CHAPTER 2

Photorelease Techniques for Raising or Lowering Intracellular Ca^{2+}

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I. Introduction

Photolabile Ca^{2+} chelators, sometimes called caged Ca^{2+} chelators, are used to control $[Ca^{2+}]_i$ in cells rapidly and quantitatively. A beam of light is aimed at cells filled with a photosensitive substance that changes its affinity for binding Ca^{2+}. In the last few years, several such compounds have been invented that allow the effective manipulation of $[Ca^{2+}]_i$ in cells. These compounds offer tremendous advantages over the alternative methods of microinjecting Ca^{2+} salts, pharmacologically releasing Ca^{2+} from intracellular stores, or increasing cell membrane permeability to Ca^{2+} using ionophores, detergents, electroporation, fusion with micelles, or activation of voltage-dependent channels, in terms of specificity of action, repeatability and reliability of effect, maintenance of cellular integrity, definition of spatial extent, and rapidity of effect, all combined with the ability to maintain the $[Ca^{2+}]_i$ change for sufficient time to measure its biochemical or physiological consequences. Only photosensitive chelators allow the concentration of Ca^{2+} in the cytoplasm of intact cells to be changed rapidly by a predefined amount over a selected region or over the whole cell. Since loading can precede photolysis by a substantial amount of time, cells can recover from the adverse effects of the loading procedure before the experiments begin. The ideal photosensitive Ca^{2+} chelator does not exist, but would have the following properties.

1. The compound could be introduced easily into cells, by microinjection or by loading a membrane-permeating derivative that would be altered enzymatically to an impermeant version trapped in cells.

2. The compound could be loaded with Ca^{2+} to such a level that the unphotolyzed form would buffer the $[Ca^{2+}]_i$ to near the normal resting level, so its introduction into cells would not perturb the resting Ca^{2+} level. Additionally, by adjusting the Ca^{2+} loading or selecting chelator variants, the initial resting Ca^{2+} level could be set to somewhat higher or lower than the normal resting concentration.

3. The chelator should be chemically and photolytically stable.

4. Photolysis by a bright flash of light should allow rapid changes in the free Ca^{2+} level; this characteristic requires rapid photochemical and subsequent dark reactions of the chelator.

5. Photolysis should be achievable with biologically appropriate wavelengths, which requires a high quantum efficiency and absorbance at wavelengths that readily penetrate cytoplasm but cause little biological damage, that is, that are not highly ionizing. For the chelator to be protected from photolysis by light needed to view the preparation would also be useful.

6. The photoproducts, or post-photolysis buffer mixture, should continue to buffer Ca^{2+}, and so hold it at the new level in the face of homeostatic pressure from membrane pumps and transport processes.

7. Neither the unphotolyzed chelator nor its photoproducts should be toxic, but rather should be inert with respect to all ongoing cellular molecular and physiological processes. Three classes of compounds, the nitr series, DM-nitrophen, and the diazo series share enough of these properties to have generated intense interest and widespread popularity, and form the subjects of this review.

Numerous more general reviews of photolabile or caged compounds, which contain some information on photolabile Ca^{2+} chelators, have appeared (Ogden, 1988; Kaplan and Somlyo, 1989; McCray and Trentham, 1989; Walker, 1991; Parker, 1992; Adams and Tsien, 1993; Gurney, 1993; Kao and Adams, 1993). Reviews focused more on photosensitive Ca^{2+} chelators may be consulted also (Kaplan, 1990; Ashley *et al.*, 1991a; Gurney, 1991).

II. Nitr Compounds

A. Chemical Properties

The first useful class of photosensitive Ca^{2+} chelators to be developed was the series of nitr compounds. These compounds rely on the substitution of a photosensitive nitrobenzyl group on one or both of the aromatic rings of the Ca^{2+} chelator 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetracetic acid (BAPTA; Tsien and Zucker, 1986; Adams *et al.*, 1988; Adams and Tsien, 1993). Light absorption results in the abstraction of the benzylic hydrogen atom by the excited nitro group and oxidation of the alcohol group to a ketone. The resulting nitrosobenzoyl group is strongly electron withdrawing, reducing the electron density around the metal-coordinating nitrogens and reducing the affinity of the tetracarboxylate chelator for Ca^{2+}. In the first member of this series, nitr-2, methanol is formed as a by-product of photolysis, but in subsequent members (nitr-5, nitr-7, and nitr-8) only water is produced. Photolysis of nitr-2 is also slow (200 msec time constant). For the other nitr chelators, the dominant photolysis pathway is much faster (nitr-7, 1.8 msec; nitr-5, 0.27 msec; nitr-8, not reported). For these reasons, nitr-2 is no longer used. For the three remaining nitr compounds, photolysis is most efficient at the absorbance maximum for the nitrobenzhydrol group, about 360 nm, although light between 330 and 380 nm is nearly as effective. The quantum efficiency of the Ca^{2+}-bound form is about 1/25 (nitr-5, 0.035; nitr-7, 0.042), and is somewhat less in the Ca^{2+}-free form (0.012 and 0.011). The absorbance at this wavelength is 5500 $M^{-1}cm^{-1}$ (decadic molar extinction coefficient) for nitr-5 and nitr-7, and 11,000 $M^{-1}cm^{-1}$ for nitr-8. The structures of the nitr series of compounds are given in Fig. 1; the photochemical reaction of the most popular member of this group, nitr-5, is shown in Fig. 2.

These chelators share the advantages of the parent BAPTA chelator: high specificity for Ca^{2+} over H^+ and Mg^{2+} (Mg^{2+} affinities, 5–8 mM), lack of
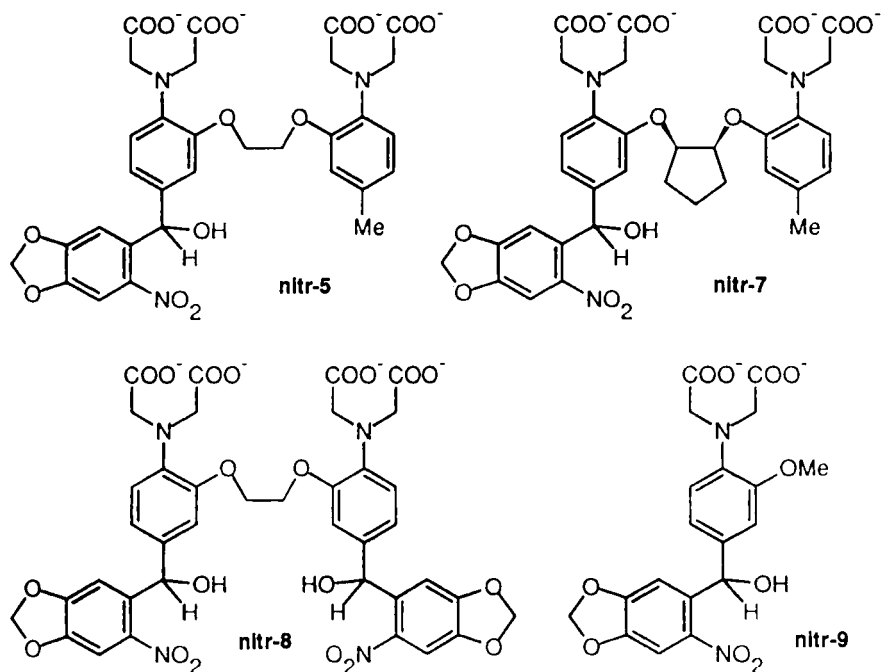
Fig. 1   Structures of the nitr series of photolabile chelators, which release calcium on exposure to light.
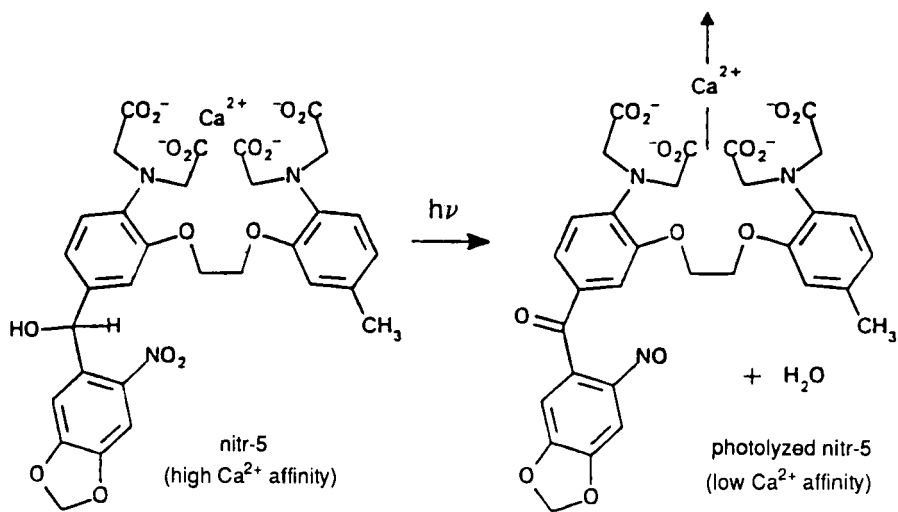
Fig. 2   Reaction scheme for the photolysis of nitr-5.

dependence of Ca^{2+} affinity on pH near pH 7, and fast buffering kinetics. One limitation is that the drop in affinity in the nitr compounds after photolysis is relatively modest, about 40-fold for nitr-5 and nitr-7. The Ca^{2+} affinity of nitr-5 drops from 0.15 to 6 μM at 120 mM ionic strength after complete photolysis. These affinities must be reduced at higher ionic strength, roughly in proportion to the tonicity (Tsien and Zucker, 1986). By incorporating a cis-cyclopentane ring into the bridge between the chelating ether oxygens of BAPTA, nitr-7 was created with significantly higher Ca^{2+} affinities (54 nM, decreasing to 3 μM after photolysis at 120 mM ionic strength). To increase the change in Ca^{2+} binding affinity on photolysis, nitr-8 was created with a 2-nitrobenzyl group on each aromatic ring of BAPTA. Photolysis of each group reduces affinity only about 40-fold, as for nitr-5 and nitr-7, but photolysis of both nitrobenzyl groups reduces affinity nearly 3000-fold, to 1.37 mM, with a quantum efficiency of 0.026. Finally, nitr-9 is a dicarboxylate 2-nitrobenzhydrol with a low Ca^{2+} affinity that is unaffected by photolysis; this compound can be used to control for nonspecific effects of the photoproducts.

