

A new caged Ca²⁺, azid-1, is far more photosensitive than nitrobenzyl-based chelators

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Background: Photolabile chelators that release Ca²⁺ upon illumination have been used extensively to dissect the role of this important second messenger in cellular processes such as muscle contraction and synaptic transmission. The caged calcium chelators that are presently available are often limited by their inadequate changes in Ca²⁺ affinity, selectivity for Ca²⁺ over Mg²⁺ and sensitivity to light. As these chelators are all based on nitrobenzyl photochemistry, we explored the use of other photosensitive moieties to generate a new caged calcium with improved properties.

Results: Azid-1 is a novel caged calcium in which a fluorescent Ca²⁺ indicator, fura-2, has been modified with an azide substituent on the benzofuran 3-position. Azid-1 binds Ca²⁺ with a dissociation constant (K_d) of ~230 nM, which changes to 120 μ M after photolysis with ultraviolet light (330–380 nm). Mg²⁺ binding is weak (8–9 mM K_d) before or after photolysis. Azid-1 photolyzes with unit quantum efficiency, making it 40–170-fold more sensitive to light than caged calciums used previously. The photolysis of azid-1 probably releases N₂ to form a nitrenium ion that adds water to yield an amidoxime cation; the electron-withdrawing ability of the amidoxime cation reduces the chelator's Ca²⁺ affinity within at most 2 ms following a light flash. The ability of azid-1 to function as a caged calcium in living cells was demonstrated in cerebellar Purkinje cells, in which Ca²⁺ photolytically released from azid-1 could replace the normal depolarization-induced Ca²⁺ transient in triggering synaptic plasticity.

Conclusions: Azid-1 promises to be a useful tool for generating highly controlled spatial and temporal increases of Ca²⁺ in studies of the many Ca²⁺-dependent biological processes. Unlike other caged calciums, azid-1 has a substantial cross section or shows a high susceptibility for two-photon photolysis, the only technique that confines the photochemistry to a focal spot that is localized in three dimensions. Azide photolysis could be a useful and more photosensitive alternative to nitrobenzyl photochemistry.

Introduction

The generation of controllable increases in intracellular free Ca²⁺, [Ca²⁺]_i, by photolysis of caged calcium chelators has become a standard technique in physiology and cell biology [1–4]. A high affinity photolabile Ca²⁺ chelator is introduced into the cell as the predominant Ca²⁺ buffer (at concentrations of a few millimolar) and irradiated with ultra-violet (UV) light to produce a weaker binding photoproduct, thus releasing Ca²⁺. The starting affinity and the selectivity of the chelator for Ca²⁺, degree of loading with Ca²⁺, change in affinity for Ca²⁺ upon photolysis, and the photosensitivity of the caged Ca²⁺ govern the magnitude of the elevation in Ca²⁺ attainable per flash of UV light. These properties of the currently available caged Ca²⁺ chelators, nitr-5 [5], nitr-7 [5], dimethoxy (DM)-nitrophen [6], and nitrophenyl (NP)-EGTA [7] are summarized in Table 1.

All the currently available Ca²⁺ chelators exploit the two-nitrobenzyl photochemistry [2,8,9] used by most caged compounds to either electronically (e.g. the nitr series of caged Ca²⁺) or sterically (DM-nitrophen and NP-EGTA) reduce the binding of Ca²⁺ to the chelator. This leads to a limit on the overall light sensitivity of such chelators as these groups have low absorbance ($\epsilon \sim 5000 \text{ M}^{-1} \text{ cm}^{-1}$) and modest quantum efficiencies (0.20 or less). Low photosensitivities limit the magnitude and duration of the Ca²⁺ jump attainable, particularly because unphotolyzed Ca²⁺-free chelator can rebind the photoreleased Ca²⁺. Higher light intensities to compensate for low photosensitivity are often intrinsically damaging to the cells or technically difficult to deliver. Low photosensitivity is a major problem with two-photon uncaging of photoreleasable compounds [10], a technique, in which two infra-red photons replace a single ultra-violet photon, that promises far greater spatial

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Table 1

Properties of nitr-5, nitr-7, DM-nitrophen, NP-EGTA and azid-1.*

Photolabile chelator	K_d for Ca^{2+} (μM) before $h\nu$	K_d for Ca^{2+} (μM) after $h\nu$	K_d for Mg^{2+} (mM) before $h\nu$	K_d for Mg^{2+} (mM) after $h\nu$	Quantum yield [†] Q	Extinction coefficient ^{††} ϵ ($\text{M}^{-1} \text{cm}^{-1}$)	Light sensitivity [§] ϵQ ($\text{M}^{-1} \text{cm}^{-1}$)
nitr-5 [#]	0.145	6.3	8.5	8	0.035	5500	190
nitr-7 [#]	0.054	3.0	5.4	5	0.042	5500	231
DM-nitrophen [¶]	0.005	3000	0.005	3	0.18	4330	780
NP-EGTA [*]	0.080	1000	9.0	~9	0.23	974	224
azid-1	0.23	120	7.6	~9	1	33,000	33,000

*Dissociation constants (K_d) values before and after photolysis are given for pH 7.0–7.5 and 0.1–0.15 M ionic strength and $22 \pm 2^\circ\text{C}$, except for DM-nitrophen and NP-EGTA which have values that are pH sensitive in this range so they were determined at pH 7.2. [†]The quantum yield is the efficiency of photolysis of the Ca^{2+} -bound chelator. ^{††}The extinction

coefficient of the Ca^{2+} form of the chelator at its dominant peak at the longest wavelength, except for NP-EGTA which is the recorded value at 347 nm. [§]The product of the extinction coefficient and quantum yield is a measure of the light sensitivity of the photolabile chelator and is reported for the Ca^{2+} -bound species. [#]From [5]; [¶]from [6]; ^{*}from [7].

control but has suffered from the drastically inadequate two-photon cross section (or low photolysis susceptibility) of existing nitrobenzyl compounds.

