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The calcium concentration clamp: spikes and reversible pulses using the photolabile chelator DM-nitrophen

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Abstract — New procedures are described for producing brief transients and reversible elevations in [Ca] that can be used to quantitatively control the concentration of cytoplasmic calcium. If the photolabile calcium chelator DM-nitrophen, partially bound to calcium, is exposed to steady illumination, [Ca] can be raised from a few nM to up to 10 μM for durations of 100 ms or longer, depending on light intensity and duration. An association rate of calcium with nitrophen of 1.5 x 10^6 $M^{-1}s^{-1}$ was estimated from measurements of [Ca] using the fluorescent indicator Fluo-3, and calcium was found to speed the photolysis of nitrophen 2.5-times. Partial photolysis of DM-nitrophen partly loaded with calcium elicits a [Ca] spike of over 100 μM lasting about 1 ms, depending on intensity and duration of the light flash. Simulations of the reactions involved predict changes in Fluo-3 fluorescence measured at high time resolution with a laser scanning confocal microscope. These procedures have been applied in physiological experiments to generate cytoplasmic [Ca] spikes and pulses and study the cellular responses to them.

The 'caged calcium' chelators, nitr-5 and DM-nitrophen [1, 2], have recently been developed as tools to elevate intracellular calcium concentration. Until now, their use has been limited to step rises in [Ca] following partial photolysis of the chelator. Nitr-5 is particularly well suited to this application. A brief, intense flash of ultraviolet light (360 nm) converts the nitrobenzhydrol nitr-5, with a calcium affinity of 145 nM at 0.1–0.15 M ionic strength, to a nitrosobenzophenone with calcium affinity of 6 μM at a rate of about 3000 s^{-1} [1]. After photolysis, calcium re-equilibrates rapidly with the newly formed nitrosobenzophenone and the remaining unphotolyzed nitrobenzhydrol, with binding rates in excess of 5 x 10^8 $M^{-1}s^{-1}$ (calculated from [1]), close to the limit imposed by diffusion. Thus calcium equilibration with 10 mM nitr-5 occurs with a half-time of about 2 μs, significantly faster than the rate of photolysis, so [Ca] rises smoothly from its prephotolysis level to a new higher level after photolysis.

DM-nitrophen behaves differently. Its photolysis rate is somewhat faster, with a maximal half-time of 180 μs [3]. Although nitrophen binds calcium very strongly (5 nM affinity at 0.1 M ionic

strength), the photoproducts bind calcium only very weakly (affinity roughly 1 mM), so [Ca] will be determined almost exclusively by binding to unphotolyzed nitrophen. However, rebinding to unphotolyzed nitrophen is likely to be slow, as has been shown for related calcium chelators [4]. Thus calcium released rapidly from photolyzed nitrophen will remain relatively free for some time, until re-equilibration with unphotolyzed nitrophen is complete. This should result in a calcium concentration 'spike' following partial flash photolysis of nitrophen [5].

Another consequence of the slow binding of calcium to nitrophen is that [Ca] should rise significantly during exposure to steady light. This is due to the lag in rebinding of calcium as it is released steadily by continual photolysis. If not too much nitrophen has been photolyzed (as in the case of sufficiently dim or brief exposures), the concentration of nitrophen will change little during illumination, and the rates of release and rebinding of calcium will be constant. This should result in a steady level of [Ca], which will drop back to very low levels as soon as the light is extinguished, so long as unphotolyzed nitrophen remains in excess of total calcium. This should produce a calcium concentration 'pulse' or a reversible rise in calcium to a level determined by the light intensity, and duration determined by the light duration. This might be regarded as a sort of 'calcium clamp', that could be a valuable tool for investigating calcium action in cellular processes.

The purpose of this study was to confirm the ability of DM-nitrophen to generate 'calcium concentration spikes' and 'calcium concentration pulses' under appropriate conditions of photic stimulation, and to get some idea of the range of calcium concentrations over which calcium can be manipulated in these ways.

Materials and Methods

Micro-cuvettes and test solutions

Calcium released from photolysis of DM-nitrophen was measured using the fluorescent calcium indicator Fluo–3, in an ionic environment resembling cell cytoplasm and in micro-cuvettes small enough that the intensities of the photolysis and fluorescence excitation lights were reasonably constant. Cuvettes of 50 μm path length had an absorbance of 0.22 at 360 nm when filled with 10 mM DM-nitrophen. These cuvettes (from Vitro Dynamics, Rockaway, NJ, USA), were 0.5 mm wide and cut to 2 mm lengths, to be smaller than the focused light spot of our light sources and so be exposed to uniform light intensities. 10 mM tetrasodium nitrophen (Calbiochem, La Jolla, CA, USA) and 0.3 mM Fluo–3 (Molecular Probes, Eugene, OR, USA) were dissolved in 95 mM K-glutamate, and 40 mM K-MOPS (Sigma, St Louis, MO, USA), pH 7.2. Nitrophen was loaded 30% with calcium (i.e. 3 mM $CaCl_2$ was added to the solution), and the uncomplexed nitrophen was neutralized with 7 mM HCl. This resulted in 7 mM of trisodium nitrophen, 3 mM of calcium disodium nitrophen, and 13 mM NaCl. The final ionic strength of the solution was calculated to be 180 mM. Cuvettes filled with this solution were placed in a glass bottom Petri dish under a thin layer of light mineral oil to prevent evaporation.

Since the composition of this solution was important, the purity of the nitrophen had to be assessed. This was done by adding 5–10 μl aliquots of 1 mM $CaCl_2$ to 1 ml of nominally 100 μM tetrasodium nitrophen and measuring [Ca] with a calcium-sensitive electrode (Orion 93-20, Cambridge, MA, USA; Ag-AgCl reference electrode MI-402 from Microelectrodes, Inc., Londonderry, NH, USA). For 100% pure nitrophen, [Ca] should rise suddenly from undetectable levels on adding 100 μl of the $CaCl_2$, and reach 10 μM at 110 μl. For fresh solutions of nitrophen, these transitions occurred at 90 and 100 μl respectively, indicating 90% purity. For month-old solutions that had been stored in the dark at –5°C, but thawed, opened, and exposed to room light about 30-times, purity dropped to 75%. The degree of impurity was taken into account when mixing solutions.

[Ca] measurement

[Ca] was measured by monitoring Fluo–3 fluorescence using an MRC-600 laser scanning confocal microscope (Bio-Rad Microscience, Cambridge,

MA, USA) and the fluorescein (BHS) filter set. This arrangement prevents photolysis of nitrophen by the fluorescence excitation beam [6]. Cuvettes were viewed with a Nikon (Garden City, NJ) Plan 10x objective, 0.30 n.a. The confocal aperture was adjusted to 30% maximum for a 20 μm confocal depth, and a focal plane at or slightly above the middle of the cuvette was used. Bleaching of Fluo–3 was minimized by using a 3% neutral density filter in the laser beam. The display field was filled with the cuvette image using a zoom of 2.5. Black level was set with auto-black off, to give a background reading adjacent to a cuvette of 15 bits, while a linear gain was chosen to give a high-calcium reading of 215 bits. High speed [Ca] measurements were obtained using the microscope in line scanning mode, with no delay between adjacent scans. Images were stored digitally, and hardcopy was produced with a Sony (Tokyo, Japan) UP- 811 video graphic printer having a 64-step grey scale.

