells using the AM esters. Fig. 5*b* shows a record of the fluorescence of a REF52 fibroblast which had been incubated for 1 h at 23 °C with 20 μM nitr-5/AM and 10 μM fluo-3/AM before observation. The record is basically similar to that shown in Fig. 5*a* in that a 1-s UV flash released $Ca^{2+}$ from nitr-5 and subsequent application of 2.5 μM ionomycin produced a rise in $[Ca^{2+}]_i$, although the trace is noisier. $Mn^{2+}$ equilibration and digitonin lysis yielded data for constructing the $[Ca^{2+}]_i$ calibrations indicated on the *right axis* of Fig. 5*b*.

*Intracellular $Ca^{2+}$ Mobilization by Photorelease of Caged $InsP_3$*—Suddenly liberation of $InsP_3$ inside a fibroblast also produced a spike in $[Ca^{2+}]_i$ (Fig. 6). In this experiment, the biologically inert but photolabile 4 (or 5)-1-(2-nitrophenyl)ethyl ester of $InsP_3$ was pre-injected into the cell along with fluo-3. At the time indicated, UV from the xenon lamp was allowed to illuminate the cell for 1 s, thereby photolyzing the ester to $InsP_3$, which released $Ca^{2+}$ presumably from internal stores. $[Ca^{2+}]_i$ transiently rose high enough nearly to saturate the dye (>5 μM) and then fell again very rapidly, considerably faster than the recovery after illuminating nitr-5. Further delivery of two 1-s UV pulses elicited no further transient rises in $[Ca^{2+}]_i$ (Fig. 6), suggesting that all the caged $InsP_3$ had been photolyzed by the first UV flash. Indeed, in separate *in vitro* calibration tests we have found that, at the light intensities used in these experiments, a 1-s UV flash photolyzed >90% of the caged $InsP_3$ injected (data not shown). In Fig. 6 the rate of recovery of $[Ca^{2+}]_i$ reflects the conversion of the $InsP_3$ to less active metabolites and the rate of $Ca^{2+}$ pumping back into depleted storage organelles, whereas in the nitr-5 experiments (Fig. 5, *a* and *b*) the rate of recovery reflects $Ca^{2+}$ pumping that has to fight the buffering of the photolyzed chelator. Evidently, $InsP_3$ metabolism imposes less kinetic limitation than excess $Ca^{2+}$ buffering or increased repletion of the $Ca^{2+}$ stores.

[3] A. Harootunian, unpublished results.

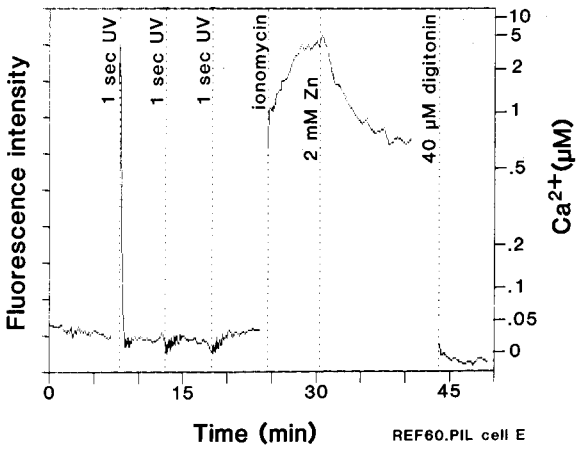


FIG. 6. **Fluo-3 monitoring of $Ca^{2+}$ mobilization by photoreleased $InsP_3$ in REF52 fibroblasts.** Cells were co-injected with caged $InsP_3$ and fluo-3. At the time indicated, 2.5 μM ionomycin was added to the medium. A $Zn^{2+}$ calibration yielded the $[Ca^{2+}]_i$ values indicated on the *right axis*.

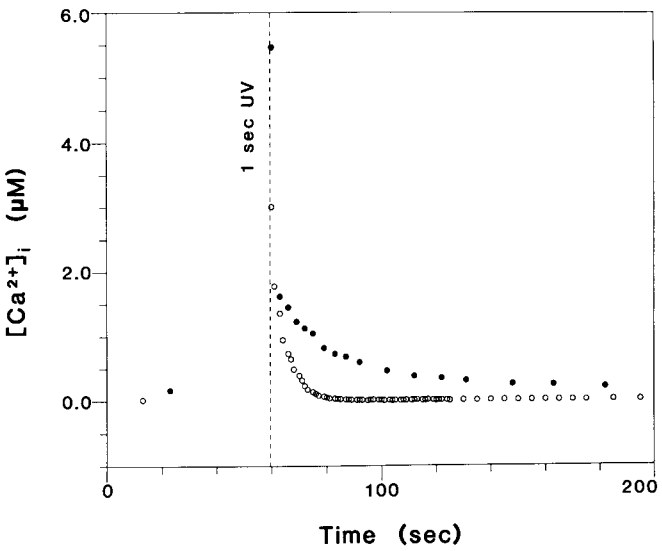


FIG. 7. **Comparison of the kinetics of intracellular $Ca^{2+}$ sequestration after flash photolysis of nitr-5 (●) and caged $InsP_3$ (○).** The plot is a superposition of the records from Fig. 5*a* and Fig. 6 expanded around the time of UV flash photolysis.