The ideal caged Ca^{2+} would combine the high $\text{Ca}^{2+}:\text{Mg}^{2+}$ selectivity, pH insensitivity and fast buffering kinetics of the BAPTA (1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid)-based nitr series of chelators, the large change in Ca^{2+} affinity of DM-nitrophen and NP-EGTA with increased light sensitivity through higher quantum efficiencies and/or extinction coefficient. The Ca^{2+} affinity of the unphotolyzed chelator should match or be slightly less than typical $[\text{Ca}^{2+}]_i$ resting levels to provide maximal buffering power and therefore control of pre-illumination Ca^{2+} levels. After photolysis, final $[\text{Ca}^{2+}]_i$ of tens to hundreds of micromolar should be attainable to match the physiological levels reached in microdomains immediately below membrane Ca^{2+} channels [11].

A newly developed caged Ca^{2+} , azid-1, appears to fulfil most of these criteria. Azid-1, the 3-azido derivative of fura-2 [12], has the high $\text{Ca}^{2+}:\text{Mg}^{2+}$ selectivity of BAPTA chelators and decreases its Ca^{2+} affinity 500-fold upon photolysis (Figure 1). It has an extinction coefficient of $33,000 \text{ M}^{-1}\text{cm}^{-1}$ and quantum yield of 1.0, making it

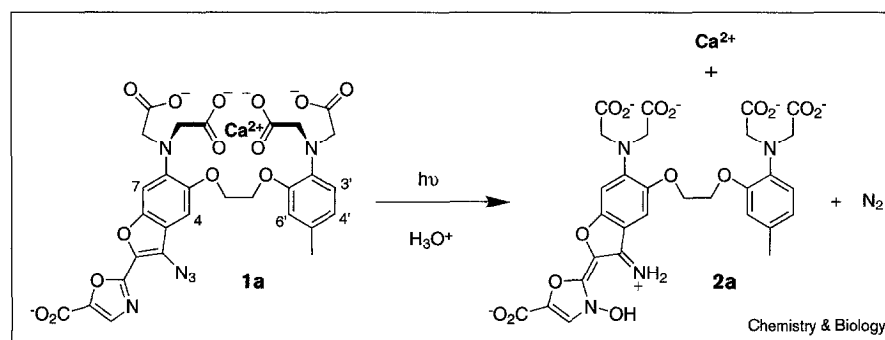
40–170 times more light sensitive than previous caged Ca^{2+} and it is also capable of releasing Ca^{2+} at rates sufficient for most biological experiments.