Nitrophen is known to affect the sensitivity of Fluo–3 to calcium [7]. The Fluo–3/nitrophen mixture was calibrated for calcium sensitivity in the series of buffers shown in the Table. All had pH adjusted to 6.9 with K-PIPES (Sigma), and ionic strength of 180 mM. The free calcium concentration in each solution was calculated using the effective dissociation constants for EGTA given by Tsien and Pozzan [8], and the hydrogen and calcium binding constants for HEEDTA given by Martell and Smith [9].

Nitrophen photolysis

Two light sources were used to photolyze nitrophen. The first was an ILC Technology (Sunnyvale, CA, USA) 150 W Cermax xenon arc lamp, focused through an f/1.5 75-mm focal length quartz lens. Light duration was controlled with a Vincent Associates (Rochester, NY, USA) Uniblitz electrical shutter. The second illuminator was a Chadwick-Helmuth (El Monte, CA, USA) Strobex 238 200-joule xenon arc flash lamp system. Light from the bulb was collected with an elliptical reflector [10]. A new high-inductance pulse extender (Chadwick-Helmuth 11122) was added to produce light flashes of about 1 ms duration with more efficient electrical-to-UV energy conversion. Light intensity was monitored with a photodiode and current-to-voltage converter. Records were digitized and stored on a Nicolet (Madison, WI, USA) 4094 oscilloscope and subsequently transferred to an IBM PC using Vu-Point software (S-Cubed, La Jolla, CA, USA). Infrared and far UV were removed from both sources with a liquid filter as described earlier [11].

Absorbance measurements

The calcium-dependence of nitrophen photolysis was determined by measuring the absorbance spectra of 100 mM ionic strength solutions containing 200 mM of either K-MOPS (pH 7.2), K-Trizma

Table Composition of calibration solutions

	Free [Ca] (μM)					
Salts	*0*	*0.29*	*0.59*	*1.2*	*6.6*	*2000*
Na_4NP	10	10	10	10	10	10
HCl	10	–	–	–	–	–
$CaCl_2$	–	10	10	10	10	12
PIPES	40	40	40	40	40	40
KCl	78	10	10	10	10	92
Fluo–3	0.3	0.3	0.3	0.3	0.3	0.3
CaEGTA	–	14.5	29	58	–	–
K_2EGTA	–	29	29	29	–	–
CaHEEDTA	–	–	–	–	29	–
$K_2HEEDTA$	–	–	–	–	29	–

All concentrations are in millimolar

(pH 8.3) or K-MES (pH 6.1), plus 60 μM DM-nitrophen and either 0 or 2 mM calcium, following different periods of exposure to the steady UV light. All buffers were obtained from Sigma. Absorbance spectra were obtained using 1 mm pathlength quartz cuvettes on a Beckman (Fullerton, CA, USA) DU-70 scanning spectrophotometer and transferred to an IBM PC using a Beckman Data Transporter.

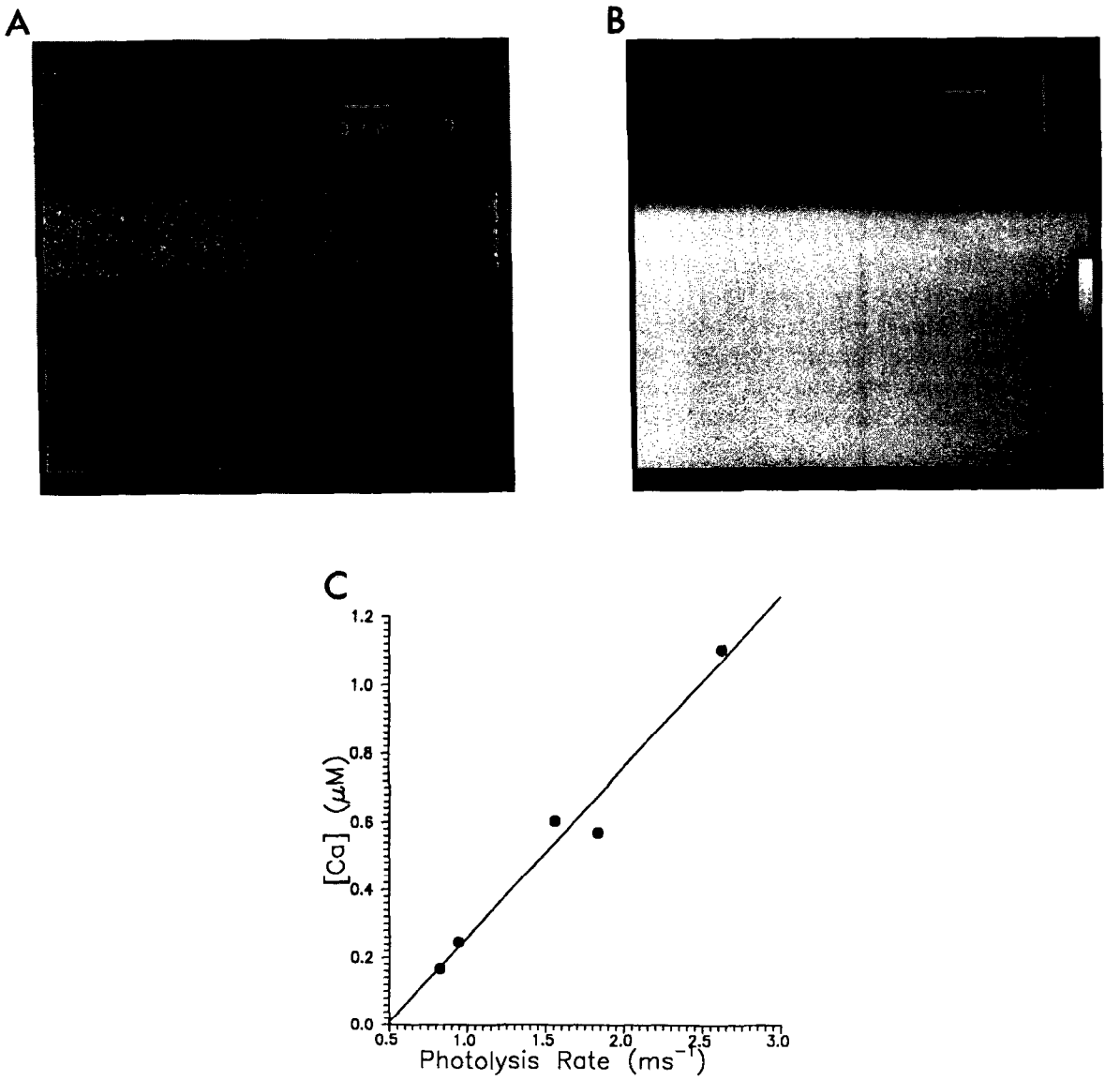

Fig. 1 A steady light elevates [Ca] for the duration of illumination. A, a laser scanning confocal microscope was focused on a cuvette containing DM-nitrophen and Fluo–3. The laser beam was repeatedly scanned along a single line, and successive lines are stacked vertically. Therefore, the horizontal axis represents fast time during single scans, and the vertical axis represents slower time between scans. The light was turned on for 0.2 s, and the average fluorescence increased by 9.2 bits during light exposure. This has been accentuated by compressing the intensity scale. B, the same cuvette was scanned at a slower speed, and the light was turned on again after 1 s. Fluorescence again increased a small amount, until 70% of the nitrophen was photolyzed, whereupon [Ca] rose to very high levels. The light was turned off 1 s before the bottom of the panel. The intensity scale shows fluorescence intensities corresponding to [Ca] levels in μM. C, [Ca] during light exposure plotted vs. photolysis rate of calcium-bound DM-nitrophen