Our intracellular testing of fluo-3 in fibroblasts showed that the dye seemed fairly well behaved as a $[Ca^{2+}]_i$ indicator, responding well to mitogen and peptide hormone stimulation or ionophore treatment. Dye bleaching and leakage were low enough to give reasonably stable base lines for an hour or so and to permit retrospective calibration of single-cell records by addition of ionomycin-$Mn^{2+}$ followed by lysis at the end of the experiment. The two major advantages of fluo-3 over fura-2 and quin-2 are both reflected in Figs. 5–7: fluo-3 avoids UV excitation that would photolyze caged compounds, and it resolves $[Ca^{2+}]_i$ levels of 2–10 μM that would saturate fura-2 and quin-2. The decay of $[Ca^{2+}]_i$ after nitr-5 photolysis is surprisingly fast, considering that the cell contained millimolar levels of nitr-5 about half-saturated with $Ca^{2+}$. Nearly all that $Ca^{2+}$ would have to be sequestered or extruded from the cell in order to return $[Ca^{2+}]_i$ to its pre-flash resting value, yet the task was accomplished in a minute or two. This result is quite different from the behavior of invertebrate giant neurons, in which comparable photolyses of nitr-5 result in

step increases of $[Ca^{2+}]_i$ that are maintained for minutes to tens of minutes (17). The decay may be faster in mammalian fibroblasts because of more active $Ca^{2+}$ uptake systems as well as greater surface-to-volume ratio; the kinetics are consistent with roles for both high capacity, low affinity and low capacity, high affinity $Ca^{2+}$ removal systems. Even faster kinetics were seen in the recovery of $[Ca^{2+}]_i$ after photochemical generation of inositol 1,4,5-trisphosphate, believed to be the messenger for release of $[Ca^{2+}]_i$ from internal stores. The simplest interpretation is that the $InsP_3$ is very rapidly metabolized, so that the emptied internal stores quickly switch back to $Ca^{2+}$ reuptake. By contrast, $Ca^{2+}$ sequestration into the internal stores may be less effective in the nitr-5 experiment because the stores start partly loaded with $Ca^{2+}$ and would become even more heavily loaded once they start to absorb the $Ca^{2+}$ shed from the chelator.

In a recent study on the cooperative binding of $Ins(1,4,5)P_3$ to its intracellular receptors, Meyer *et al.* (18) set an upper bound of 4 s for the kinetics of binding of $InsP_3$ to its receptor. The same authors suggested that flash photolysis of caged $InsP_3$ would be an ideal way to probe the kinetics of binding of $InsP_3$ to its receptors, since the kinetics of photorelease of $InsP_3$ from the photolabile precursor is known to be quite fast ($>231\ s^{-1}$, Ref. 2). In our instrument we were able to deliver UV flashes of ~1/60 s duration to REF52 cells pre-injected with fluo-3 and caged $InsP_3$. Subsequent acquisition of fluo-3 fluorescence intensity data was always complete within 0.75 s, yet we have never observed a rising phase in the $[Ca^{2+}]_i$-sensitive fluo-3 signal following photolysis of intracellular caged $InsP_3$. These observations suggest that the binding of $InsP_3$ to intracellular receptors as well as the resultant release of $Ca^{2+}$ from internal stores are essentially complete within 0.75 s. This upper bound holds for the range 25–37 °C, within which all our measurements have been made.

Fluorescence monitoring and photochemical generation of second messengers are powerful techniques that synergize well as long as their respective illumination wavelengths can be kept separate. Fluorescence measurements by themselves all too often result merely in descriptive accounts of $[Ca^{2+}]_i$ changes without clear evidence for the necessity or sufficiency of such events in cell function. Photochemical perturbations of $[Ca^{2+}]_i$ can help remedy that failing, but themselves need quantification to enable comparison with endogenous transients. Elaborate pulsed lasers or capacitance-discharge lamps are not necessary if time resolutions of a second or so are adequate. Instead, shutter-controlled illumination with an ordinary xenon lamp fitted with a broadband UV filter is quite sufficient. The relatively simple photochemical instrumentation coupled with fluorescent $Ca^{2+}$ indicators such as fluo-3, whose excitation and emission wavelengths fall within the visible, should permit more quantitative intracellular application of caged compounds which perturb $Ca^{2+}$ mobilization and in so doing enhance the power and versatility of photorelease methods.

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