Results

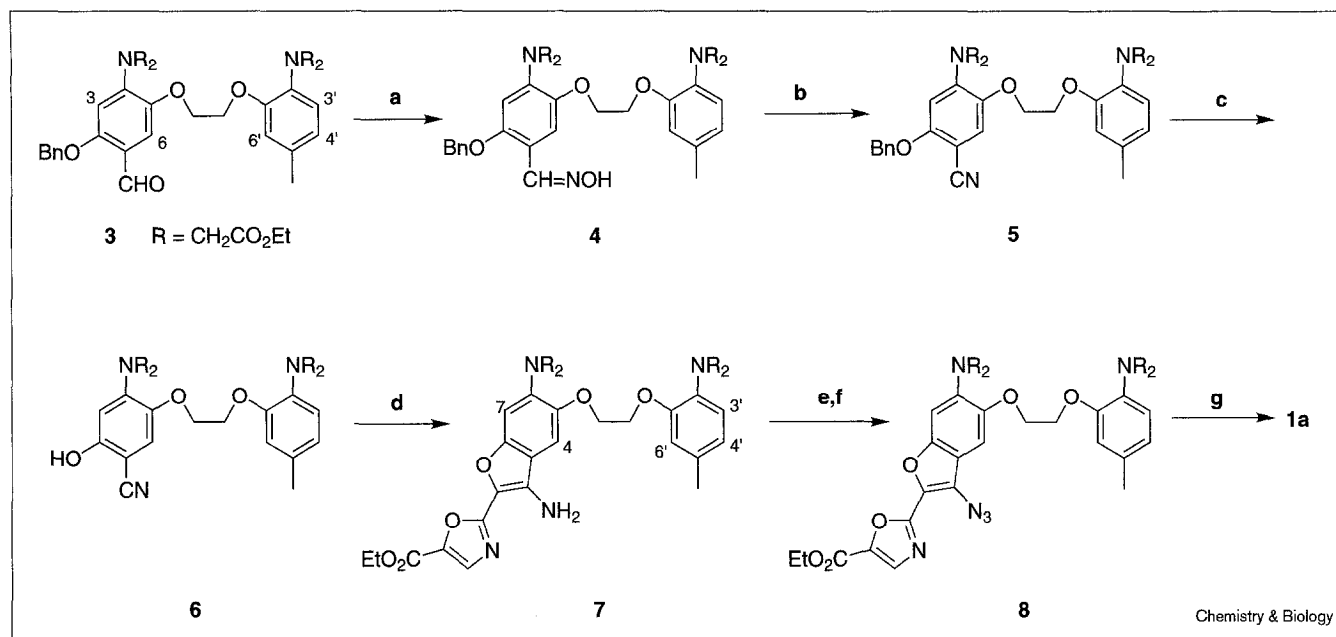
Photochemical and synthetic strategy

To investigate a wider range of photochemical reactions in the manipulation and measurement of Ca^{2+} -dependent processes in biology, we explored the properties of aromatic azides. Ca^{2+} chelators containing an azido substituent in the 3-position of a benzofuran were selected for their similarity to the fura family of fluorescent Ca^{2+} indicators [12] and because of their comparative ease of synthesis. An azide derivative of fura-2, named azid-1, was prepared by the steps shown in Figure 2. A key intermediate was the salicylnitrile (6) derivative of BAPTA which was synthesized from the salicylaldehyde benzyl ether (3) via dehydration of the intermediate oxime (4) by phosgene iminium chloride. Reaction of the salicylnitrile with ethyl 2-(chloromethyl)oxazole-5-carboxylate generated a derivative (7) of fura-2 containing a 3-amino group on the furan ring. Two interesting products were produced from 7. Simple saponification gave 3-amino-fura-2, a Ca^{2+} indicator with strong fluorescence like its parent fura-2 but with a large blue shift in emission from 485 nm to 433 nm upon

Figure 1



Photolysis of the Ca^{2+} complex of azid-1 (1a) leading to the formation of an amidinium product (2a) and photorelease of Ca^{2+} .

Figure 2

Synthesis of azid-1 (**1a**, see Figure 1). Reagents: **a**, NH₂OH, NaOAc, dioxane-MeOH; **b**, Me₂N⁺ = CCl₂ Cl⁻, CHCl₃; **c**, H₂, Pd-C, HOAc; **d**, ethyl 2-(chloromethyl)oxazole-5-carboxylate, K₂CO₃, DMF; **e**, NOHSO₄, HOAc-H₂SO₄; **f**, NaN₃, H₂O; **g**, KOH, dioxane-MeOH.

binding Ca²⁺ (with a dissociation constant of 0.1–0.2 μM), analogous to indo-1 [12] rather than fura-2 which has a negligible emission shift. The sensitivity of the emission wavelengths of 3-amino-fura-2 to Ca²⁺ binding probably results from the electron-donating properties of the amino substituent, which makes the excited state relatively electron-rich, like indo-1, so that the Ca²⁺-complex is not so prone to dissociate in the excited state.

Diazotization of ester **7** to form a diazonium salt and displacement with azide gave the pentaethyl ester of azid-1 (**8**) in good yield. Saponification of the esters with potassium hydroxide gave the desired product (**1a**), which was particularly light sensitive and unexpectedly lost affinity for Ca²⁺ when photolyzed. Azid-1 was therefore investigated further as a potential caged Ca²⁺.

Spectral properties, Ca²⁺ binding, photolysis and quantum yield of azid-1

The absorbance spectra of azid-1 with and without Ca²⁺ were similar to those of the parent chelator fura-2, but were red-shifted by about 10 nm as would be expected from the incorporation of an azide group into an aromatic system [13]. Ca²⁺-free azid-1 absorbed maximally at 372 nm with an extinction coefficient of 27,000 M⁻¹ cm⁻¹, compared to values of 362 nm and 27,000 M⁻¹ cm⁻¹ for fura-2 [12]. In the presence of Ca²⁺, the spectrum of azid-1 shifted 30 nm to shorter wavelengths giving a maximal absorbance of 33,000 M⁻¹ cm⁻¹ at 342 nm compared to 33,000 M⁻¹ cm⁻¹ at 335 nm for fura-2. The Ca²⁺ affinity of

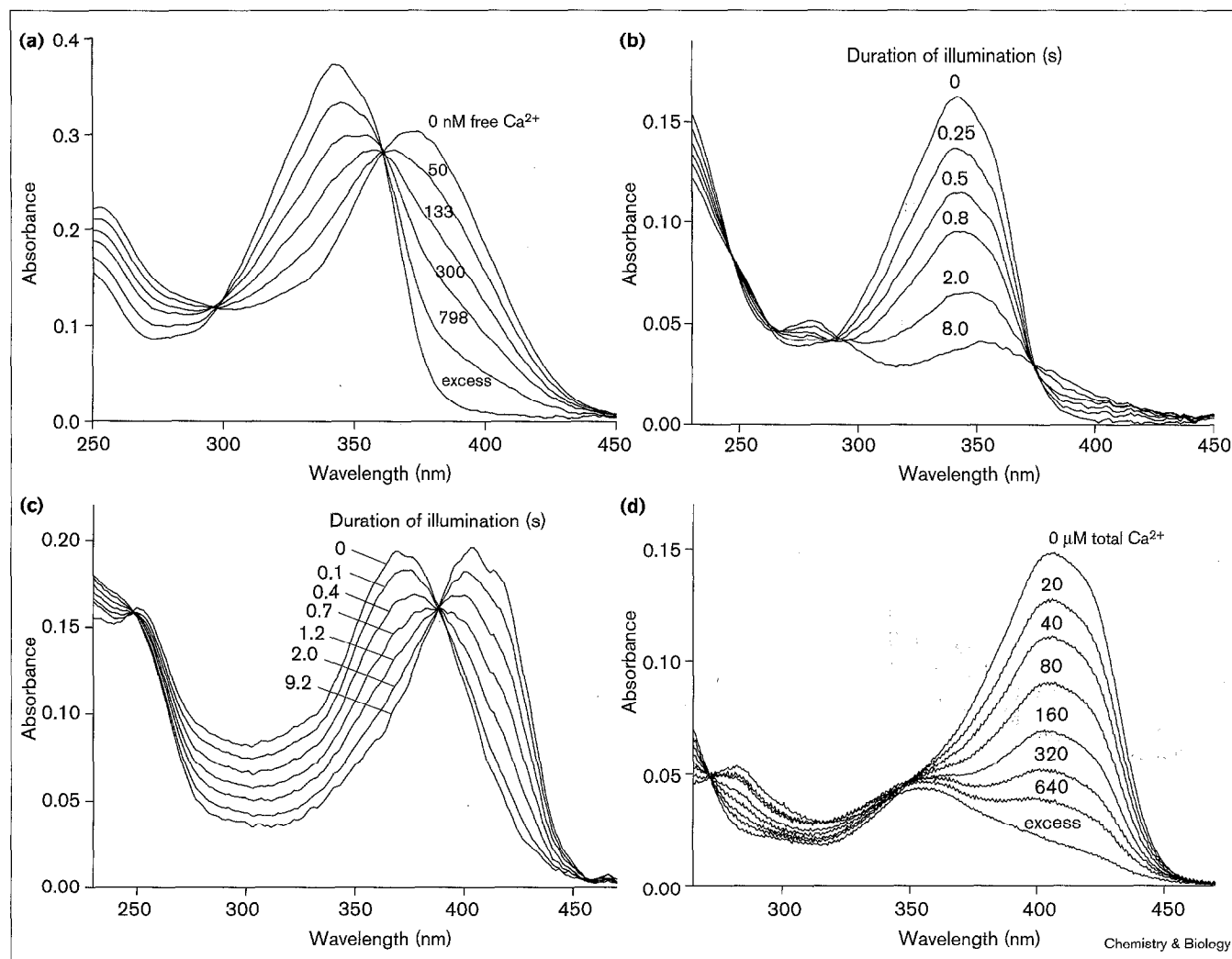
azid-1 before photolysis was determined to be 230 nM by measuring absorbance spectra in an EGTA-buffered solution that was set to various free Ca²⁺ concentrations (Figure 3a). A Hill plot of this spectral change gave a gradient of 1, indicating a 1:1 complex is formed between azid-1 and Ca²⁺. The slight weakening of Ca²⁺ binding probably results from increased electron withdrawal at the chelating amino group by the conjugated electronegative azide. The replacement of the benzofuran 3-hydrogen of fura-2 by an azido group therefore had only small and rationalizable effects upon the absorbance spectra and metal binding of the resulting molecule, azid-1.