Nitr-5 is the substance most often applied in biological experiments, largely because it was the first photolabile chelator to have most of the qualities of the ideal substance. The limited affinity for Ca^{2+} of this substance in the unphotolyzed form requires that it be lightly loaded with Ca^{2+} when introduced into cells; otherwise the resting $[Ca^{2+}]_i$ will be too high. However, the compound in a lightly loaded state contains little Ca^{2+} to be released on photolysis. Nitr-7 alleviates this problem with an affinity closer to that of normal resting $[Ca^{2+}]_i$, but its synthesis is more difficult and its photochemical kinetics are significantly slower. Both compounds permit less than two orders of magnitude increase in $[Ca^{2+}]_i$, generally to only the low micromolar range, and then only with very bright flashes or prolonged exposures to steady light to achieve complete photolysis. Nitr-8 permits a very large change in $[Ca^{2+}]_i$, but requires a brighter flash. Photolysis kinetics for this compound have not yet been reported. Neither nitr-8 nor the control compound nitr-9 is presently commercially available; nitr-5 and nitr-7 are supplied by CalBiochem (La Jolla, California).

B. Calculating $[Ca^{2+}]_i$ Changes in Cells

If a nitr compound is photolyzed partially by a flash of light, the reduction in Ca^{2+} affinity of a portion of the nitr requires ~0.3 msec. During this period of photolysis, low affinity buffer is being formed and high affinity buffer is vanishing while the total amount of Ca^{2+} remains unchanged. As the buffer concentrations change, Ca^{2+} ions re-equilibrate among the new buffer concentrations by shifting from the newly formed low affinity nitrosobenzophenone to the remaining unphotolyzed high affinity nitrobenzhydrol. Since the on-rate of binding is close to the diffusion limit (as calculated from Adams et al., 1988; see also Ashley et al., 1991b), this equilibration occurs much faster than pho-

tolysis, and Ca^{2+} remains in quasi-equilibrium throughout the photolysis period. The $[Ca^{2+}]_i$ in a cell rises smoothly in a step-like fashion over a period of 0.3 msec from the low level determined by the initial total concentrations of Ca^{2+} and nitrobenzhydrol to a higher level determined by the final concentrations of all the chelator species after partial photolysis. $[Ca^{2+}]_i$ remains under the control of the low and high affinity nitr species, so the elevated Ca^{2+} is removed only gradually by extrusion and uptake into organelles. Thus, the nitr compounds are well suited to producing a modest but quantifiable step-like rise in $[Ca^{2+}]_i$ in response to a partially photolyzing light flash, or a gradually increasing $[Ca^{2+}]_i$ during exposure to steady light. Subsequent flashes cause further increments in $[Ca^{2+}]_i$. These increments actually increase because, with each successive flash, the remaining unphotolyzed nitr is loaded more heavily with Ca^{2+}. Eventually, the unphotolyzed nitr is fully Ca^{2+} bound, and subsequent flashes elevate Ca^{2+} by smaller increments as the amount of unphotolyzed nitr drops.

If a calibrated light source is used that photolyzes a known fraction of nitr in the light path, or in cells filled with nitr and exposed to the light, then the mixture of unphotolyzed nitr and photoproducts may be calculated with each flash (Landò and Zucker, 1989; Lea and Ashley, 1990). The different quantum efficiencies of free and Ca^{2+}-bound chelators must be taken into account. Simultaneous solution of the buffer equations for low and high affinity nitr species and native Ca^{2+} buffers predicts the $[Ca^{2+}]_i$. For sufficiently high nitr concentration (above 5 mM), the native buffers have little effect and usually may be ignored in the calculation. Further, since $[Ca^{2+}]_i$ depends on the ratio of the different nitr species, the exact concentration of total nitr in the cell makes little difference, at least in small cells or cell processes.

If the cell is large, the light intensity will drop as it passes from the front to the rear of the cell. Knowing the absorbance of cytoplasm and nitr species at 360 nm, and the nitr concentrations before a flash, the light intensity and photolysis rate at any point in the cytoplasm may be calculated. A complication in this calculation is that the nitr photoproducts have very high absorbance (Ca^{2+}-free nitr-5 nitrosobenzophenone, 24,000 $M^{-1}cm^{-1}$; Ca^{2+}-bound nitr-5 nitrosobenzophenone, 10,000 $M^{-1}cm^{-1}$; Adams et al., 1988). As photolysis proceeds, the cell darkens and photolysis efficiency is reduced by self-screening. Nevertheless, with estimation of the spatial distribution of light intensity, the spatial concentrations of photolyzed and unphotolyzed chelator can be computed; from this calculation follows the distribution of the rise in $[Ca^{2+}]_i$. The subsequent spatial equilibration of $[Ca^{2+}]_i$ can be calculated by solving diffusion equations, using the initial $[Ca^{2+}]_i$ and chelator distributions as the boundary conditions. Effects of endogenous buffers, uptake, and extrusion mechanisms on the rise in $[Ca^{2+}]_i$ can be included in such models. Simulations of the temporal and spatial distribution of $[Ca^{2+}]_i$ have been devised (Zucker, 1989) and applied to experimental data on physiological effects of $[Ca^{2+}]_i$; the predicted changes in $[Ca^{2+}]_i$ have been confirmed with Ca^{2+}-

sensitive dyes (Landò and Zucker, 1989). Simplified and approximate models using the volume-average light intensity to calculate volume-average photolysis rate and average $[Ca^{2+}]_i$ changes often suffice when the spatial distribution of $[Ca^{2+}]_i$ is not important, for example, when estimating the change in $[Ca^{2+}]_i$ in a cell after diffusional equilibration has occurred.

III. DM-Nitrophen

A. Chemical Properties

A different strategy for releasing Ca^{2+} involves attaching a 2-nitrobenzyl group to one of the chelating amines of ethylenediaminetetraacetic acid (EDTA) to form the photosensitive chelator DM-nitrophen (Ellis-Davies and Kaplan, 1988; Kaplan and Ellis-Davies, 1988). Photolysis by UV light in the wavelength range 330–380 nm cleaves the DM-nitrophen through a series of intermediates (McCray *et al.*, 1992) to form iminodiacetic acid and a 2-nitrosoacetophenone derivative within less than 180 μsec. The reaction is shown in Fig. 3. Although DM-nitrophen binds Ca^{2+} with an affinity of 5 nM at pH 7.2, the products bind with affinities of about 3 mM and 0.25 mM at an ionic strength of 150 mM (Kaplan and Ellis-Davies, 1988; Neher and Zucker, 1993). Thus, complete photolysis of DM-nitrophen can elevate Ca^{2+} 50,000-fold, much more than photolysis of the nitr compounds. This significant advantage is counterbalanced to some extent by the facts that the photoproducts buffer Ca^{2+} so weakly that the final $[Ca^{2+}]_i$ will be determined largely by native cytoplasmic buffers, and that the Ca^{2+} liberated by photolysis of DM-nitrophen will be removed more readily by extrusion and uptake pumps. The

DM-nitrophen
(high Ca²⁺ affinity)

DM-nitrophen photolysis products
(low Ca²⁺ affinity)

Fig. 3 Structure of and reaction scheme for DM-nitrophen, which releases calcium on exposure to light.

absorbance and quantum efficiency of Ca^{2+}-saturated DM-nitrophen are 4300 $M^{-1}cm^{-1}$ and 0.18. The Ca^{2+}-free and photolyzed forms have nearly the same absorbance, but the quantum efficiency of the unbound chelator is 40% that of the bound form (Zucker, 1993a). Photolysis of Ca^{2+}-loaded DM-nitrophen produces a different spectrum than photolysis of the Ca^{2+}-free form followed by addition of Ca^{2+}. Therefore, multiple photochemical pathways are involved; the photochemistry of DM-nitrophen is still not well understood.

A serious drawback of DM-nitrophen is that it shares the cation-binding properties of its parent molecule EDTA. In particular, H^+ and Mg^{2+} compete for Ca^{2+} at the hexacoordinate binding site. The affinity of DM-nitrophen for Mg^{2+} at pH 7.2 is 0.8 μM, whereas the photoproducts bind Mg^{2+} with affinities of about 3 mM. Further, both the Ca^{2+} and Mg^{2+} affinities of DM-nitrophen are highly pH dependent (Grell *et al.*, 1989), changing by a factor of 2 for 0.3 pH units. Thus, in the presence of typical $[Mg^{2+}]_i$ levels of 1–3 mM, DM-nitrophen that is not already bound to Ca^{2+} will be largely in the Mg^{2+} form. Further, excess DM-nitrophen will pull Mg^{2+} off of ATP, which binds it substantially more weakly, compromising the ability of ATP to serve as an energy source or as a substrate for ATPases. Finally, photolysis of DM-nitrophen will lead to a jump in $[Mg^{2+}]_i$ as well as $[Ca^{2+}]_i$, and to a rise in pH. Unless controlled by native or exogenous pH buffers, this pH change can alter the Ca^{2+} and Mg^{2+} affinities of the remaining DM-nitrophen. In the absence of Ca^{2+}, DM-nitrophen even may be used as a caged Mg^{2+} chelator. Attributing physiological responses to a $[Ca^{2+}]_i$ jump requires control experiments in which DM-nitrophen is not loaded with Ca^{2+}. DM-nitrophen currently is sold by CalBiochem.