Programming

All analysis programs were written in Microsoft (Redmond, WA, USA) BASIC 7.0 on an IBM (Armonk, NY, USA) PS/2 Model 70 computer. Results were plotted using Grapher (Golden Software, Golden, CO, USA).

Results

Light pulses evoke reversible [Ca] pulses

When ultraviolet light illuminates a solution of 10 mM nitrophen and 3 mM calcium, the free calcium concentration rises from 2 nM to a new steady level for the duration of illumination, and rapidly returns to a level near the starting point when the light is turned off. Figure 1A shows the rise in [Ca], detected as an increase in fluorescence of the 0.3 mM Fluo–3 included in the solution, in response to a 0.2 s light pulse from the ILC Technology lamp. In this experiment, Fluo–3 fluorescence increased by 9 bits. Control experiments to measure direct excitation of Fluo–3 as well as reflected light passed through the fluorescein filter set used an identical solution with no added calcium. The maximum rise in apparent fluorescence in such controls was always less than 0.3 bits (data not shown).

In order to translate the increase in fluorescence to units of [Ca], mixtures of 10 mM nitrophen and 0.3 mM Fluo–3 were buffered to various levels of [Ca] as described in Materials and Methods, and the fluorescence of each mixture was measured. The results are plotted in Figure 2A. A dotted line fitted to the points provides a calibration curve for the nitrophen/Fluo–3 mixture used in these experiments, from which a [Ca] level of 0.6 μM is calculated for Figure 1A.

The rate of calcium binding to nitrophen

In a nitrophen/Fluo–3 mixture, the rate of change of calcium depends on the rate of photolysis of nitrophen, the bimolecular association rates of calcium binding to nitrophen and Fluo–3, and the dissociation rates of Ca—Fluo–3 and Ca—nitrophen:

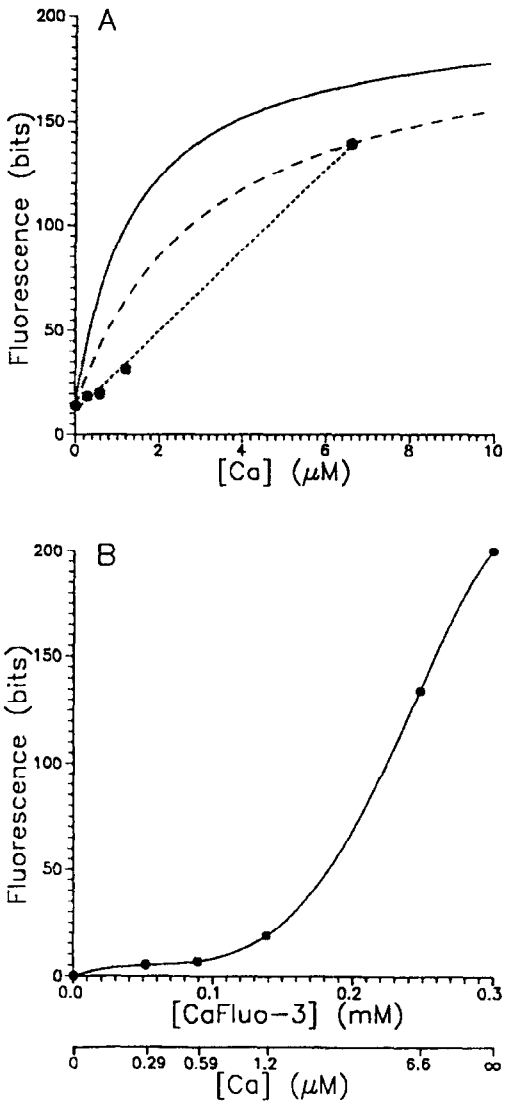

Fig. 2 Calibration of the calcium sensitivity of 0.3 mM Fluo–3 in the presence of 10 mM DM-nitrophen. A, circles show the fluorescence of calibrating solutions listed in Table 1. The solution with [Ca] = 2 mM had a fluorescence of 200. The dotted line is fitted to the points. The solid line shows the expected fluorescence of Fluo–3 if its affinity for calcium is 1.4 μM, as was measured for dilute mixtures of Fluo–3 and nitrophen [6]. Points deviate from this curve, the more the lower the [Ca], and using a higher affinity (3.2 μM) derived from the point at 6.6 μM (dashed line) does not improve the fit to the data, suggesting that Fluo–3 fluorescence is quenched at low [Ca]. B, fluorescence plotted vs. the concentration of calcium-bound Fluo–3, assuming an affinity of 1.4 μM. Fluorescence has been scaled to a change of 200 bits between [Ca] = 0 and [Ca] = ∞

$$dCa/dt = R \cdot CaNP - \alpha_1 \cdot Ca \cdot NP + \beta_1 \cdot CaNP - \alpha_2 \cdot Ca \cdot F + \beta_2 \cdot CaF \quad \text{Eq. 1}$$

where NP, CaNP, F and CaF are free and calcium-bound nitrophen and Fluo–3 respectively, R is the rate of photolysis of CaNP, α_1 and β_1 are the association and dissociation rates for Ca binding to nitrophen, and α_2 and β_2 are the rates for Ca binding to Fluo–3. In steady state, dCa/dt is zero. When photolysis is not too rapid, CaNP, NP, CaF, and F change little from their starting values. β_1 can be replaced by $\alpha_1 \cdot K_D$, where K_D is the dissociation constant for the calcium-nitrophen complex (5 nM). If the photolysis rate of CaNP and the kinetics of Fluo–3 binding to calcium are known, this equation can be solved for α_1.

Photolysis rates for nitrophen

A simple way to estimate the photolysis rate of nitrophen, R, is to leave the steady light on, and observe when [Ca] escapes from nitrophen buffering and rises rapidly to high levels. This will occur when the amount of unphotolyzed nitrophen still remaining falls below the total amount of calcium. Figure 1B shows that this occurred in the cuvette of Figure 1A when the light was turned on for an additional 0.86 s. If NP and CaNP are photolyzed at the same rate, then this result would indicate that 70% of the nitrophen was photolyzed in 1.06 s. Since the concentration of unphotolyzed nitrophen drops at the rate e^{-Rt}, this reaches 30% in 1.06 s if $R = 1.14\ s^{-1}$.