Upon UV illumination, however, azid-1 did not fluoresce like fura-2 but underwent the irreversible spectral changes shown in Figure 3b and c. Thus, following irradiation at 365 nm, in zero Ca²⁺, the main absorbance peak shifted cleanly (with sharp isosbestic points) to a longer wavelength (400 nm) with a similar high extinction coefficient. The subsequent addition of Ca²⁺ produced a shift to the blue (to about 350 nm) and a large decrease in absorbance. A Ca²⁺ titration of the photoproduct revealed, unexpectedly, that the Ca²⁺ affinity was reduced over 500-fold to about 120 μM (Figure 3d). Photolysis of azid-1 in Ca²⁺ resulted in a similar spectrum indicating the same photoproduct was formed with and without Ca²⁺ (Figure 3b). The efficiency of this photochemical reaction was very high; quantum yields of 1.3 and 0.9 were measured from the absorbance changes in the presence or absence of Ca²⁺ respectively. The value of >1 (i.e. more than 1 product molecule formed per photon

absorbed) probably reflects errors or uncertainties in the UV-light intensity measurement, the finite spectral width

of the irradiating beam at 365 nm, or in the extinction coefficient of Ca^{2+} bound azid-1 at 365 nm, at the edge of

Figure 3



(a) Absorbance spectra of unphotolyzed azid-1 as a function of free $[\text{Ca}^{2+}]$. The titration was performed at 22°C by using 10 ml of 100 mM KCl, 10 mM K-MOPS, 10 mM $\text{K}_2\text{H}_2\text{EGTA}$ and 11 μM azid-1 as starting materials, adjusting the pH to 7.14, recording the spectrum, and then discarding 1.0 ml of this solution and replacing it with 1.0 ml of 100 mM KCl, 10 mM K-MOPS, 10 mM $\text{K}_2\text{Ca-EGTA}$ and 11 μM azid-1, readjusting the pH to 7.14, and recording the spectrum which was then in 9 mM $\text{K}_2\text{H}_2\text{EGTA}$. Subsequent iterations to reach n mM $\text{K}_2\text{Ca-EGTA}$, $(10-n)$ mM $\text{K}_2\text{H}_2\text{EGTA}$, $n = 2-10$, were performed by discarding $10.0/(11-n)$ ml and replacing it with equal volumes of the 10 mM $\text{K}_2\text{Ca-EGTA}$, 11 μM azid-1 stock. After $n = 10$ had been reached to give a free Ca^{2+} between 10^{-5} and 10^{-4} M, addition of excess 1 mM CaCl_2 had a small additional effect on the spectrum. For clarity, only six spectra are included in the figure, $n = 0, 2, 4, 6, 8$ and excess. Each spectrum is labeled with the calculated free $[\text{Ca}^{2+}]$ imposed by the EGTA buffer, assuming a log effective stability constant [35] of 6.70 at pH 7.14. **(b)** Absorbance spectra of azid-1 undergoing photolysis in the presence of Ca^{2+} . Azid-1 was dissolved at 5 μM in 100 mM KCl, 10 mM MOPS, 12 mM Tris, 1 mM $\text{Tris}_3\text{-EDTA}$, 3 mM CaCl_2 pH 7.5. Spectra were obtained after 0.05, 0.1, 0.25, 0.4, 0.5, 0.8, 1.3, 2.0, 4.0 and 8.0 s of 365 nm illumination at 1.6×10^{-8} einstein $\text{cm}^2 \text{s}^{-1}$ from the Spectroline