B. Calculating Changes in $[Ca^{2+}]_i$

Quantifying changes in $[Ca^{2+}]_i$ caused by photolysis is much more difficult for DM-nitrophen than for the nitr compounds. The initial level of $[Ca^{2+}]_i$ before photolysis is dependent on the total concentrations of Mg^{2+}, Ca^{2+}, DM-nitrophen, ATP, and native Ca^{2+} buffers, because two buffers (DM-nitrophen and native buffer) compete for Ca^{2+}, two (ATP and DM-nitrophen) compete for Mg^{2+}, and, after partial photolysis, both cations also bind to the photoproducts. Calculating equilibrium Ca^{2+} levels involves simultaneous solution of six nonlinear buffer equations (Delaney and Zucker, 1990), which is a tedious chore at best. Also, the various dissociation constants depend on ionic strength, and have been measured only at 150 mM. The high affinity of DM-nitrophen for Ca^{2+} might appear to dominate the buffering of Ca^{2+} in cytoplasm, but this idea is misleading. A solution of DM-nitrophen that is 50% saturated with Ca^{2+} will hold the free $[Ca^{2+}]_i$ at 5 nM at pH 7.2; this action will be independent of the total DM-nitrophen concentration. However, 5 mM DM-nitrophen with 2.5 mM Ca^{2+} and 5 mM Mg^{2+} will buffer free $[Ca^{2+}]_i$ to 2.5 μM; now doubling all concentrations results in a final $[Ca^{2+}]_i$ of 5 μM.

Since the total $[Mg^{2+}]_i$ available, free or weakly bound to ATP, is several millimolar, clearly partially Ca^{2+}-loaded DM-nitrophen may bring the resting Ca^{2+} level to a surprisingly high level. Because the solution is still buffered, this Ca^{2+} may be reduced only gradually by pumps and uptake, but eventually Ca^{2+} will be pumped off the DM-nitrophen until the $[Ca^{2+}]_i$ is restored to its normal level. Then photolysis may lead to rather small jumps in $[Ca^{2+}]_i$. On the other hand, if a large amount of DM-nitrophen is introduced into a cell relative to the total $[Mg^{2+}]_i$, the compound may be loaded heavily with Ca^{2+} and still buffer free $[Ca^{2+}]_i$ to low levels, while releasing a large amount on photolysis. In fact, if DM-nitrophen is introduced into cells with no added Ca^{2+}, it may absorb Ca^{2+} from cytoplasm and intracellular stores gradually, so photolysis still produces a jump in $[Ca^{2+}]_i$. Therefore, both the resting and the postphotolysis levels of Ca^{2+} may vary over very wide ranges, depending on [DM-nitrophen], $[Mg^{2+}]_i$, and cellular $[Ca^{2+}]_i$ control processes, all of which are difficult to estimate or control. Thus, quantification of changes in $[Ca^{2+}]_i$ is not easy to achieve.

The situation may be simplified by perfusing cells with Ca^{2+}–DM-nitrophen solutions while dialyzing out all Mg^{2+} and native buffer (Neher and Zucker, 1993; Thomas *et al.*, 1993). Of course, this procedure will not work in studies of cell processes requiring Mg^{2+}-ATP or if perfusion through whole-cell patch pipettes is not possible.

Another consequence of Mg^{2+} binding by DM-nitrophen is that cytoplasmic Mg^{2+} may displace Ca^{2+} from DM-nitrophen early in the injection or perfusion procedure, leading to a transient rise in $[Ca^{2+}]_i$ before sufficient DM-nitrophen is introduced into the cell. Such a "loading transient" has been calculated from models of changes of the concentrations of total $[Ca^{2+}]_i$, $[Mg^{2+}]_i$, ATP, native buffer, and DM-nitrophen during filling from a whole-cell patch electrode (R. S. Zucker, unpublished), and has been confirmed by measurements with Ca^{2+} indicators introduced into the cell with the DM-nitrophen (Neher and Zucker, 1993). Since this process may have important physiological consequences, controlling it is important. The process may be eliminated largely by separating the Ca^{2+}–DM-nitrophen filling solution in the pipette from the cytoplasm by an intermediate column of neutral solution [such as dilute ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) or BAPTA] in the tip of the pipette, which allows most of the Mg^{2+} to escape from the cell before the DM-nitrophen begins to enter. Then most of the loading transient occurs within the tip of the pipette.

One method of better controlling the change in $[Ca^{2+}]_i$ in DM-nitrophen experiments is filling cells with a mixture of Ca^{2+}–DM-nitrophen and another weak Ca^{2+} buffer such as N-hydroxyethylethylenediaminetriacetic acid (HEEDTA) or 1,3-diaminopropan-2-ol-tetraacetic acid (DPTA). These tetracarboxylate Ca^{2+} chelators have Ca^{2+} affinities in the micromolar or tens of micromolar range. If cells are filled with such a mixture without Mg^{2+}, the initial Ca^{2+} level can be set by saturating the DM-nitrophen and adding appropriate Ca^{2+} to the other buffer. Then photolysis of DM-nitrophen releases its

Ca^{2+} onto the other buffer; the final Ca^{2+} can be calculated from the final buffer mixture in the same fashion as for the nitr compounds. Since all the constituent affinities are highly pH dependent, a large amount of pH buffer (e.g., 100 mM) should be included in the perfusion solution, and the pH of the final solution adjusted carefully.

The kinetic behavior of DM-nitrophen is even more complex than its equilibrium reactions. Photolysis of DM-nitrophen is as fast as or faster than that of the nitr compounds, but the on-rate of Ca^{2+} binding is much slower, about 1.5 mM^{-1}msec^{-1} (Zucker, 1993a). This characteristic has particularly interesting consequences for partial photolysis of partially Ca^{2+}-loaded DM-nitrophen. A flash of light will release some Ca^{2+}, which initially will be totally free. If the remaining unphotolyzed and unbound DM-nitrophen concentration exceeds that of the released Ca^{2+}, this Ca^{2+} will rebind to the DM-nitrophen by displacing H^+ within milliseconds, producing a brief intense $[Ca^{2+}]_i$ "spike" (Grell *et al.*, 1989; Kaplan, 1990; McCray *et al.*, 1992). If Mg^{2+} is also present, a secondary relaxation of $[Ca^{2+}]_i$ follows because of the slower displacement of Mg^{2+} from DM-nitrophen (Delaney and Zucker, 1990). Moreover, if a steady UV source is used to photolyze the DM-nitrophen, Ca^{2+} rebinding continually lags release, leading to a low (micromolar range) free $[Ca^{2+}]_i$ while the illumination persists. When the light is extinguished, the $[Ca^{2+}]_i$ drops rapidly to a low level under control of the remaining DM-nitrophen buffer. If the remaining DM-nitrophen is bound to Mg^{2+}, achievement of equilibrium is somewhat slower (tens of milliseconds). Thus a reversible "pulse" of $[Ca^{2+}]_i$ is generated, the amplitude of which depends on light intensity and the duration of which is controlled by the length of the illumination. This situation remains so until the remaining DM-nitrophen becomes fully saturated with Ca^{2+}, whereupon $[Ca^{2+}]_i$ escapes from the control of the chelator, imposing a practical limit on the product of $[Ca^{2+}]_i$ and duration of about 0.75 $\mu M \cdot$ sec. Model calculations of the "spike" and "pulse" behavior of DM-nitrophen have been confirmed with experimental measurements (Zucker, 1993a; R. Zucker, unpublished results). Similar kinetic considerations apply when Ca^{2+} is passed by photolysis from DM-nitrophen to another slow buffer such as HEEDTA or DPTA. If this behavior is considered undesirable, it may be avoided by using only fully Ca^{2+}-saturated DM-nitrophen, for which rebinding to unphotolyzed chelator is impossible. Thus, the kinetic complexity of DM-nitrophen can be turned to experimental advantage, increasing the flexibility of control of $[Ca^{2+}]_i$.

IV. Diazo Compounds

A. Chemical Properties

In some experiments, being able to lower the $[Ca^{2+}]_i$ rapidly, rather than raise it, is desirable. For this purpose, the caged calcium chelators were developed. Initial attempts involved attachment of a variety of photosensitive pro-

tecting groups to mask one of the carboxyl groups of BAPTA, thus reducing its Ca^{2+} affinity until restored by photolysis. Such compounds displayed low quantum efficiency (Adams *et al.*, 1989; Ferenczi *et al.*, 1989) and their development has not been pursued. A more successful approach (Adams *et al.*, 1989) involved substituting one (diazo-2) or both (diazo-4) of the aromatic rings of BAPTA with an electron-withdrawing diazoketone that reduces Ca^{2+} affinity, much like the photoproducts of the nitr compounds. Figure 4 shows the structures of the diazo series of chelators. Photolysis converts the substituent to an electron-donating carboxymethyl group while releasing a proton; the Ca^{2+} affinity of the photoproduct is, thereby, increased. The reaction is illustrated in Fig. 5.

Diazo-2 absorbs one photon with quantum efficiency 0.03 to increase affinity, in 433 μsec, from 2.2 μM to 73 nM at 120 mM ionic strength (150 nM at 250 mM ionic strength). The absorbance maximum of the photosensitive group is 22,200 M^{-1}cm^{-1} at 370 nm, and drops to negligible levels at this wavelength after photolysis. A small remaining absorbance reflects formation of a side product of unenhanced affinity and unchanged molar extinction coefficient in 10% of the instances of effective photon absorption. This "inactivated" diazo still binds Ca^{2+} (with some reduction in absorbance), but is incapable of further photolysis. The Ca^{2+}-bound form of diazo-2 has about one-tenth the absorbance of the free form, dropping to negligible levels after photolysis, with quantum efficiency of 0.057 and a time constant of 134 μsec. Binding of Ca^{2+} to photolyzed diazo-2 is fast, with an on-rate of 8 × 10^8 M^{-1}sec^{-1}. Mg^{2+} binding is weak, dropping from 5.5 to 3.4 mM after photolysis, and pH interference is small with this class of compounds.

