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 photo-release experiments. Fluo-3 permits continuous monitoring of [Ca\(^{2+}\)] without interference with use of UV-sensitive caged compounds. A procedure for combined use of ionophore and heavy metal ions in end-of-experiment calcium 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\(_3\) (2)), or exogenous Ca\(^{2+}\) buffer, 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\(^{2+}\)], oscillations (5–8)). Heretofore, monitoring [Ca\(^{2+}\)], 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 ionic 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 fluorescent Ca\(^{2+}\) indicator in its own right, also facilitates quantitative application of photorelease methodology to the study of cellular phenomena.

**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 ~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 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 fluorometric 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/v) Triton X-100. EL4-BU lymphocytes were loaded in a 37°C incubator, at a cell density of 5-10 x 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 µM 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 bi-lactate in lysis buffer.
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 mM CaCl2 was added to the sample and control lysates to yield 2 mM free Ca2+. 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 DMEM 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 potassium-stimulation studies, the REF52 cells were loaded with fluo-3 by incubation at 22 °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 non-ionic surfactant, Pluronic F-127 (BASF Wyandotte Corp., Wyandotte, MI), in the absence or presence of 10 mM 490-nm excitation light through a grating monochromator to cells. The fluorescence microscope was essentially as described and the intracellular pH was clamped to well defined values (13, 14) by equilibrating the cells with solutions containing 110 mM nitr-5/AM and 10 mM 490-nm excitation light through a grating monochromator to cells. The fluorescence microscope was essentially as described and the intracellular pH was clamped to well defined values (13, 14) by equilibrating the cells with solutions containing 110 mM nitr-5/AM and 10 mM 490-nm excitation light through a grating monochromator to cells.

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 ester into aqueous medium. Non-ionic surfactant solutions containing 5 μM nigericin, 1.5 mM CaCl2, 1 μg/mL Pluronic F-127, and 25 mM glucose, and buffer to known pH values in the range of 6.6-7.4 were used. The cells were then allowed 20-40 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 110 mM nitr-5/AM and 10 mM 490-nm excitation light through a grating monochromator to cells. The fluorescence microscope was essentially as described and the intracellular pH was clamped to well defined values (13, 14) by equilibrating the cells with solutions containing 110 mM nitr-5/AM and 10 mM 490-nm excitation light through a grating monochromator to cells.

Fluo-3 and its AM ester were initially synthesized as described previously (15, 14) and were subsequently supplied by Molecular Probes, Inc. (Eugene, OR). 50 mM fluoro-3/AM was dissolved in dry dimethyl sulfoxide for use. For fluorescence measurements, the AM esters were dissolved in dry dimethyl sulfoxide for use. For fluorescence measurements, the AM esters were dissolved in dry dimethyl sulfoxide for use.

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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 was more diffuse.

After lysis of the cells with the plasma membrane-selective detergent digitonin, the plasma membrane-selective surrounding cytoplasm, indicating greater cell thickness or homogeneous and diffuse. The nucleus was brighter than the surrounding cytoplasm. In the present illustration, 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 [Ca2+]i-pH experiment using the well established fluorescent Ca2+ indicator fura-2. The fura-2 measurements parallel the fluo-3 results: for 11 cells loaded with fura-2, the mean fluorescence not immediately releasable by digitonin remained with the cell remnants (Fig. 4f).

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 [Ca2+], in these cells rose very slightly upon acidification of the cytoplasm. In situ Mn2+ calibration (see below) of the fluo-3 intensity data from 15 NIH 3T3 cells showed that the average [Ca2+], changed from 51 nM at pH = 7.4 to 93 nM at pH = 6.6. The pH dependence is very weak, as a drop of 0.8 pH unit resulted in an apparent change in mean [Ca2+], of only 42 nM. The dependence of resting [Ca2+], on pH, 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 [Ca2+]i-pH experiment using the well established fluorescent Ca2+ indicator fura-2. The fura-2 measurements parallel the fluo-3 results: for 11 cells loaded with fura-2, the mean [Ca2+], changed from 72 nM at pH = 7.4 to 144 nM at pH = 6.6.

**Calibration**—Because fluo-3 responds to Ca2+ 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 Mn2+ or Zn2+ brings fluo-3 to fluences about 20 and 61%, respectively, of that of the Ca2+-saturated dye, simulating 100 and 500 nM Ca2+, respectively. At the end of an experiment, we usually added micromolar levels of ionomycin, which produced a large Ca2+ rise on its own. Enough of a 1 M stock solution of divalent heavy metal (MnSO4 or ZnCl2) 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,
Photochemical Manipulations of Ca²⁺ Viewed with Fluo-3

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 MnSO₄ in the continued presence of 2.5 μM ionomycin (e); and 34 s after adding 2.5 μM ionomycin (f).