line. For clarity, only the recorded at spectra 0, 0.25, 0.5, 0.8, 2.0 and 8.0 s have been reproduced here. The 4.0 s and 8.0 s spectra were identical, confirming that photolysis was complete after those times. Solutions were at $22 \pm 2^\circ\text{C}$. **(c)** Absorbance spectra during photolysis of azid-1 in the absence of Ca^{2+} . The method was as described in (b) except that the solution contained no CaCl_2 , and the spectra were measured after 0, 0.1, 0.2, 0.4, 0.7, 1.2, 2.0, 3.0, 5.0 and 9.2 s of illumination at 1.6×10^{-8} einstein $\text{cm}^2 \text{s}^{-1}$ from the Spectroline line. The last two spectra were identical confirming completion of photolysis. For clarity, the 3.0 and 5.0 s spectra have been omitted. **(d)** Absorbance spectra of photolyzed azid-1 as a function of total $[\text{Ca}^{2+}]$. The photoproduct was produced by irradiating a 4 μM solution of azid-1 in 100 mM KCl (Ultrex grade; J.T. Baker Chem Co., Phillipsburg, NJ), 10 mM K-MOPS, pH 7.30, using the Spectroline lamp at 365 nm until completion. The titration was performed by the stepwise addition of concentrated CaCl_2 solution to attain a total $[\text{Ca}^{2+}]$ in the cuvet of 5, 10, 20, 40, 80, 160, 320, 640, 21,600 and 61,600 μM , and successively recording the absorbance spectrum. For clarity, only the spectra corresponding to 0, 20, 40, 80, 160, 320, 640 and 21,600 μM are shown here. The spectra at 21,600 and 61,600 μM were almost identical indicating complete saturation of the photoproduct by Ca^{2+} was achieved.

the absorbance peak. In contrast to the large change in Ca^{2+} affinity upon photolysis, the Mg^{2+} affinity remained essentially unchanged at about 8 mM. The photoproduct itself was not fluorescent, although slight impurities of 3-amino-fura-2 sometimes made solutions of azid-1 appear fluorescent before and after photolysis. In different preparations of azid-1, the concentration of this contaminant varies from 3–4% of the azid-1, that is 30–40 μM of indo-1-like indicator per 1 mM azid-1. Like most aromatic azides [14], azid-1 is susceptible to reduction to the fluorescent 3-amino-fura-2 by thiol anions at alkaline pH, particularly by dithiols such as dithiothreitol. Azid-1 reacts with 2-mercaptoethanol (10 mM) at physiological pH and room temperature with a half-life of 1–2 h (data not shown). Contact with such thiols should therefore be kept to a minimum.

Nature of the photoproducts

As the photolysis of aromatic azides can generate a variety and mixture of photoproducts via the highly reactive nitrene intermediate, it was important to analyze and identify the product(s) resulting from irradiation of azid-1. Separation of the reaction mixture produced by photolysis of 50 μM to 10 mM azid-1 pentapotassium salt at pH 7 with or without Ca^{2+} by reverse-phase thin layer chromatography (TLC), reverse-phase high performance liquid chromatography (HPLC) or capillary electrophoresis indicated only one major product, that was non-fluorescent was formed (data not shown). This product was stable at physiological pH for at least several hours, considerably more polar than azid-1 as judged by retention on reverse-phase HPLC, and slightly less negatively charged at pH 7 as judged by its electrophoretic behavior. Raising the pH caused an initially reversible spectral shift of the photoproduct to shorter wavelengths; this transition had a pK_a of about 10.5. Prolonged standing at high pH made the hypsochromic shift irreversible. A similar product appeared to result from hydrolysis of the photoproduct at pH 4.5 at room temperature over several hours. We hoped to separate and identify the products more easily by photolyzing large amounts of azid-1 pentaethyl ester in organic solvents, but more complex and different products were produced. Photolysis of azid-1 pentapotassium salt in methanol gave similar spectral shifts and loss of Ca^{2+} affinity as occurred in water, but the product was still highly charged and difficult to analyze by chromatography and mass spectroscopy.