One limitation of diazo-2 is that the unphotolyzed chelator has sufficient Ca^{2+} affinity that its incorporation into cytoplasm is likely to reduce resting levels to some degree, and certainly will have some effect on [Ca^{2+}]$_i$ rises that occur physiologically. To obviate this problem, diazo-4 was developed with two photolyzable diazoketones. Absorption of one photon increases the Ca^{2+}
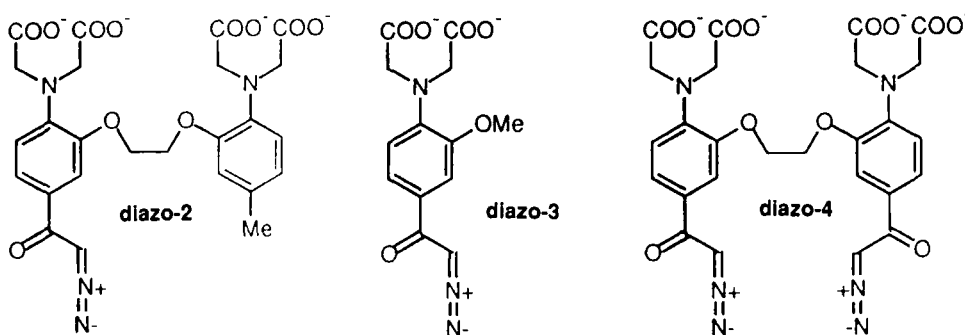

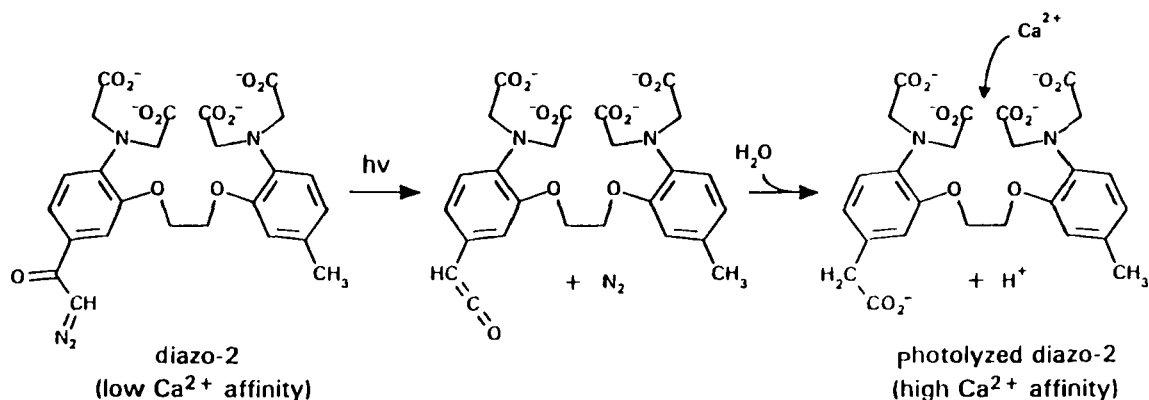Fig. 4 Structures of the diazo series of photolabile chelators, which take up calcium on exposure to light.

Fig. 5 Reaction scheme for the photolysis of diazo-2.

affinity from 89 μM to 2.2 μM (with a 10% probability of producing a side-product with one inactivated group). Absorption of two photons (with a probability assumed to equal the square of the probability of one group absorbing one photon, and with a measured quantum efficiency of 0.015) results in further increase of the affinity to 55 nM, a total increase of 1,600-fold. This large increase in affinity is, to some extent, offset by the small fraction of diazo-4 that can be doubly photolyzed readily. Thus, a flash of light produces a variety of species: unphotolyzed, singly photolyzed, doubly photolyzed, singly inactivated, doubly inactivated, and singly photolyzed–singly inactivated, with a variety of transition probabilities among species (Fryer and Zucker, 1993). Unphotolyzed diazo-4 is highly absorbant (46,000 $M^{-1}cm^{-1}$ at 371 nm for the free form; about 4,600 $M^{-1}cm^{-1}$ for the Ca^{2+}-bound form). The singly photolyzed species have absorbances of half these values; doubly photolyzed diazo-4 has negligible absorbance at this wavelength. Inactivation causes little change in absorbance.

A third member of this series, diazo-3, has a diazoketone attached to half the cation-coordinating structure of BAPTA, and has negligible Ca^{2+} affinity. On photolysis, diazo-3 produces the photochemical intermediates of diazo-2 plus a proton, and may be used to control for these effects of photolysis of the diazo series. At this time, only diazo-2 and diazo-3 are commercially available (Molecular Probes, Eugene, Oregon).

B. Calculating Effects of Photolysis

As for the nitr compounds, equilibration is faster than photolysis, so a flash of light leads to a smooth step transition in the concentration of Ca^{2+} chelator species. If the percentage of photolysis caused by a light flash is known, the

proportions of photolyzed and inactivated diazo-2, or of the six species of diazo-4, can be calculated. Usually, diazo is injected without any added Ca^{2+}, so the effect of photoreleased buffers is to reduce the $[Ca^{2+}]_i$ from its resting value. This change can be calculated only if the total Ca^{2+} bound to the native buffer in cytoplasm as well as the characteristics of that buffer are known. These characteristics often can be inferred from available measurements on cytoplasmic Ca^{2+} buffer ratio and the normal resting $[Ca^{2+}]_i$ level. The more usual application of these substances is to reduce the effect of a physiologically imposed rise in $[Ca^{2+}]_i$. In many cases, the magnitude of the source of this Ca^{2+} is known, as in the case of a Ca^{2+} influx measured as a Ca^{2+} current under voltage clamp or the influx through single channels estimated from single channel conductances. Also, the magnitude of the total Ca^{2+} increase in a response can be estimated from measured increases in $[Ca^{2+}]_i$ and estimates of cytoplasmic buffering. With this information, the expected effect of newly formed diazo photoproducts on a physiological rise in $[Ca^{2+}]_i$ can be calculated by solving diffusion equations that are appropriate for the distribution of Ca^{2+} sources before and after changing the composition of the mixture of buffers in the cytoplasm. Examples of such solutions of the diffusion equation exist for spherical diffusion inward from the cell surface (Sala and Hernandez-Cruz, 1990; Nowycky and Pinter, 1993), cylindrical diffusion inward from membranes of nerve processes (Zucker and Stockbridge, 1983; Stockbridge and Moore, 1984), diffusion from a point source (Stern, 1992; Fryer and Zucker, 1993), and diffusion from arrays of point sources (Fogelson and Zucker, 1985; Simon and Llinás, 1985). For large cells, the spatial nonuniformity of light intensity and photolysis rate also must be considered, taking into account the absorbances of all the species of diazo and the changes in their concentration with photolysis. Unlike the nitr chelators, the self-screening imposed by diazo chelators is reduced with photolysis, so successive flashes (or prolonged illumination) are progressively more effective.

V. Introduction into Cells

The photolabile chelators are introduced into cells by pressure injection from micropipettes, perfusion from whole-cell patch pipettes, or permeabilization of the cell membrane. Iontophoresis is also suitable for diazo compounds, since this procedure inserts only the Ca^{2+}-free form. For the caged Ca^{2+} substances, this method of introduction requires that the chelator load itself with Ca^{2+} by absorbing it from cytoplasm or intracellular stores. Filling cells from a patch pipette has the special property that, if the photolysis light is confined to the cell and excludes all but the tip of the pipette, the pipette acts as an infinite reservoir of unphotolyzed chelator. Then the initial conditions of solutions in the pipette can be restored within minutes after photolysis of the chelator in the cell. The nitr and diazo compounds are soluble at concentra-

tions over 100 mM and DM-nitrophen is soluble at 75 mM, so levels in cytoplasm exceeding 10 mM can be achieved relatively easily, even by microinjection, making the exogenous chelator compound the dominant Ca^{2+} buffer.

Nitr and diazo chelators also have been produced as membrane-permeant acetoxymethyl (AM) esters (Kao *et al.,* 1989). Exposure of intact cells to medium containing these esters (available from CalBiochem and Molecular Probes, respectively) might result in the loading of cells with up to millimolar concentrations, if sufficient activity of intracellular esterase is present to liberate the membrane-impermeant chelator. However, nitr-5 or nitr-7 introduced in this manner is not bound to Ca^{2+}, so it must sequester Ca^{2+} from cytoplasm, from intracellular stores, or after Ca^{2+} influx is enhanced, for example, by depolarizing excitable cells. The final concentration, level of Ca^{2+} loading, and localization of the chelator are uncertain, so this method of incorporation does not lend itself to quantification of effects of photolysis.

During loading and other preparatory procedures, the photolabile chelators may be protected from photolysis with low pass UV-blocking filters in the light path of the tungsten or quartz halide beams used for viewing. For more detail on these filling procedures, the reader is referred to Gurney (1991). Other methods of loading cells, used primarily with other sorts of caged compounds, are discussed by Adams and Tsien (1993).

VI. Light Sources

Photolysis of caged Ca^{2+} chelators requires a bright source of near UV light. If time resolution is unimportant, an ordinary mercury or xenon arc lamp may be used. Mercury lamps have a convenient emission line at 366 nm. Exposure can be controlled with a shutter, using MgF-coated Teflon blades for particularly bright sources. Lamps of 100–150 W power with collimating quartz lenses provide sufficient energy to photolyze ~25% of caged Ca^{2+} compounds in ~2 sec. Bulbs of larger power only generate bigger arcs, with more energy in a larger spot of similar intensity. With additional focusing, photolysis can be achieved in one-tenth the time or even less. These light sources are the appropriate choice in applications using reversible $[Ca^{2+}]_i$ elevation with DM-nitrophen.

Fast events require the use of a laser or xenon arc flashlamp. The xenon lamps are less expensive and cumbersome; convenient commercial systems are available from Chadwick Helmuth (El Monte, California) and Rapp Optoelektronik (Hamburg, Germany). Both flashlamps discharge up to 200 J electrical energy across the bulb to provide a pulse of ~1-msec duration with up to 300 mJ energy in the 330- to 380-nm band. The Chadwick-Helmuth unit includes only a power supply and lamp socket, so a housing with focusing optics must be constructed (see Rapp and Guth, 1988). Focusing can be accomplished with a UV-optimized elliptical reflector or with quartz refractive op-

tics. The reflector can be designed to capture more light (i.e., have a larger effective numerical aperture), but reflectors have greater physical distortion than well-made lenses. In practice, the reflector generates a larger spot with more total energy, but somewhat less intensity, than refractive methods. One advantage of reflectors is that they are not subject to chromatic aberration—focusing is independent of wavelength—so the UV will be focused in the same spot as visual light. This is not true of refractive lenses. To focus and aim them accurately at the sample, a UV filter must be used to block visual light and the beam must be focused on a fluorescent surface. Both types of housing are available from Rapp Optoelektronik. Using either system, photolysis rates approaching 80–90% in one flash are achievable. This rate may be reduced by imposing neutral density filters or reducing discharge energy, but the relationship between electrical and light energy is not linear and should be measured with a photometer. Flashlamps can be reactivated only after their storage capacitors have recharged, setting the minimal interval between successive flashes at 10 sec or more.