This calculation depends on the assumption that calcium does not affect the rate of photolysis of nitrophen. To test this, absorbance spectra of calcium-free and calcium-bound nitrophen were measured after different exposures to UV light (Fig. 3). The results show that at pH 7.2, CaNP is photolyzed 2.5-times as fast as NP. Since the absorbance of nitrophen is little affected by binding to calcium [2], this implies that the quantum efficiency of CaNP is 2.5-times that of NP. Nitr-5 has a similar calcium dependence of quantum efficiency [1].

This forces a revision to the simple method for estimating the rate of photolysis of CaNP. [Ca] will jump up to high levels when the total nitrophen concentration (NPT = CaNP + NP) drops to less than the total calcium concentration (CaT = Ca + CaNP + CaF). If R′ is the rate of photolysis for NP

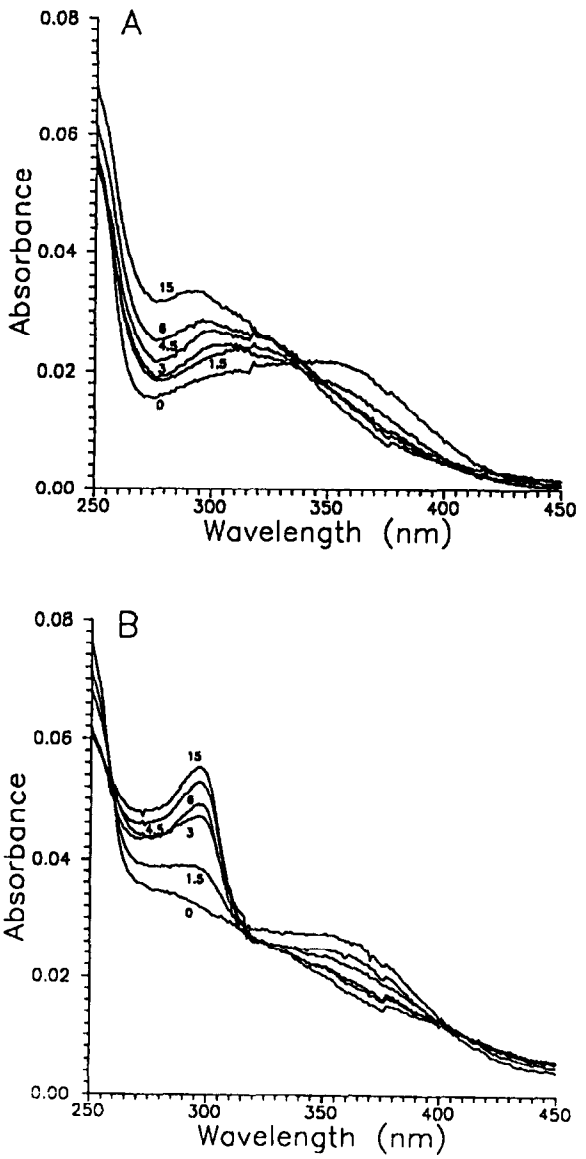

Fig. 3 Photolysis rates for free (A) and calcium-bound (B) DM-nitrophen. Absorbance spectra were obtained for 60 μM nitrophen in 1 mm cuvettes after exposure to UV light for the time in seconds shown adjacent to each curve. The spectrum after 30 s light exposure was the same as that after 15 s in B, and about 10% higher in A. The data fit exponential approaches to steady state with time constants of 8.3 s in A and 3.3 s in B

and R the rate of photolysis for CaNP, then the rate of loss of total nitrophen will be

$$dNPT/dt = -R' \cdot NP - R \cdot CaNP$$
$$= -R' \cdot NPT + (R' - R) \cdot CaNP \quad \ldots . \text{Eq. 2}$$

As long as NP remains in excess of CaT, almost all of the calcium will be bound to NP as CaNP (i.e. CaNP = CaT), because nitrophen's affinity for calcium (5 nM) is so much higher than that of Fluo–3 (about 1 μM; *see* [7]). If $R = K \cdot R'$ ($K = 2.5$), then Equation 2 may be solved to give

$$NPT(t) = [NPT_0 - (1 - K) \cdot CaT]\, e^{-R't} + (1 - K) \cdot CaT \quad \ldots . \text{Eq. 3}$$

where NPT_0 is the initial total nitrophen concentration. Substituting NPT_0 = 10 mM, CaT = 3 mM, K = 2.5, and NPT(t) = 3 mM at t = 1.06 s yields $R' = 0.62\ s^{-1}$ and $R = 1.55\ s^{-1}$ for the experiment of Figure 1.

Calcium binding kinetics to Fluo–3

The only remaining parameters of Equation 1 are α_2 and β_2, the CaF association and dissociation rates. These have been measured as $1.4 \times 10^9\ M^{-1}s^{-1}$ and $580\ s^{-1}$ [12]. However, the presence of 10 mM nitrophen appears to reduce the calcium affinity of Fluo–3 to 1.4 μM, and seems to have an additional effect of quenching the fluorescence of Fluo–3 when [Ca] is low [6]. This behaviour may be seen in Figure 2A, in which the solid line shows the expected form of the fluorescence (Fl) vs. [Ca] curve (*see* Eq. 6 of [13]), for K_D = 1.4 μM,

$$Fl = \frac{(Ca \cdot F_{max} + K_D \cdot F_{min})}{(K_D + Ca)} \quad \ldots . \text{Eq. 4}$$

where F_{max} is the fluorescence of 100% CaF and F_{min} is the fluorescence of 100% F. It is evident that the data points fall below this line. Choosing K_D = 3.2 μM, the value derived from the fluorescence measurement at [Ca] = 6.6 μM, another curve is plotted as a dashed line. The low [Ca] points still fall below this line, indicating that the data cannot be fit simply by raising K_D. Rather, the lower the value of [Ca], the higher the value of K_D derived from that point. This suggests that nitrophen is somehow quenching the fluorescence of Fluo–3, especially at low [Ca]. In that case, the real Fluo–3 affinity for calcium may be about 1.4 μM, as it is in more dilute nitrophen/Fluo–3 mixtures with similar relative amounts of nitrophen and Fluo–3 [7]. It is assumed that this is the case, and that the reduced K_D of Fluo–3 of 1.4 μM, compared to its usual value of 0.4 μM, corresponds to β_2 remaining equal to $580\ s^{-1}$ and α_2 being reduced to $0.414 \times 10^9\ M^{-1}s^{-1}$ by some interference from nitrophen of calcium binding to Fluo–3.