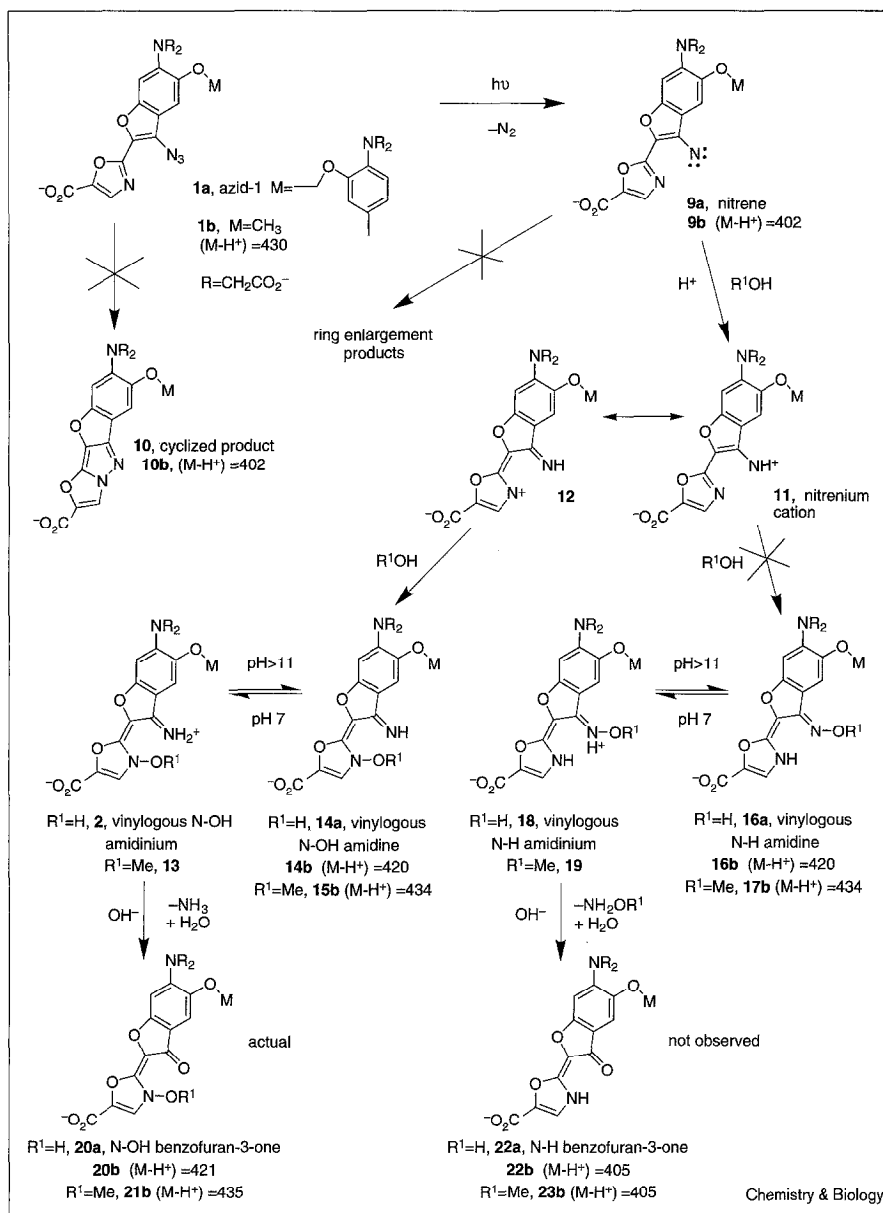
The reaction could also be conveniently monitored by ^1H -nuclear magnetic resonance (NMR) by photolyzing 5 mM azid-1 at pH 7 in D_2O in the absence of Ca^{2+} in a NMR tube and acquiring spectra at various time points. Again, a fairly clean transformation to one product was observed with most proton resonances only undergoing modest shifts. Two major shifts occurred, involving the aromatic proton (H-7) and acetate protons ($\text{N}-\text{CH}_2\text{CO}_2^-$) adjacent to the benzofuran amino group with an upfield shift of

1.1 ppm and a downfield shift of 0.5 ppm respectively. The resulting spectrum is similar to those of BAPTA chelators bearing strong electron-withdrawing groups (such as aldehydes or nitrile groups e.g. **6**) para to the amino group and is consistent with the observed weakening of Ca^{2+} binding. There was no indication of nitrene insertion (formed by loss of N_2 from the azido group) into the Ca^{2+} binding site or the ring expansions found with many phenyl azides.

The most revealing approach eventually was to apply electrospray mass spectrometry to **1b** (Figure 4, $\text{M} = \text{CH}_3$), a truncated model of azid-1 in which the photochemically irrelevant bis(carboxymethyl)aminophenoxyethoxy moiety was replaced by a simple methoxy substituent, to aid volatilization and remove any complications of Ca^{2+} binding. Compound **1b** showed the same changes in absorbance upon photolysis as azid-1, but it bound Ca^{2+} with only millimolar affinity before photolysis. The compound **1b** was prepared by an analogous synthetic route [12] to that described for azid-1 but starting with 2-methoxy-5-(benzyloxy)nitrobenzene. Before photolysis, the major peak had a molecular ion of 430.2, corresponding to the singly charged parent molecule; a minor peak at 402.1 was interpreted as a loss of N_2 , presumably by thermolysis during volatilization. Photolysis of **1b** in water gave a peak at 420.1, consistent with addition of water after initial expulsion of N_2 . When the photolysis was conducted in methanol, the product had a mass of 434, confirming that a molecule of solvent had been added. Treatment of the 420.1 Da aqueous photoproduct with aqueous 1 M-triethylamine at room temperature overnight yielded the final hydrolysis product with a mass of 421.1. The methanol photoproduct was stable to aqueous triethylamine but under mildly acidic conditions (1 M acetic acid at room temperature overnight) gave a 435 Da product.

A reaction scheme consistent with all these results is shown in Figure 4 and involves initial loss of N_2 to generate a nitrene **9**. This highly reactive intermediate reacts with solvent water, probably via nitrenium ion **11**, to form a vinylogous N-hydroxyamidinc **14** that protonates at pH 7 to give **2**. The delocalized positive charge of **2** is conjugated with and withdraws electron density from the amino group involved in Ca^{2+} binding, leading to a large decrease in affinity. It seems reasonable that amidinium **2** should have a pK_a of 10.5 and absorb at longer wavelengths than its conjugate base **14**. The positive charge on **2** is consistent with its reduced retention on reverse-phase HPLC or TLC and its decreased overall negative charge on capillary electrophoresis. Upon prolonged alkaline hydrolysis, the imine should hydrolyze to a carbonyl group in **20** with a gain of 1 Da as observed. Generation of the nitrene **9** in methanol should give the analogous N-methoxyamidinc **15** which hydrolyzes to **21**, consistent with the mass spectra.

Figure 4



Flash photolysis kinetics *in vitro*

For use as a caged Ca²⁺ in many biological systems, azid-1 should release Ca²⁺ rapidly (within a millisecond) following a light flash. The proposed mechanism for generating decreased Ca²⁺ binding involves dark reactions which could be rate-limiting. To determine the rate of Ca²⁺ release following flash photolysis, we used the fluorescent Ca²⁺ indicator fluo-3 which permits monitoring of the Ca²⁺ concentration at wavelengths that do not photolyze azid-1. The kinetics of Ca²⁺ binding to fluo-3 have been estimated to be $>10^9 M^{-1}s^{-1}$ [15] and therefore should not limit the detection of Ca²⁺ release under the conditions used (fluo-3 concentration of 0.1 mM). Following a brief flash (duration <1 ms) of a mixture of azid-1, fluo-3 and

Ca²⁺, fluo-3 fluorescence increased to a new level with kinetics that were faster than the time-resolution of the instrument indicating the release of Ca²⁺ from photolyzed azid-1 is complete within 2 ms (data not shown). Control experiments omitting azid-1 and/or Ca²⁺ failed to give any changes in fluorescence.