Flashlamps are prone to generating a number of artifacts. The discharge causes electrical artifacts that can burn out semiconductors and op amps, and reset or clear digital memory in other nearby equipment. Careful electrostatic shielding, wrapping inductors with paramagnetic metal, power source isolation, and using isolation circuits in trigger pulse connections to other equipment prevent most problems. The discharge generates a mechanical thump at the coil used to shape the current pulse through the bulb; this thump can dislodge electrodes from cells or otherwise damage the sample. Mechanical isolation of the offending coil solves the problem. The light pulse also generates a movement artifact at electrodes, which can be seen to oscillate violently for a fraction of a second when videotaped during a flash. This movement can damage cells severely, especially those impaled with multiple electrodes. Small cells sealed to the end of a patch pipette often fare better against such mistreatment. To reduce this source of injury, the light can be filtered to eliminate all but the near UV. Commercial Schott filters (UG-1, UG-11), coated to reflect infrared (IR) light, serve well for this purpose, but can cut the 330- to 380-nm energy to 30% or less. Liquid filters to remove IR and far UV also have been described (Tsien and Zucker, 1986). Removing IR reduces temperature changes, which otherwise can exceed 1°C, whereas removing far UV prevents the damaging effects of this ionizing radiation. Chlorided silver pellets and wires often used in electrophysiological recording constitute a final source of artifact. These components must be shielded from the light source or they will generate large photochemical signals.

To simply aim and focus the light beam directly onto the preparation is easiest. If isolating the lamp from the preparation is necessary, the light beam may be transmitted by a fiber optic or liquid light guide, with some loss of intensity. If a microscope is being used already, the photolysis beam may be directed through the epifluorescence port of the microscope. The lamp itself, or a light guide, may be mounted onto this port. Microscope objectives having

high numerical aperture and good UV transmission will focus the light quite effectively onto a small area, which can be delimited further by a field stop aperture. With the right choice of objectives and direct coupling of the lamp to the microscope port, light intensities similar to those obtained by simply aiming the focused steady lamp or flashlamp can be achieved. Half reflective mirrors can be used to combine the photolysis beam with other light sources, such as those used for $[Ca^{2+}]_i$ measurement. However, as the optical arrangement becomes more complex, photolysis intensity inevitably decreases.

Lasers provide an alternative source of light, with the advantages of a coherent collimated beam that is focused easily to a very small spot. Pulsed lasers such the frequency-doubled ruby laser or the XeF excimer laser provide at least 200 mJ energy at 347 or 351 nm in 50 and 10 nsec, with possible repetition rates of 1 and 80 Hz, respectively. Liquid coumarin-dye lasers, with up to 100 mJ tunable energy in the UV and 1-μsec pulse duration, are also available. New and inexpensive nitrogen lasers providing lower pulse energies (0.25 mJ) in 3-nsec pulses at 337 nm also have been developed (Laser Science, Cambridge, Massachusetts) and, with appropriate focusing, might be useful. To date, lasers have found their widest application in studies of muscle contraction. More information on these laser options is contained in discussions by Goldman *et al.* (1984) and McCray and Trentham (1989).

An adaptation of laser photolysis is the two-photon absorption technique (Denk *et al.*, 1990). A colliding-pulse mode-locked laser generating 100-fsec pulses of 630-nm light at 80 MHz is focused through a confocal scanning microscope. Photolysis of UV-sensitive caged compounds requires simultaneous absorption of two red photons, so photolysis occurs only in the focal plane of the scanning beam. This behavior restricts photolysis in three dimensions, but the photolysis rate is so slow that several minutes of exposure are required with currently available equipment. This technique is expensive and specialized, and is still under development, but may have practical application after undergoing further refinement.

Near UV light alone seems to have little effect on most biological tissues, with the obvious exception of photoreceptors and the less obvious case of smooth muscle (Gurney, 1993). Control experiments on the effects of light on unloaded cells, and on the normal physiological response under study, can be used to ascertain the absence of photic effects.

VII. Calibration

When designing a new optical system or trying a new caged compound, being able to estimate the rate of photolysis of the apparatus used is important. This information is necessary to adjust the light intensity or duration for the desired degree of photolysis, and to insure that photolysis is occurring at all.

In principle, the fraction (F) of a substance photolyzed by a light exposure of energy J can be computed from the formula $e^{-(J - J')} = (1 - F)/0.1$, where J' is the energy needed to photolyze 90% of the substance and is given by $J' = hcA/Q\varepsilon\lambda$, where h is Planck's constant, c is the speed of light, A is Avogardro's number, Q is the quantum efficiency, ε is the decadic molar excinction coefficient, and λ is the wavelength of the light. In practice, however, this equation is rarely useful, for the following reasons.

1. Measuring the energy of the incident light on a cell accurately is difficult, especially for light of broad bandwidth with varying intensity at different wavelengths.
2. The quantum efficiency, although provided for all the photolabile Ca^{2+} chelators, is not such a well-defined quantity. The value depends critically on how it is measured, which is not always reported. In particular, the effective quantum efficiency for a pulse of light of moderate duration (e.g., from a flashlamp) is often greater than that of either weak steady illumination or a very brief pulse (e.g., from a laser), because of the possibility of multiple photon absorptions of higher efficiency by photochemical intermediates. This phenomenon has been noted to play a particularly strong role in nitr-5 photolysis (McCray and Trentham, 1989). Thus, apparent differences in quantum efficiencies between different classes of chelators may be mainly the results of different measurement procedures.
3. Finally, the quantum efficiency is a function of wavelength, which is rarely given.

A more practical and commonly adopted approach is mixing a partially Ca^{2+}-loaded photolabile chelator with a Ca^{2+} indicator in a solution with appropriate ionic strength and pH buffering, and measuring the [Ca^{2+}] change in a small volume of this solution, the net absorbance of which is sufficiently small to minimize inner filtering of the photolyzing radiation. Suitable indicators include fura-2, indo-1 (Grynkiewicz *et al.*, 1985), furaptra (Konishi *et al.*, 1991), fluo-3, rhod-2 (Minta *et al.*, 1989), Calcium Green™, Orange™, and Crimson™ (Eberhard and Erne, 1991), arsenazo III (Scarpa *et al.*, 1978), and fura-red (Kurebayashi *et al.*, 1993). The choice depends largely on available equipment. Fura-2, indo-1, and furaptra are dual-excitation or -emission wavelength fluorescent dyes, allowing more accurate ratiometric measurement of [Ca^{2+}], but they require excitation at wavelengths that photolyze the photolabile Ca^{2+} chelators and are subject to bleaching by the photolysis light. The former problem may be minimized by using low intensity measuring light with a high sensitivity detection system. Furaptra is especially useful for DM-nitrophen, because of its lower Ca^{2+} affinity. Fluo-3 and rhod-2 were designed specifically for use with photolabile chelators (Kao *et al.*, 1989), being excited at wavelengths different from those used to photolyze the chelators, but they

are not ratiometric dyes and are difficult to calibrate accurately. Calcium Green, Orange, and Crimson suffer the same limitation. Arsenazo and antipyralazo are metallochromic dyes that change absorbance on binding Ca^{2+}, fortunately at wavelengths different from those at which the photolabile chelators show any significant absorbance. However, these dyes are also difficult to calibrate for absolute levels of $[Ca^{2+}]$, although changes in $[Ca^{2+}]$ may be determined fairly accurately. Fura-red is a new ratiometric dye that is excited by visible light, so it might have some application in calibrating photolysis. A problem common to all the fluorescent indicators is that their fluorescent properties may be altered by the presence of photolabile chelators, which generally are used at millimolar levels whereas the indicators are present at 100 μM or less. The photolabile chelators often produce contaminating fluorescence, which also may be Ca^{2+}- dependent and may partially quench the fluorescence of the indicators (Zucker, 1992a). Thus, the indicators must be calibrated in the presence of photolabile chelator at three well-controlled $[Ca^{2+}]$ levels, preferably before and after exposure to the photolysis flash, before they can be used to measure the effects of photolysis on $[Ca^{2+}]$ (Neher and Zucker, 1993). The low and high $[Ca^{2+}]$ calibrating solutions may be made with excess Ca^{2+} or another buffer such as EGTA or BAPTA, but the intermediate $[Ca^{2+}]$ solution is more difficult to generate, since photolysis of the chelator will release some Ca^{2+} and change the $[Ca^{2+}]_i$ and pH in this solution unless it contains a very high concentration of controlling chelator and pH buffer.

The calibration procedure is generally the same for any combination of chelator and indicator. A small sample of the mixture is placed in a 1-mm length of microcuvette with a 20- to 100-μm pathlength (In Vitro Dynamics, Rockaway, New Jersey) under mineral oil to prevent evaporation. This cuvette is exposed repeatedly to the photolysis light or to flashes, which should illuminate the whole cuvette uniformly, and the $[Ca^{2+}]$ after each flash or exposure is measured using a microscope-based fluorescence or absorbance photometer. A small droplet of solution under mineral oil alone would work, and may be necessary if the photolysis beam is directed through the microscope and illuminates a very small area, but sometimes the fluorescent properties of the indicators are affected by the mineral oil. This effect would be detected in the procedure for calibrating the chelator–indicator mixture, but is best avoided using the microcuvettes, in which contact with oil is only at the edges, the fluorescence or absorbance change of which need not be measured. In some applications, such as whole-cell patch clamping of cultured cells, using the cell as a calibration chamber can be easier than any other procedure.