In 5 experiments, α_1 was determined by measuring [Ca] during exposure to steady light for 0.2 s as in Figure 1A, and R was measured from the time required for [Ca] to escape from nitrophen buffering as in Figure 1B. Using Equation 1, the association rate of calcium binding to nitrophen was calculated as $1.47 \pm 0.21 \times 10^6\ M^{-1}s^{-1}$ (mean ± SE). This may be compared to the value estimated for HEEDTA, $3 \times 10^5\ M^{-1}s^{-1}$ [1], which should be the same order of magnitude.

In these 5 experiments, the light intensity was not always the same. Brighter lights should photolyze nitrophen faster, and excite a proportionately larger [Ca]. That this was the case is illustrated in Figure 1C, where the level of [Ca] during the light is plotted against the rate of photolysis.

Light flashes evoke [Ca] spikes

A brief intense flash of light should rapidly photolyze some nitrophen, leading to a sudden increase in [Ca] released from the photolyzed CaNP. This calcium will then drop rapidly as it rebinds to unphotolyzed free nitrophen. This 'spike' in [Ca] should be reflected as a transient increase in Fluo–3 fluorescence. Figure 4A shows the response of a cuvette containing 10 mM nitrophen, 3 mM calcium, and 0.3 mM Fluo–3 to a flash caused by discharging 200 J of electrical energy in the Chadwick-Helmuth flashlamp. Figure 4B shows the flash artefact observed when calcium is omitted from the solution. The artefact begins at the instant of the flash, and lasts for the remainder of the 2 ms long scan line. Therefore, the first scan line of a flash response should be ignored.

Figure 4A shows that Fluo–3 fluorescence rises to about 150 bits right after the flash, and declines in about 20 ms to near its starting level. From Figure 2A, the equilibrium calibration curve for 0.3 mM Fluo–3 in the presence of 10 mM nitrophen, the peak rise in [Ca] after a flash would be estimated as 6 μM. However, the constituents of the cuvette in the experiment of Figure 4A are **not** in equilibrium. Calcium is rising rapidly during the flash, and falling rapidly afterwards as it binds to nitrophen. Fluo-3 will not be able to capture the calcium released by photolysis, and equilibrium will not be

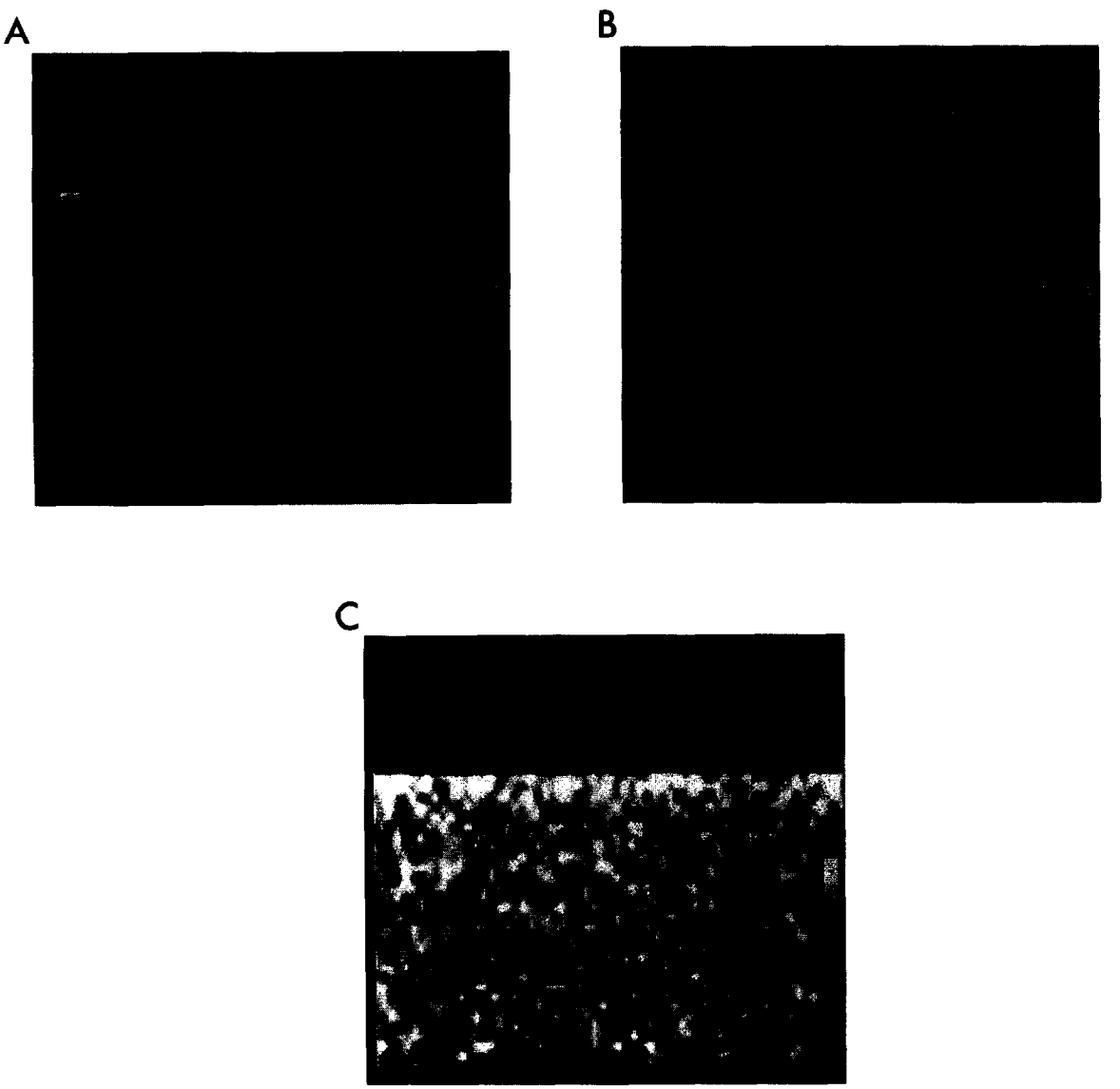

Fig. 4 Light flashes transiently elevate [Ca]. A, magnified line scans of the effect of a brief intense flash on fluorescence of a mixture of DM-nitrophen and Fluo–3. B, flash artefact measured with a mixture containing no calcium. C, effect of the third flash on fluorescence. The time scales and [Ca] calibration bar in B apply to all panels; the [Ca] concentrations (in μM) are based on the steady state calibration data of Figure 2A. Fluorescence ranges from 15 bits above background for [Ca] = 0 to 200 bits above background for [Ca] = ∞

achieved until calcium is fully rebound. Therefore, Fluo–3 fluorescence will not accurately represent the magnitude or time course of changes in [Ca].

The only way to estimate the effect of flash photolysis on [Ca] is to model computationally the reactions in the cuvette, and predict the changes in [Ca], CaNP, NP, CaF, and F. If the predicted changes in Fluo–3 fluorescence match those expected from the predicted changes in CaF and F, this can be taken as confirmation of the model and its prediction of the change in [Ca].