Biological testing of azid-1

Azid-1 is biologically useful because it can be used to demonstrate the role of Ca²⁺ in synaptic plasticity of the cerebellum. Long-term changes in synaptic efficacy are thought to be the neuronal basis for learning and memory. In the cerebellum, long-term depression (LTD) is defined as the reduction in synaptic transmission between parallel

fibers and Purkinje cells resulting from simultaneous pre-synaptic activity and postsynaptic depolarization. We studied LTD in acute rat cerebellar slices using the whole-cell patch technique [16] which permits monitoring of the synaptic currents of the Purkinje cell while simultaneously introducing azid-1 by perfusion. Previously, we have demonstrated that NO is an essential participant in LTD induction and that it is produced outside the Purkinje cells as a result of parallel fiber activation. NO acts inside the Purkinje cell in concert with a depolarization-induced Ca^{2+} transient to induce LTD [16]. Simultaneous parallel-fiber stimulation and depolarization (at a frequency of 1 Hz) for 30 s (Figure 5, protocol i) were unable to induce LTD as they normally would have in the absence of azid-1 [16,17] because unphotolyzed azid-1 is a high affinity Ca^{2+} buffer and should mimic BAPTA in preventing LTD induction. When uncaging was synchronized with parallel-fiber stimulation over a similar 30 s period (protocol ii), however, LTD promptly resulted ($n = 4$). Thus, photoreleased Ca^{2+} is sufficient to replace climbing fiber activity and depolarization, and the elevation of $[\text{Ca}^{2+}]_i$ is the only essential function of depolarization in inducing LTD in mature Purkinje cells in slices. Experiments using azid-1 gave similar results to those using another caged calcium, nitr-7 [5] except light flashes of much shorter duration (50–100 ms compared to 500 ms for nitr-7) were sufficient to induce LTD.

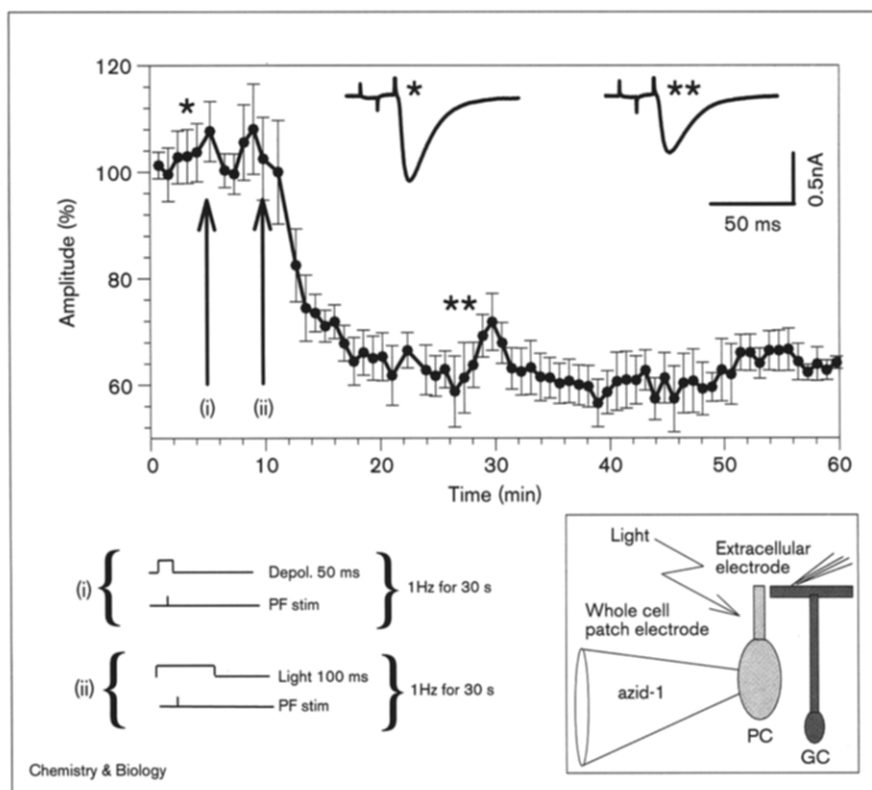
Discussion

Photochemical mechanism of azid-1

The majority of caged compounds (and all previous versions of caged Ca^{2+}) utilize the photochemistry of 2-nitrobenzyl groups in their photorelease mechanism. Other photochemical groups have been used more rarely. For example, diazo chelators [18] use the Wolff rearrangement of diazoketones to generate a Ca^{2+} chelator with an increased affinity upon photolysis. Benzoin esters of ATP [19] release the nucleotide triphosphate upon UV illumination with the formation of a phenylbenzofuran photoproduct. The photochemistry of azides has been extensively studied from a physical and chemical standpoint but few applications have been reported to be pertinent to designing new caged compounds. Photo-affinity labeling [14,20], a technique used extensively in biology, frequently involves the photolysis of an azido group in a ligand to covalently tag a macromolecular receptor. Most aromatic azides react upon photolysis to produce a wide variety of end products resulting from ring expansion, direct insertion into saturated and unsaturated bonds, or self-reaction, however. Such behavior results from the highly reactive nitrene intermediate which rapidly reacts with many neighboring chemical groups [21]. Coupling such photochemistry to the photorelease of biologically active compounds is difficult, although an azide-based