The expected changes in $[Ca^{2+}]$ depend on the chelator used. The nitr and diazo chelators should lead to a stepwise rise or fall in $[Ca^{2+}]$ after each exposure; the results can be fit to models of the chelators and their photoproducts, using their affinities and the relative quantum efficiencies of free and bound chelators (Landò and Zucker, 1989; Fryer and Zucker, 1993). The percentage

photolysis of the chelator in response to each light exposure is the only free parameter, and is varied until the model fits the results. In the case of the high affinity DM-nitrophen, little rise in [Ca²⁺] will occur until the total amount of remaining unphotolyzed chelator equals the total amount of Ca²⁺ in the solution, whereupon the [Ca²⁺] will increase suddenly. Equations relating initial and final concentrations of DM-nitrophen, total [Ca²⁺], and photolysis rate (Zucker, 1993a) then may be used to calculate photolysis rate per flash or per second of steady light exposure.

The photosensitive compounds also undergo substantial absorbance changes after photolysis. These changes can be monitored during repeated exposure to the light source without a Ca²⁺ indicator; the number of flashes or the duration of light exposure required to reach a given percentage photolysis then can be determined. Realizing that photolysis proceeds exponentially to completion (Zucker, 1993a), these data can be used to determine the photolysis rate directly. Ideally, both methods should be used to check for consistent results. A final method for determining photolysis rate is using high pressure liquid chromatography (HPLC) to separate and quantify parent chelators and photoproducts in the reaction solution after partial photolysis (Walker, 1991).

VIII. Purity and Toxicity

When experiments do not work as planned, the first suspected source of error is the integrity of the photolabile chelator. Different procedures have proved most useful for testing the different classes of compounds. The nitr and diazo compounds undergo large absorbance changes on binding calcium and on photolysis. A 100 μM solution (nominally) of the chelator is mixed with 50 μM Ca²⁺ in 100 mM chelexed HEPES solution (pH 7.2), and 0.3 ml is scanned in a 1-mm pathlength spectrometer. Then 1 μl 1 M K₂EGTA is added to bring the [Ca²⁺] to 0, and the sample is scanned again. Finally, 1 μl 5 M CaCl₂ is added to provide excess Ca²⁺, and a third scan is recorded. The first scan should be midway between the other two. If the first scan is closer to the excess Ca²⁺ scan, it is indicative of a lower than expected concentration of the chelator, probably because of an impurity. Alternatively, Ca²⁺ may have been present with the chelator, which may be checked by running a scan on the chelator with no added Ca²⁺ and comparing the result with a scan with added EGTA; they should be identical. Ca²⁺-free and Ca²⁺-saturated chelator solutions also are scanned before and after exposure to UV light sufficient to cause complete photolysis; the spectra are compared with published figures (Adams et al., 1988,1989; Kaplan and Ellis-Davies, 1988) to determine whether the sample was partially photolyzed at the outset. The Ca²⁺ affinities of unphotolyzed and photolyzed chelators can be checked by measuring [Ca²⁺] of 50%-loaded chelators with a Ca²⁺-selective electrode.

The absorbance of DM-nitrophen is almost Ca²⁺ independent, so these pro-

cedures are not effective for this chelator. A solution of DM-nitrophen nominally of 2 mM concentration is titrated with concentrated $CaCl_2$ until the $[Ca^{2+}]$ measured with ion-selective electrodes suddenly increases; this change indicates the actual concentration of the chelator and gives an estimate of purity. The affinity of the photolysis products can be measured as for the other chelators; spectra before and after photolysis indicate whether the sample was already partially photolyzed.

Purities of 80–90% are typical for commercial samples of all the chelators, but occasional batches of 60% purity or less have been seen; these also sometimes show high degrees of toxicity. Whether such low purity is the result of poor synthesis or storage is unclear. Nitr compounds decompose detectably after only 1 day at room temperature, and exposure to ambient fluorescent lighting for 1 day causes detectable photolysis. Chelators should be shipped on dry ice and stored at −80°C in the dark; even under these conditions they might not last forever. Repeated thawing and freezing also may degrade the compounds.

Some of the photolabile Ca^{2+} chelators display a degree of biological toxicity in some preparations. Commercial samples of nitr-5 have been seen to lyse sea urchin eggs (R. S. Zucker and L. F. Jaffe, unpublished results) and leech blastomeres (K. R. Delaney and B. Nelson, unpublished results) within minutes. Zucker and Haydon (1988) found that nitr-5 blocked transmitter release within 10 min of perfusion in snail neurons, whereas DM-nitrophen has no similar effect (P. Haydon, unpublished results). These effects are not caused by the photoproducts, since photolysis is not necessary for the problems to occur. DM-nitrophen has been observed to reduce secretion in chromaffin cells; higher chelator concentrations, photolyzed to give the same final $[Ca^{2+}]_i$ level, caused less secretion (C. Heinemann and E. Neher, unpublished results). The effect was overcome partially by inclusion of glutathione in the perfusion solution, as reported for the photoproducts of other 2-nitrobenzhydrol-based caged compounds (Kaplan *et al.*, 1978). These signs of toxicity have been observed sporadically; whether they are properties of the chelators themselves or of impurities in the samples used is unclear. The chelators have been applied successfully to a wide range of preparations without obvious deleterious results, although subtle effects may have been missed.

IX. Biological Applications

A brief synopsis of the biological applications of the caged Ca^{2+} chelators follows, and is included in this chapter because many of the original papers include a wealth of detail about methodology and interpretation of these new techniques.

A. Ion Channel Modulation

1. Potassium and Nonspecific Cation Channels

The first and still one of the major applications of photosensitive Ca^{2+} chelators is studying the properties of Ca^{2+}-dependent ion channels in excitable cells. In 1987, Gurney and colleagues used nitr-2, -5, and -7 to activate Ca^{2+}-dependent K^+ current in rat sympathetic neurons. These researchers found that a single Ca^{2+} ion binds to the channel with rapid kinetics and 350 nM affinity.

The next application of the nitr chelators was in an analysis of Ca^{2+}-activated currents in *Aplysia* neurons (Landò and Zucker, 1989). These investigators found that Ca^{2+}-activated K^+ and nonspecific cation currents in bursting neurons were linearly dependent on $[Ca^{2+}]_i$ jumps in the micromolar range, as measured by arsenazo spectrophotometry and modeling studies. Both currents relaxed at similar rates after photolysis of nitr-5 or nitr-7, reflecting diffusional equilibration of $[Ca^{2+}]_i$ near the front membrane surface facing the light source. Potassium current relaxed more quickly than nonspecific cation curent, after activation by Ca^{2+} entry during a depolarizing pulse, because of the additional voltage sensitivity of the K^+ channels. This difference was responsible for the more rapid decay of hyperpolarizing after-potentials than of depolarizing after-potentials.

The role of Ca^{2+}-activated K^+ current in shaping plateau potentials in gastric smooth muscle was explored by Carl *et al.* (1990). In fibers loaded with nitr-5/AM, Ca^{2+} photorelease accelerated repolarization during plateau potentials and delayed the time to subsequent plateau potentials, suggesting a role for changes in $[Ca^{2+}]_i$ and Ca^{2+}-activated K^+ current in slow wave generation.

Another current modulated by $[Ca^{2+}]_i$ is the so-called M current, a muscarine-blocked K^+ current in frog sympathetic neurons. Although inhibition is mediated by an as yet unidentified second messenger other than Ca^{2+}, resting M current is enhanced by modest elevation of $[Ca^{2+}]_i$ (some tens of nanomolar) and reduced by greater elevation of $[Ca^{2+}]_i$, which also suppresses the response to muscarine (Marrion *et al.*, 1991). As for ventricular I_{Ca} (see subsequent discussion), apparently several sites of modulation of M current by $[Ca^{2+}]_i$ exist. In these experiments, $[Ca^{2+}]_i$ was elevated by photorelease from nitr-5 and simultaneously measured with fura-2.

The after-hyperpolarization that follows spikes in rat hippocampal pyramidal neurons is caused by a class of Ca^{2+}-dependent K^+ channels called I_{AHP} channels. This after-hyperpolarization and the current underlying it rise slowly to a peak 0.5 sec after the end of a brief burst of spikes. Photorelease of Ca^{2+} from either nitr-5 or DM-nitrophen activates this current without delay; the current may be terminated rapidly by photolysis of diazo-4 (Lancaster and Zucker, 1991; Zucker, 1992b), suggesting that the delay in its activation following action potentials is caused by a diffusion delay between points of Ca^{2+} entry and the I_{AHP} channels.

The Ca^{2+} sensitivity of the mechanoelectrical transduction current in chick cochlear hair cells was studied using nitr-5 introduced by hydrolysis of the AM form (Kimitsuki and Ohmori, 1992). Elevation of $[Ca^{2+}]_i$ to 0.5 μM (measured with fluo-3) diminished responses to displacement of the hair bundle, and accelerated adaptation during displacement when Ca^{2+} entry occurred. Preventing Ca^{2+} influx blocked adaptation. Evidently, adaptation of this current was the result of an action of Ca^{2+} ions entering through the transduction channels.

In guinea pig hepatocytes, noradrenaline evokes a rise in K^+ conductance after a seconds-long delay. Photorelease of Ca^{2+} from nitr-5 and use of caged inositol 1,4,5-trisphosphate (IP_3) show that this delay arises from steps prior to or during generation of IP_3 (Ogden et al., 1990), which releases Ca^{2+} from intracellular stores to activate K^+ current.

2. Ca^{2+} Channels

The first application of DM-nitrophen was in a study of Ca^{2+} channels in chick dorsal root ganglion neurons (Morad et al., 1988). With divalent charge carriers, inactivation by photorelease of intracellular Ca^{2+} occurred within 7 msec, whereas with monovalent charge carriers a nearly instantaneous block occurred, especially when Ca^{2+} was released extracellularly. A similar rapid block of monovalent current through Ca^{2+} channels was observed in response to photorelease of extracellular Ca^{2+} in frog ventricular cells (Näbauer et al., 1989). Different Ca^{2+} binding sites may be exposed if altered conformational states are induced in the channels by the presence of different permeant ions.