Model for flash photolysis of nitrophen/Fluo–3 mixtures

The model calculates changes in chemical species in two steps. In each time increment, the change in NP and CaNP due to photolysis are calculated. It is assumed that photolyzed nitrophen is converted to a species that does not bind calcium, since the affinity of the nitrophen photoproducts for calcium is very weak. Then the changes in Ca, NP, CaNP, F, and CaF are calculated from the association and dissociation of calcium with nitrophen and Fluo–3.

The rate of photolysis of CaNP during a flash, P(t), was calculated from the convolution of the time course of the light intensity, L(t), and the rate of photolysis of nitrophen following absorption of a photon, A(t),

$$P(t) = P\int_0^t L(T)\,A(t-T)\,dt \quad \text{.....Eq. 5}$$

where P is the fraction of CaNP photolyzed by the flash and $A(t) = \tau e^{-t/\tau}$. The time constant of photolysis, τ, was taken as 260 μs, corresponding to a half time of 180 μs [3]. The time course of a light flash was measured with a photodiode (thin line in Fig. 5A) and fitted with the equation $e^{-r_1 t} - e^{-r_2 t}$, with $r_1 = 2500\ s^{-1}$ and $r_2 = 4000\ s^{-1}$ (thick line in Fig. 5A).

The fraction of CaNP photolyzed in a flash was calculated from a procedure similar to that used for steady light. If N flashes are given, Equation 3 can be modified by replacing R't with P'N, where P' is the fraction of free NP photolyzed by each flash,

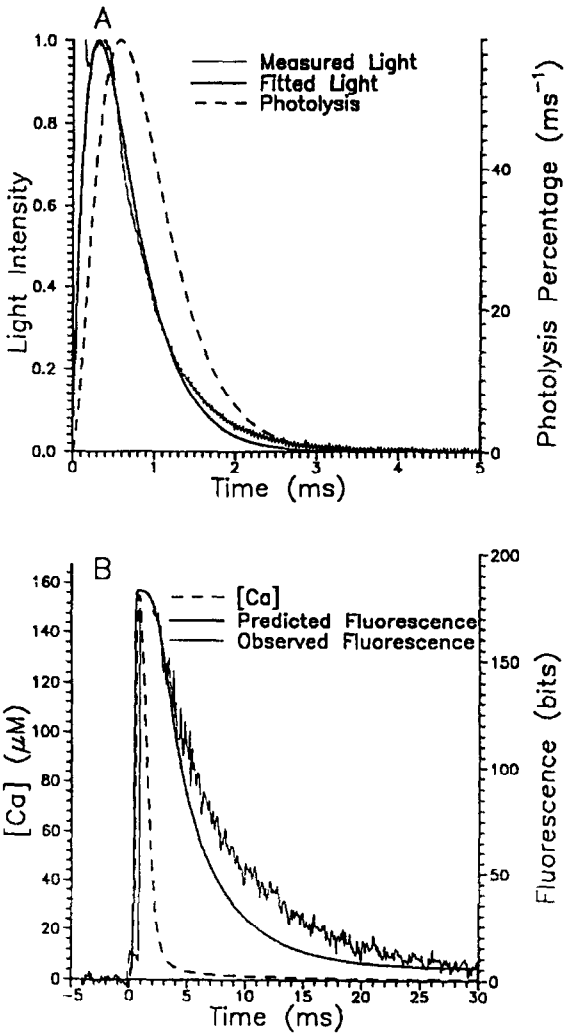

Fig. 5 Model of flash-evoked [Ca] spike. A, measured time course of light flash (thin line) and fitted function (thick line). The predicted time course of photolysis is shown as a dashed line. B, predicted effect of flash on [Ca] (dashed line), and on fluorescence of Fluo–3 (thick line) compared to observed fluorescence following flash (thin line). [Ca] rises to 150 μM, saturating Fluo–3; the fluorescence decays more slowly than [Ca]

$$NPT(N) = [NPT_0 - (1 - K) \cdot CaT]\, e^{-P'N} + (1 - K) \cdot CaT \quad \text{.....Eq. 6}$$

Then Equation 6 will give the total amount of nitrophen remaining after each flash, NPT(N). When total nitrophen, NPT, drops below total

calcium, CaT, [Ca] will remain elevated after a flash. In the cuvette used in Figure 4A, this occurred on the third flash (Fig. 4B). In the solutions used, this occurs when NPT drops from NPT_0 = 10 mM to NPT = 7 mM, and substituting CaT = 3 mM, K = 2.5, and N = 3 into Equation 6 yields an estimate for P′ of 0.26 and of P = KP′ of 0.66.

The predicted time course of photolysis using this value of P in Equation 5 is plotted as a dashed line in Figure 5A. Photolysis, P(t), lags the light, L(t), by about 1/4 ms, but is otherwise largely determined by the time course of the light intensity during the flash.

The changes in the constituents of a cuvette are calculated in each time increment, dt, by first reducing NP and CaNP and increasing Ca by photolysis, and then allowing for changes in CaNP, NP, CaF, F, and Ca due to association and dissociation of calcium with nitrophen and Fluo–3,

$$dCaNP = -dCa = -CaNP \cdot P(t) \cdot dt$$

$$dNP = -NP \cdot P'(t) \cdot dt$$

$$dNPT = dCaNP + dNP$$

$$dCaNP = -dNP = (\alpha_1 \cdot Ca \cdot NP - \beta_1 \cdot CaNP) \cdot dt$$

$$dCaF = -dF = (\alpha_2 \cdot Ca \cdot F - \beta_2 \cdot CaF) \cdot dt$$

$$dCa = -dCaNP - dCaF \quad \text{Eq. 7}$$

To predict changes in fluorescence, a relationship was derived for its dependence on the concentration of CaF. This was done by calculating the amount of CaF in each of the calibration solutions used for Figure 2A from the calculated [Ca] in each solution, assuming an affinity of Fluo–3 for calcium of 1.4 μM in the presence of 10 mM nitrophen. The relationship between fluorescence and CaF obtained in this manner was fitted with a 5th order polynomial (Fig. 2B), and this was used to translate changes in CaF following a flash to changes in fluorescence.

The predicted changes in fluorescence intensity following a single flash are plotted as a thick line in Figure 5B. These are compared to measured changes in fluorescence (thin line in Fig. 5B). The latter were extracted from the line scans of the cuvette produced by the confocal microscope by averaging successive groups of 38 horizontal pixels (100 μs), and subtracting the average pedestal before the flash.

The agreement between prediction and observation is good, but not perfect. Likely sources of error include the measurement of photolysis percentage by a flash (could easily have required only 2 and a fraction of flashes to photolyze 70% of the nitrophen), fluctuations in flash intensity, the estimates of DM-nitrophen and Fluo–3 binding and dissociation rates, and the model used to translate CaF into fluorescence. Considering the rather large uncertainty in some of these estimates, the agreement is surprisingly good.