Fig. 5. Fluo-3 monitoring of Ca²⁺ photorelease 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 Ca²⁺ was deliberately added to the pipette filling solution, the fluorescence of the cells just after injection (not shown) was high, indicating that enough Ca²⁺ 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 [Ca²⁺], and [Na⁺], drastically.³ However, the elevated [Ca²⁺] soon declined as the impalement wound healed and Ca²⁺ 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 [Ca²⁺], (Fig. 5a). This elevation was surprisingly transient, as shown in Fig. 7, which is an expansion of the time interval around the photolysis. The recovery of [Ca²⁺] 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. 5a) 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 [Ca²⁺], 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 Ca²⁺ across the plasma membrane. The pre-illumination resting value of [Ca²⁺], was about 170 nM, which would be sufficient for the nitr-5 (Kₐ = 150 nM) to have been about half-saturated with Ca²⁺. Upon complete photolysis of the nitr-5, the [Ca²⁺], should therefore have jumped to ~6 μM, the Kₛ 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. 5b 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. 5a in that a 1-s UV flash released Ca²⁺ from nitr-5 and subsequent application of 2.5 μM ionomycin produced a rise in [Ca²⁺], although the trace is noisier. Mn²⁺ equilibration and digitonin lysis yielded data for constructing the [Ca²⁺], calibration indicated on the right axis of Fig. 5b.

Intracellular Ca²⁺ Mobilization by Photorelease of Caged InsP₃. Suddenly liberation of InsP₃ inside fibroblast also produced a spike in [Ca²⁺], (Fig. 6). In this experiment, the biologically inert but photolabile 4-(or 5)-1-(2-nitrophenyl)ethyl ester of InsP₃ 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 InsP₃, which released Ca²⁺ presumably from internal stores. [Ca²⁺], 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 [Ca²⁺], (Fig. 6), suggesting that all the caged InsP₃ 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 InsP₃ injected (data not shown). In Fig. 6 the rate of recovery of [Ca²⁺], reflects the conversion of the InsP₃ to less active metabolites and the rate of Ca²⁺ pumping back into depleted storage organelles, whereas in the nitr-5 experiments (Fig. 5, a and b) the rate of recovery reflects Ca²⁺ pumping that has to fight the buffering of the photolyzed chelator. Evidently, InsP₃ metabolism imposes less kinetic limitation than excess Ca²⁺ buffering or increased repletion of the Ca²⁺ stores.

Our intracellular testing of fluo-3 in fibroblasts showed that the dye seemed fairly well behaved as a [Ca²⁺], 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-Mn²⁺ 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 [Ca²⁺], levels of 2–10 μM that would saturate fura-2 and quin-2. The decay of [Ca²⁺], after nitr-5 photolysis is surprisingly fast, considering that the cell contained millimolar levels of nitr-5 about half-saturated with Ca²⁺. Nearly all that Ca²⁺ would have to be sequestered or extruded from the cell in order to return [Ca²⁺], 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

³ A. Harootunian, unpublished results.
step increases of [Ca\textsuperscript{2+}], that are maintained for minutes to tens of minutes (17). The decay may be faster in mammalian fibroblasts because of more active Ca\textsuperscript{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\textsuperscript{2+} removal systems. Even faster kinetics were seen in the recovery of [Ca\textsuperscript{2+}] after photochemical generation of inositol 1,4,5-trisphosphate, believed to be the messenger for release of [Ca\textsuperscript{2+}], from internal stores. The simplest interpretation is that the InsP\textsubscript{3} is very rapidly metabolized, so that the emptied internal stores quickly switch back to Ca\textsuperscript{2+} reuptake. By contrast, Ca\textsuperscript{2+} sequestration into the internal stores may be less effective in the nitr-5 experiment because the stores part load with Ca\textsuperscript{2+} and would become even more heavily loaded once they start to absorb the Ca\textsuperscript{2+} shed from the chelator.

In a recent study on the cooperative binding of Ins(1,4,5)P\textsubscript{3} to its intracellular receptors, Meyer et al. (18) set an upper bound of 4 s for the kinetics of binding of InsP\textsubscript{3} to its receptor. The same authors suggested that flash photolysis of caged InsP\textsubscript{3} would be an ideal way to probe the kinetics of binding of InsP\textsubscript{3} to its receptors, since the kinetics of photorelease of InsP\textsubscript{3} from the photolabile precursor is known to be quite fast (>231 s\textsuperscript{-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\textsubscript{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\textsuperscript{2+}]\textsubscript{i} sensitive fluo-3 signal following photolysis of intracellular caged InsP\textsubscript{3}. These observations suggest that the binding of InsP\textsubscript{3} to intracellular receptors as well as the resultant release of Ca\textsuperscript{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\textsuperscript{2+}], changes without clear evidence for the necessity or sufficiency of such events in cell function. Photochemical perturbations of [Ca\textsuperscript{2+}], 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\textsuperscript{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\textsuperscript{2+} mobilization and in so doing enhance the power and versatility of photo-release methods.

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