The regulation of Ca^{2+} current (I_{Ca}) in frog atrial cells by $[Ca^{2+}]_i$ also has been studied with nitr-5 (Gurney et al., 1989; Charnet et al., 1991). Rapid elevation of $[Ca^{2+}]_i$ potentiated high-voltage-activated or L-type I_{Ca} and slowed its deactivation rate when Ba^{2+} was the charge carrier, after a delay of several seconds. Inclusion of BAPTA in the patch pipette solution blocked the effect of nitr-5 photolysis. The similarity of effect of Ca^{2+} and cAMP and their mutual occlusion suggest a common phosphorylation mechanism.

Regulation of I_{Ca} in guinea pig ventricular cells appears to be more complex (Hadley and Lederer, 1991; Bates and Gurney, 1993). A fast phase of inactivation seems to be the result of direct action of $[Ca^{2+}]_i$ on Ca^{2+} channels, since I_{Ca} inactivation caused by photorelease of Ca^{2+} from nitr-5 is independent of the phosphorylation state of the channels. Ca^{2+} inactivates the current through the channels without affecting the gating current, perhaps indicating an effect on permeation rather than on voltage-dependent gating. A slower phase of potentiation is also present, the magnitude of which depends on the flash intensity delivered during a depolarizing pulse, but not on the initial $[Ca^{2+}]_i$ level, the degree of loading of nitr-5, or the presence of BAPTA in the patch pipette. This result suggests that, during a depolarization, nitr-5 becomes locally loaded by Ca^{2+} entering through Ca^{2+} channels, and that the

Ca^{2+} binding site regulating potentiation is near the channel mouth. Larger [Ca^{2+}]$_i$ jumps elicited by photolysis of DM-nitrophen evoke greater I$_{Ca}$ inactivation, but no potentiation, perhaps because of the more transient rise in [Ca^{2+}]$_i$ when DM-nitrophen is photolyzed. Differences in Ca^{2+} sensitivity of the effects are also likely. An effect of resting [Ca^{2+}]$_i$ on the kinetics of potentiation is explained in terms of a third Ca^{2+} binding site that inhibits potentiation. As in atrial cells, potentiation is suppressed by isoprenaline, which leads to cAMP-dependent channel phosphorylation. However, kinase inhibitors block the response to isoprenaline but not Ca^{2+}-dependent potentiation, suggesting the involvement of different pathways.

DM-nitrophen loaded with magnesium in the absence of Ca^{2+} was used to study the magnesium-nucleotide regulation of L-type I$_{Ca}$ in guinea pig cardiac cells (Backx *et al.*, 1991; O'Rourke *et al.*, 1992). In the presence of ATP, a rise in [Mg^{2+}]$_i$ to 50–200 μM led to a near doubling of the magnitude of I$_{Ca}$ in a few seconds. Omitting ATP prevented this effect, although the rise in [Mg^{2+}]$_i$ still blocked inwardly rectifying K$^+$ channels. Release of caged ATP also increased I$_{Ca}$. Therefore, the effect on Ca^{2+} channels was caused by a rise in Mg^{2+}–ATP. Nonhydrolyzable ATP analogs worked as well as ATP and kinase inhibitors failed to block the potentiation, so Mg^{2+}–ATP seems to modulate Ca^{2+} channels directly.

In another study, microinjection of nitr-5, DM-nitrophen, and diazo-4 was used to characterize Ca^{2+}-dependent inactivation of Ca^{2+} current in *Aplysia* central neurons (Fryer and Zucker, 1993). Elevation of [Ca^{2+}]$_i$ to the low micromolar range with nitr-5 caused little inactivation, but photolysis of DM-nitrophen rapidly inactivated half the I$_{Ca}$, presumably that in the half of the cell facing the light source. Thus, inactivation requires high [Ca^{2+}]$_i$ levels and occurs rapidly in all channels, even if they are closed. Experiments with diazo-4 showed that an increase in buffering power reduced the rate of inactivation of I$_{Ca}$ only modestly. Simulations of the effects of the calculated change in Ca^{2+} buffering on diffusion of Ca^{2+} ions from the channel mouth indicated that Ca^{2+} appeared to act at a site with a mean free path 25 μm from the channel mouth to cause I$_{Ca}$ inactivation (see also Johnson and Byerly, 1993).

B. Muscle Contraction

One of the earliest applications of photolabile Ca^{2+} chelators was initiating muscle contraction in frog cardiac ventricular cells by photorelease of extracellular Ca^{2+} from DM-nitrophen (Näbauer *et al.*, 1989). The strength of contraction elicited by a stepwise rise in [Ca^{2+}]$_e$ showed a membrane potential dependence that was indicative of entry through voltage-dependent Ca^{2+} channels rather than of transport by Na$^+$–Ca^{2+} exchange.

Several laboratories have used caged Ca^{2+} chelators to study Ca^{2+}-dependent Ca^{2+} release from the sarcoplasmic reticulum in rat ventricular

myocytes. Valdeolmillos *et al.* (1989) loaded intact cells with the AM form of nitr-5, Kentish *et al.* (1990) subjected saponin-skinned fibers to solutions containing Ca^{2+}-loaded nitr-5, and Näbauer and Morad (1990) perfused single myocytes with DM-nitrophen loaded with Ca^{2+}. Photolysis of the chelator elicited a contraction that was blocked by ryanodine or caffeine pretreatment, procedures that prevent release of Ca^{2+} from the sarcoplasmic reticulum, so the investigators concluded that the contractions resulted from Ca^{2+}-induced Ca^{2+} release. When Ca^{2+} release was confined to a portion of a fiber (O'Neill *et al.*, 1990), contraction remained localized, indicating that Ca^{2+}-induced Ca^{2+} release does not invariably lead to propagation of and rise in $[Ca^{2+}]_i$ throughout myocytes.

Recently, Györke and Fill (1993) used Ca^{2+}-DM-nitrophen to show that the ryanodine receptor in cardiac muscle adapts to a maintained elevation of $[Ca^{2+}]_i$ in the micromolar range, remaining sensitive to larger $[Ca^{2+}]$ changes and responding by releasing still more Ca^{2+}. In smooth muscle from guinea-pig portal vein, the IP_3-dependent release of Ca^{2+} was also found to depend on $[Ca^{2+}]_i$ (Iino and Endo, 1992). Elevation of $[Ca^{2+}]_i$ by photolysis of Ca^{2+}-DM-nitrophen and detected with fluo-3 accelerated the release of Ca^{2+} from a ryanodine-insensitive, $InsP_3$-activated store.

Ca^{2+}-loaded nitr-5 was used in skinned muscle fibers of the scallop and the frog to show that, for both myosin- and actin-regulated muscles, the rate-limiting step in contraction is not the time course of the rise in $[Ca^{2+}]_i$ but the response time of the contractile machinery (Lea *et al.*, 1990; Ashley *et al.*, 1991b). Using isolated myofibrillar bundles from barnacle muscle, Lea and Ashley (1990) showed that photorelease of 0.2–1.0 μM Ca^{2+} from Ca^{2+}-loaded nitr-5 not only activated contraction directly and rapidly (within ~200 msec) but also evoked a slower phase of contraction (~2-sec rising half-time) that was dependent on Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum.

The first biological application of the caged chelator diazo-2 was in the study of muscle relaxation. Mulligan and Ashley (1989) showed that rapid reduction in $[Ca^{2+}]_i$ in skinned frog semitendinosus muscle fibers resulted in a relaxation similar to that occurring normally in intact muscle, indicating that mechanochemical events subsequent to the fall in $[Ca^{2+}]_i$ were rate limiting. However, Lännergren and Arner (1992) reported some speeding of isometric relaxation after photolysis of diazo-2, loaded in the AM form into frog lumbrical fibers. Lowered pH slowed relaxation to a step reduction in $[Ca^{2+}]_i$ (Palmer *et al.*, 1991), perhaps accounting for a contribution of low pH to the sluggish relaxation of fatigued muscle. In contrast to frog muscle, photorelease of Ca^{2+} chelator caused a much faster relaxation in skinned scallop muscle than in intact fibers (Palmer *et al.*, 1990), suggesting that, in these cells, relaxation is rate limited primarily by $[Ca^{2+}]_i$ homeostatic processes.

C. Synaptic Function

Action potentials evoke transmitter release in neurons by admitting Ca^{2+} through Ca^{2+} channels. Because of the usual coupling between depolarization and Ca^{2+} entry, assessing the possibility of an additional direct action of membrane potential on the secretory apparatus has been difficult. Photolytic release of presynaptic Ca^{2+} by nitr-5 perfused into a presynaptic snail neuron in culture was combined with voltage clamp of the presynaptic membrane potential to distinguish the roles of $[Ca^{2+}]_i$ and potential in neurosecretion (Zucker and Haydon, 1988). These researchers found no direct effect of membrane potential on the rate of transmitter release triggered by a rise in $[Ca^{2+}]_i$.

Hochner et al. (1989) injected Ca^{2+}-loaded nitr-5 into the preterminal axon of a crayfish motor neuron, and used a low $[Ca^{2+}]$ medium to block normal synaptic transmission. These investigators found that action potentials transiently accelerated the transmitter release that was evoked at a low level by photolysis of the nitr-5. However, Mulkey and Zucker (1991) used fura-2 to show that the extracellular solutions used by Hochner et al. (1989) failed to block Ca^{2+} influx through voltage-dependent Ca^{2+} channels. When external Ca^{2+} chelators or more effective channel blockers were used to eliminate Ca^{2+} influx completely, spikes failed to have any influence on transmitter release, even when it was activated strongly by photolysis of intracellularly injected Ca^{2+}-loaded DM-nitrophen.