If this is taken as confirmation of the computational model used, then its prediction of the magnitude and time course of changes in [Ca] may be accepted. This is plotted as a dashed line in Figure 5B, and indicates that [Ca] rises to a peak of about 150 μM, but that this subsides rapidly, with a half width of only 1.2 ms. The Fluo–3 fluorescence greatly outlasts the [Ca] spike, and cannot sense its magnitude. The persistent fluorescence is due to the time it takes for calcium bound to Fluo–3 to be passed to nitrophen. During this time, [Ca] remains slightly above its steady state level, which it would achieve much more rapidly in the absence of Fluo–3. This was confirmed by simulations that left out Fluo–3 (data not shown).

Two further predictions of the formulations presented here were undertaken. If a steady light is left on, [Ca] should rise gradually until CaT exceeds NPT, whereupon it should rise sharply. From Equation 1, setting d[Ca]/dt to zero (approximately), the level of [Ca] can be calculated when most of the free nitrophen has been photolyzed by setting NP = 1 mM, and substituting $CaF = Ca \cdot FT/(K_D + Ca)$ and $F = K_D \cdot FT/(K_D + Ca)$ into the equation, where FT and K_D are the total Fluo–3 concentration and its calcium dissociation constant. The result is that [Ca] is calculated to rise to 1.9 μM in about 6/7 the time to photolysis of 70% of the nitrophen, when calcium escapes from buffering. Figure 6A plots averages of 100 pixels from each scan line of

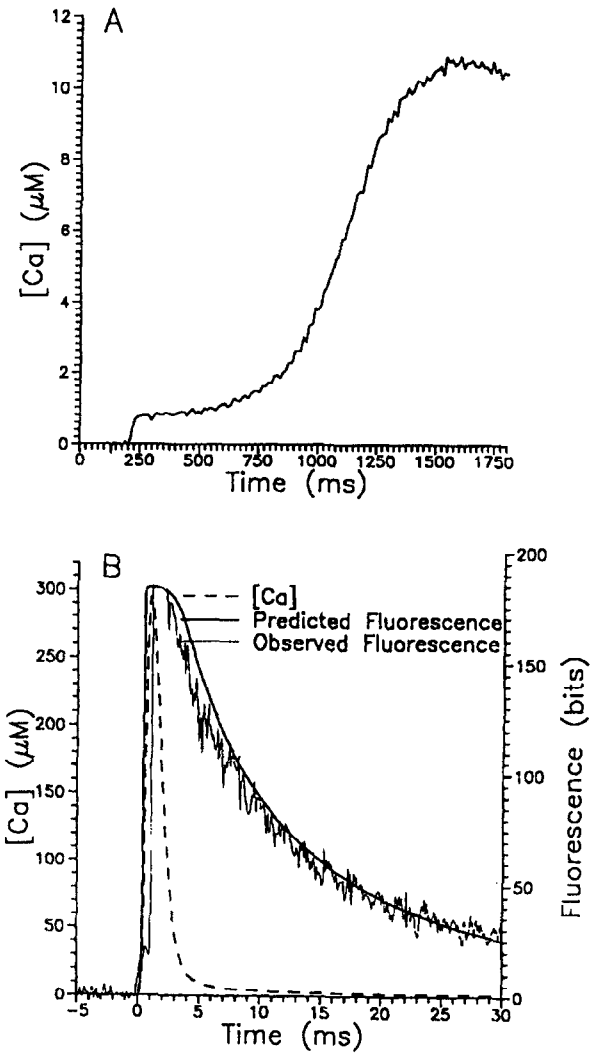

Fig. 6 Additional characteristics of light responses. **A**, rise in [Ca] during steady light exposure (following prior 0.2 s exposure). [Ca] should rise to about 2 μM at 850 ms. **B**, predicted effect of second flash on [Ca] (dashed line), and on fluorescence (thick line), compared to observed fluorescence (thin line). A slower decay of fluorescence is expected and observed, while the [Ca] transient reaches 300 μM

Figure 1B vs. total time of light exposure. The light is turned on for the second time in Figure 1B after a prior exposure of 0.2 s (Fig. 1A), so the start of the second exposure is aligned at 0.2 s. It can be seen that [Ca] rises gradually, reaching 1.9 μM at about 850 ms, which is close to 6/7 of the time before [Ca] rises sharply. Note that above a few μM the calibration line of Figure 2A no longer applies, and above an apparent [Ca] of 8 μM, Fluo–3 is essentially saturated with calcium and [Ca] has risen to unmeasurably high levels.

Finally, the effect of a second flash on [Ca] and Fluo–3 fluorescence is illustrated in Figure 6B. Here, the steady state prediction of the effect of one flash was taken as the starting point for predicting the effect of another. The figure compares measured to predicted fluorescence changes, and again the agreement is good. The calculations indicate that this flash elicits a 300 μM [Ca] spike.

Effects of hydrogen and magnesium

Hydrogen and magnesium compete for calcium at the same binding sites of nitrophen, and so might be expected to affect its behavior to photolytic stimuli. When pH was raised to 8.3, substituting a Trizma buffer for MOPS, or reduced to 6.9 using PIPES, no difference was observed in either the responses to a steady light or to repeated flashes (data not shown). This indicated little effect on either photolysis rate or on calcium binding. Measurements of photolysis of nitrophen by absorbance changes, as in Figure 3, also showed no effect of elevating pH. From the functional form of the pH dependence of the calcium binding rate on pH [4], one might observe significant effects only at low pH or only at high pH. Unfortunately, when pH was lowered to 6.1 using MES buffer, responses to light pulses and flashes were undetectable. This is probably due to the known effect of low pH on the sensitivity of Fluo–3 to calcium [14]. In addition, the absorbance spectra of nitrophen were altered radically at low pH, and photolysis was slowed, suggesting a chemical modification of nitrophen (data not shown). In conclusion, over the range of pH 6.9–8.3, no differences in the behavior of nitrophen (or Fluo–3) were observed.

Magnesium binds to nitrophen with an affinity of 2.5 μM [2]. Magnesium should increase the [Ca] during steady illumination by reducing the NP available for rebinding calcium. If MgNP, like CaNP, is photolyzed more rapidly than NP, adding

magnesium should hasten the photolysis of nitrophen. Including magnesium binding in the kinetic model of flash responses slowed the decay of the fluorescence signal by an amount dependent on the kinetics of magnesium binding and dissociation. However, when magnesium was added to the cuvette solution at concentrations up to 5 mM, there was no clear effect on responses to flashes or steady light. These measures may not be sensitive enough to reveal a small effect of magnesium, or it is possible that the extent of magnesium binding has been overestimated. The results also indicate that magnesium, unlike calcium, does not accelerate the photolysis of nitrophen.

Discussion

Reversible [Ca] pulses

The results presented here illustrate a new level of flexibility and power in our ability to control intracellular [Ca]. Exposure of cells filled with nitrophen partially loaded with calcium to steady illumination results in an elevation of [Ca] for the duration of the light exposure. The [Ca] level remains nearly constant until most of the unbound nitrophen is photolyzed. With this method it is possible to raise [Ca] to about 10 μM for nearly 0.1 s, to 1 μM for nearly 1 s, or to 100 nM for nearly 10 s, from a starting level that may be as low as a few nM. The [Ca] rises sharply when the light goes on, within about 1 ms, and drops as quickly when the light is extinguished. This allows cells or cell processes to be exposed to modest but reversible and repeatable [Ca] pulses. This opens the possibility for a whole range of experimental studies that were previously impossible.

Up until now, intracellular [Ca] could be elevated only by micro-injection, exposure to drugs that trigger release from calcium stores, such as ionophores or poisons of energy metabolism, and by entry through calcium channels. These methods are neither quantitatively controllable nor rapidly reversible. Micro-injection and calcium entering through channels result in sharp spatial gradients in the region of the pipette tip or the channels, and termination of the injection or closing of the channel results in first a rapid dissipation of spatial gradient by diffusion, then a gradual removal of equilibrated calcium by active extrusion and uptake. A uniform depolarization of cell membranes, for example by modest elevation of [K] in the medium, does not uniformly elevate cytoplasmic [Ca] either, since calcium is still entering through channels, and there will be localized intense peaks in [Ca]. Perfusion of cells through patch pipettes is also slow and difficult to reverse. The technique introduced here provides an unmatched ability to uniformly, rapidly, repeatedly, and reversibly control intracellular [Ca], and quantitatively study calcium-dependent processes in cells.

The exact magnitude of [Ca] change will depend on nitrophen concentration, calcium loading, cellular magnesium concentration, light intensity, and finally on other calcium binding constituents in cytoplasm. To the extent that the latter bind calcium more rapidly than nitrophen does (1.5×10^6 $M^{-1}s^{-1}$), and are present in high concentrations, they will reduce the [Ca] to levels lower than achieved in cuvettes. In cases where the nitrophen concentration is not accurately known, for example when it is microinjected, concurrent use of a calcium indicator should aid in quantitative interpretation. In large cells, there will also be a gradient of [Ca] from the surface facing the light as light is absorbed by cytoplasm. Much of the absorbance is provided by nitrophen itself: 10 mM will cause the light intensity at 360 nm to drop to 1/3 in 100 μm.

The technique of regulating intracellular [Ca] using steady illumination of nitrophen has been applied in one physiological study so far [15]. Injection of DM-nitrophen into crayfish motor nerve terminals resulted in a reversible increase in rate of transmitter release during illumination. This technique should see wider and more quantitative applications in the near future.

The [Ca] spike

If cells are filled with nitrophen partially loaded with calcium, exposure to a brief intense light flash will evoke a [Ca] 'spike', a short lasting but high magnitude rise in cytoplasmic [Ca]. In these experiments, [Ca] rose to hundreds of μM for about 1 ms, resembling what happens near calcium channels

when they open in cell membranes [16]. Thus flash responses may be particularly relevant to the study of physiological processes that occur in the neighborhood of such channels, such as transmitter release in neurons [17].

The magnitude of the [Ca] spike can be reduced by using a dimmer flash, and with dimmer flashes it is possible to evoke several reasonably similar [Ca] spikes in a cell or cell process filled with nitrophen. In large cells, the present results will resemble what happens at the front surface of the cell, while deeper cytoplasm will experience a dimmer flash resulting in a spatial gradient in [Ca]. However, after the gradient collapses in large cells, the constituents will be restored to almost their initial condition, and even more nearly identical flash responses can be evoked before excessive photolysis of nitrophen eventually results in a step response in [Ca].

The exact magnitude of a [Ca] spike in a real cell will depend on the presence of other calcium binding constituents, in particular native cytoplasmic calcium buffers. The effectiveness of such buffers will depend critically on their calcium binding kinetics. The rebinding of calcium released by photolysis of nitrophen might also depend on [Mg] and on magnesium buffers such as ATP, since magnesium competes with calcium for binding to nitrophen. I did not observe a marked effect of magnesium on light-evoked fluorescence of Fluo–3 in these experiments, however, so its effect in cytoplasm may not be large. Experiments in which cells are perfused with solutions from a patch pipette allow the manipulation and control of intracellular constituents, and then quantitative predictions from simulations might be possible as they are in cuvettes. Simultaneous measurement of calcium-sensitive indicators might also be helpful, but it must be recognized that they suffer severe limitations in detecting brief [Ca] transients.

Re-interpretation of physiological results

Partial flash photolysis of nitrophen has been used in two previous studies to study calcium-dependent transmitter release and calcium-dependent inactivation of calcium channels in neurons [18, 19]. In both these studies, the effects of flashes were likely to be the result of sharp [Ca] spikes at the cell membrane. In one study [18], cytoplasmic [Ca] was monitored with the fluorescent indicator Fura–2. The peak of the [Ca] spike was estimated as 1 μM from the equilibrium calibration of Fura–2. However, that estimate is now realized to be in error for two reasons: (i) an interference of DM-nitrophen with Fura–2's [Ca] sensitivity [6] was not recognized; and (ii) the inapplicability of equilibrium calibration data to dynamic measurements was not appreciated. The present results indicate that [Ca] may reach a level one or two orders of magnitude higher than indicated by a calcium-sensitive dye and recorded a few ms after a flash. Since the exact magnitude depends on the kinetics of calcium binding to native buffers, it cannot be calculated until the binding rate of calcium to those buffers has been measured.

The brief [Ca] spike following a flash might evoke physiological responses of a similarly brief duration, limited by the speed of the physiological response to calcium. At the squid giant synapse, the rate of transmitter release can be estimated roughly from the slope of the postsynaptic response. This was high for only about 2–5 ms [18], not much longer than the expected duration of the [Ca] spike. A slow falling phase to the postsynaptic response, representing the persistence of a very low level of transmitter release, probably reflected the small 'tail' in [Ca] which is predicted in the present simulations, and is due to the quasi-equilibrium situation while calcium is still dissociating from the calcium indicator (and any other fast binding cytoplasmic buffers) and being absorbed by unphotolyzed nitrophen.

Another result of the present study is that the slow decay of fluorescence of a calcium-sensitive dye does not reflect the time course of [Ca] at all, but rather the time course of calcium dissociation from the dye and its transfer to unphotolyzed nitrophen in cuvettes, and to other calcium buffers in cytoplasm. This also requires reinterpretation of the slow decay of Fura–2 fluorescence reported in previous work [18]. That decay had been attributed to an actual slow decline in [Ca], due to the gradual displacement by calcium of magnesium from nitrophen, but present results indicate that magnesium need not be invoked to explain this decay, which is seen in cuvettes where no magnesium is present.

The behaviour of nitrophen lightly loaded with calcium should be contrasted to the situation where nitrophen is fully or nearly fully calcium bound [20]. Then flash photolysis will elicit a step rise in [Ca], much like that caused by photolysis of nitr-5 [10, 21], or that seen to a flash that leaves calcium in excess of nitrophen, as in Figure 4C. By proper adjustment of light and calcium loading conditions, it is thus possible to achieve steps, reversible pulses, and spikes in [Ca], providing remarkable flexibility to the use of caged calcium compounds to generate a 'calcium clamp'.

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