Delaney and Zucker (1990) confirmed that action potentials at the squid giant synapse have no effect on transmitter release triggered by a rise in $[Ca^{2+}]_i$ caused by photolysis of presynaptically injected Ca^{2+}–DM-nitrophen. Using a flashlamp to photolyze the DM-nitrophen rapidly, a transient postsynaptic response occurred that resembled the response normally caused by an action potential. The early intense phase of transmitter release probably was caused by the brief spike in $[Ca^{2+}]_i$ that followed partial flash photolysis of partially Ca^{2+}-loaded DM-nitrophen (Zucker et al., 1991; Zucker, 1993a). This response began a fraction of a millisecond after the rise in $[Ca^{2+}]_i$, a delay similar to the usual synaptic delay following Ca^{2+} influx during an action potential; both delays had the same temperature dependence. Thus, under the conditions of these experiments, photolysis of DM-nitrophen caused a $[Ca^{2+}]_i$ transient resembling that occurring normally at transmitter release sites in the vicinity of Ca^{2+} channels that open briefly during an action potential. After the 2- to 3-msec intense phase of secretion, a moderate phase of transmitter release persisted for ~15 msec, corresponding to a 60-msec relaxation in $[Ca^{2+}]_i$ measured with fura-2 that probably reflected displacement of Mg^{2+} bound to unphotolyzed DM-nitrophen by the photolytically liberated Ca^{2+} ions; the difference in time constants reflects the cooperativity of Ca^{2+} action in evoking neurosecretion. A small persistent phase of secretion, lasting a few seconds, was likely to be the result of the rise in resting $[Ca^{2+}]_i$ after partial DM-

nitrophen photolysis and of the restoring effects of Ca^{2+} sequestering and extrusion mechanisms.

Similar responses to partial flash photolysis of partially Ca^{2+}-loaded DM-nitrophen have been obtained at crayfish neuromuscular junctions (Zucker, 1993b). Transmitter release evoked by slow photolysis of Ca^{2+}–DM-nitrophen using steady illumination also has been studied at this junction (Mulkey and Zucker, 1993). The rate of quantal transmitter release, measured as the frequency of miniature excitatory junctional potentials (MEJPs), was increased ~1000-fold during the illumination. Brief illuminations (0.3–2 sec) evoked a rise in MEJP frequency that dropped abruptly back to normal when the light was extinguished, as would be expected from the reversible rise in $[Ca^{2+}]_i$ that should be evoked by such illumination, which leaves most of the DM-nitrophen unphotolyzed (Zucker, 1993a). Longer light exposures caused an increase in MEJP frequency that outlasted the light signal, as would be expected from the rise in resting $[Ca^{2+}]_i$ after photolysis of most of the DM-nitrophen. These experiments illustrate the utility of slow photolysis of partially Ca^{2+}-loaded DM-nitrophen in generating reversible changes in $[Ca^{2+}]_i$ in cells.

Caged Ca^{2+} has been used to study the modulation of transmitter release at cultured snail synapses by the neuropeptide FMRFamide (Man-Son-Hing *et al.*, 1989). This peptide had a dual effect—reducing the Ca^{2+} current during depolarization and inhibiting neurosecretion, measured as the increase in miniature inhibitory postsynaptic currents evoked by a rise in $[Ca^{2+}]_i$ from photolysis of Ca^{2+}–nitr-5 perfused into the presynaptic neuron. FMRFamide also blocked the phasic release of transmitter caused by partial flash photolysis of partially Ca^{2+}-loaded DM-nitrophen (Haydon *et al.*, 1991). As in crayfish and squid synapses, these flash-evoked postsynaptic responses resembled the spike-evoked responses and were triggered by the spike in $[Ca^{2+}]_i$ that results when DM-nitrophen is used in this fashion.

At central serotonergic synapses in the leech, a presynaptic uptake system participates in the recovery of released transmitter and the termination of postsynaptic responses. The kinetics of this process were studied (Bruns *et al.*, 1993) by recording a presynaptic serotonin transport current following the phasic activation of transmitter release by photolysis of presynaptically injected Ca^{2+}-loaded DM-nitrophen. Blocking serotonin uptake by Na^+ removal or by zimelidine, eliminated the transport current and greatly prolonged the postsynaptic response.

DM-nitrophen has been used to probe the steps involved in exocytosis in endocrine cells. Measuring $[Ca^{2+}]_i$ changes with furaptra, Neher and Zucker (1993), working with bovine chromaffin cells, and Thomas *et al.* (1993), working with rat melanotrophs, found three distinct kinetic phases in secretion in response to steps of $[Ca^{2+}]_i$ to ~100 μM. These phases were interpreted to reflect vesicles released from different pools that were more or less accessible to the release machinery. In chromaffin cells, investigators also showed that

prior exposure to a modest (micromolar) rise in [Ca^{2+}]$_i$ primed the response to a subsequent step in [Ca^{2+}]$_i$ released from DM-nitrophen, indicating that [Ca^{2+}]$_i$ not only triggers exocytosis but also mobilizes vesicles into a docked or releasable position. After exocytosis, another [Ca^{2+}]$_i$ stimulus often evoked a rapid reduction in membrane capacitance, interpreted as a phase of [Ca^{2+}]$_i$-dependent endocytosis. A similar effect was observed in rat neuronal synaptosomes from the neurohypophysis (Chernevskaya *et al.*, 1993).

Zoran *et al.* (1991) used photorelease of Ca^{2+} in a study of the developmental sequence of synapse maturation. When cultured snail neurons are brought into contact with a postsynaptic target, spike-evoked transmitter release begins only after several hours. Photorelease of Ca^{2+} from DM-nitrophen was used to show that this developmental change is the result of the delayed appearance of sensitivity of the secretory machinery to a rise in [Ca^{2+}]$_i$.

Long-term potentiation (LTP) is a complex form of synaptic plasticity thought to be involved in cognitive processes such as memory consolidation and spatial learning in the mammalian brain. Blocking a [Ca^{2+}]$_i$ rise in postsynaptic pyramidal cells in area CA1 of rat hippocampus during afferent stimulation is known to prevent the establishment of LTP. Malenka *et al.* (1988) microinjected Ca^{2+}–nitr-5 into these cells and showed that a rise in [Ca^{2+}]$_i$ was sufficient to trigger LTP in the injected neuron but in none of its neighbors. The duration of postsynaptic [Ca^{2+}]$_i$ increase necessary to induce LTP was explored by terminating the [Ca^{2+}]$_i$ rise caused by a brief afferent tetanus with photorelease of Ca^{2+} chelator from diazo-4 (Malenka *et al.*, 1992). [Ca^{2+}]$_i$ had to remain elevated for ~2 sec to generate LTP; shorter or smaller increases led only to a slowly decrementing or short-term potentiation, whereas an abbreviated [Ca^{2+}]$_i$ elevation of less than 1 sec was ineffective in generating synaptic plasticity.

D. Other Applications

In addition to the three major fields of application of caged Ca^{2+} chelators that were just described, the technique of [Ca^{2+}]$_i$ regulation has been used to address an increasingly diverse range of biological problems. Nitr and diazo compounds were inserted by AM loading into fibroblasts that were activated by mitogenic stimulation to produce [Ca^{2+}]$_i$ oscillations monitored using fluo-3 (Harootunian *et al.*, 1988). Photorelease of Ca^{2+} from nitr-5 enhanced and accelerated the oscillations, whereas release of caged chelator by photolysis of diazo-2 inhibited them. Nitr-7 photolysis caused not only an immediate rise in [Ca^{2+}]$_i$ liberated from the photolyzed chelator, but also elicited a later rise in [Ca^{2+}]$_i$ (Harootunian *et al.*, 1991). This effect was shown, pharmacologically, to be caused by IP$_3$-sensitive stores, suggesting that an interaction between [Ca^{2+}]$_i$ and these stores underlies the [Ca^{2+}]$_i$ oscillations.

Photorelease of Ca^{2+} from DM-nitrophen has been used to study the binding

kinetics of Ca^{2+} to the Ca^{2+}–ATPase of sarcoplasmic reticulum vesicles (De-Long *et al.*, 1990). The relaxation of the $[Ca^{2+}]$ step, measured by arsenazo spectrophotometry after photolysis, revealed the kinetics of binding to the ATPase. Changes in the Fourier transform infrared spectrum consequent to photorelease of Ca^{2+} from nitr-5 provided information on structural changes in the ATPase after binding Ca^{2+} (Buchet *et al.*, 1991, 1992). In a final application to the study of enzyme conformational changes, photolysis of Mg^{2+}-loaded DM-nitrophen was used to form Mg^{2+}–ATP rapidly to activate the Na^+/K^+ pump, the state of which was monitored by fluorescence of aminostyrylpyridinium dyes (Forbush and Klodos, 1991). Rate-limiting steps were measured at 45 sec^{-1} by this method.

In other applications, Gilroy *et al.* (1990) and Fricker *et al.* (1991) microinjected Ca^{2+}-loaded nitr-5 into guard cells of lily leaves and showed that photorelease of about 600 nM intracellular Ca^{2+} (measured with fluo-3) initiated stomatal pore closure. Kao *et al.* (1990) loaded Swiss 3T3 fibroblasts with nitr-5/AM, and showed that photolysis that elevated $[Ca^{2+}]_i$ by hundreds of nanomolar (measured by fluo-3) triggered nuclear envelope breakdown, an early step in mitosis, while having little effect on the metaphase to anaphase transition. Control experiments using nitr-9 showed no effect of reactive photochemical intermediates or products. Tisa and Adler (1992) used electroporation to introduce Ca^{2+}-loaded nitr-5 or DM-nitrophen into *Escherichia coli* bacteria, and showed that elevation of $[Ca^{2+}]_i$ enhanced tumbling behavior characteristic of chemotaxis whereas photorelease of caged chelator from diazo-2 decreased tumbling. Photolysis of diazo-3, which reduces pH without affecting $[Ca^{2+}]_i$, caused only a small increase in tumbling. Mutants with methyl-accepting chemotaxis receptor proteins still responded to Ca^{2+}, whereas mutants of specific Che proteins did not, indicating that the action of these proteins lay downstream of the Ca^{2+} signal.

X. Conclusions

Interest in photolabile Ca^{2+} chelators is burgeoning. Their range of application is broadening beyond the original nerve, muscle, and fibroblast preparations. The kinetic properties of the chelators are beginning to be recognized and exploited (Mulkey and Zucker, 1993; Zucker, 1993a). New techniques of photolysis, such as two-photon absorption (Denk *et al.*, 1990), are under development. New classes of chelators are being designed (Adams and Tsien, 1993). These probes offer promise of a more detailed understanding of the function of Ca^{2+} as the most common and versatile cellular second messenger.

Acknowledgments

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