Figure 5

Induction of cerebellar long-term depression by uncaged Ca^{2+} in conjunction with parallel-fiber stimulation. Excitatory postsynaptic currents (EPSCs) in a Purkinje neuron were monitored using whole-cell patch recording. Each EPSC trace is the average response from four cells and each point on the graph is an average of ten EPSC peak amplitudes from consecutive parallel-fiber stimuli delivered at 0.2 Hz (a test frequency too low to cause plasticity), normalized to the pretraining EPSC amplitude and averaged over all cells. Error bars represent the standard errors of each group. The patch pipette included 2 mM azid-1. Simultaneous parallel-fiber stimulation (PF stim) and depolarization (depol) at 1 Hz for 30 s, a standard training protocol (i) for eliciting long-term depression, was ineffective because unphotolyzed azid-1 buffered the $[\text{Ca}^{2+}]_i$ elevation. Before protocol (ii), azid-1 was reloaded with Ca^{2+} from depolarization-induced $[\text{Ca}^{2+}]_i$ transients. When photolytic release of Ca^{2+} by 100 ms flashes of 365 nm light (1 Hz for 30 s, protocol ii) coincided with parallel-fiber stimulation (1 Hz for 30 s), long-term depression was induced (i.e. a lasting reduction in EPSC amplitude to $62 \pm 11\%$ of its value just before training, a depression significant at the $p = 0.0031$ level by the one-tailed t test). Insets show typical averages of ten consecutive EPSC traces from the times indicated by *,**. PC, Purkinje cell; GC, granule cell; PF, parallel fiber.



photoremovable protecting group for carboxylic acids has been reported [22]. One class of aromatic azides containing ortho unsaturated substituents such as C = O and NO₂, however, photolyze or thermolyze to single products in high yield via a concerted pericyclic mechanism without direct formation of a nitrene. The products result from insertion into the neighboring group and such reactions have been studied extensively [23].

Azid-1 is an azide-substituted derivative of the fluorescent Ca²⁺ indicator fura-2, in which the imine bond (-C=N-) of the oxazole ring appears suitably sited for insertion. Such a photolysis mechanism would involve the initial loss of N₂ from the azido group followed by formation of a novel ring system (**10**, Figure 4) in which the benzofuran is fused to the oxazole through a pyrazole. Similar reactions have been reported for analogous classes of compounds such as 2-(2-azidophenyl)pyridine and 2-azidobenzylidene derivatives and result in the fusion of one or two 6-membered rings to a 5-membered heterocycle [13,24]. Surprisingly this mechanism apparently does not occur during photolysis of azid-1 as the photoproduct has a mass consistent with the loss of N₂ and the reaction with a solvent water molecule. Perhaps cyclization does not occur because of the excessive ring strain generated from the fusion of three 5-membered heterocycles. Loss of N₂ and addition of solvent usually indicates ring expansion with phenyl azides, but this pathway is not consistent with the NMR of the product and its decreased affinity for Ca²⁺. Ring expansion is well known to be hindered by ortho substituents as present in azid-1. Instead, rapid protonation by water of the strongly basic nitrene, **9** forms the nitrenium ion, **11** (Figure 4) within 1 ns as recently reported for aryl azides photolyzed in water [25]. Phenyl nitrenium ion reacts characteristically as the iminocyclohexadienyl cation producing 4-aminophenol in water by para attack of hydroxide or water followed by proton tautomerization [26]. A comparable resonance structure for the nitrenium ion formed from photolyzing azid-1 is **12**, in which the oxazole nitrogen bears the positive charge. Attack by hydroxide or water at the positive charge in either of these intermediates could occur resulting in either a vinylogous N-OH amidine (**14**) or a vinylogous N-H amidine (**16**) which would protonate (**2** and **18** respectively) at neutral pH with expected pK_as > 9 [27]. As these products are isomers, they cannot be distinguished by mass. Determining the site of attachment of the N-OH group by ¹H-NMR studies would be impossible in protic solvents as these protons rapidly exchange with the solvent. These two possible reaction pathways can be delineated by alkaline or mildly acidic hydrolysis of the imine bond in **14** or **16** to produce the two benzofuran-3-ones **20** or **22** respectively. Only the first route (i.e. **14** to **20**) results in the change in mass of +1 found exclusively. Attack by solvent at the non-nitrene nitrogen is consistent with previous studies of phenyl azides [26] which photolyze solely to the analogous ring-substituted product (usually at the para position). With

para-substituted phenyl azides, attack at this position leads to imine products which are susceptible to hydrolysis to substituted cyclohexadienones in a comparable manner to the photoproducts from azid-1. Further mass spectral results supporting this mechanism are peaks consistent with the addition of methanol when **1b** was photolyzed in aqueous methanol to form **15b** and subsequent hydrolysis to the corresponding N-methoxy benzofuran-3-one **21b**. These results do not rule out the possibility of subsequent rearrangement of **16** to **14** by hydroxyl migration between the imidine nitrogens although this appears unlikely as the methanol photoproduct would have to undergo intramolecular methoxy migration to produce exclusively **21b**. Similarly when azid-1 is photolyzed in methanol, the product binds Ca²⁺ with a comparable affinity to **2a** as expected for the N-methoxy amidinium product **13a**. The rapid generation of **2**, complete within 2 ms, is not surprising considering the high reactivity of nitrenes and nitrenium ions with lifetimes in the nanosecond and microsecond range respectively [25]. Transient absorbance spectroscopy following laser flash photolysis could possibly detect these intermediates as well as the formation of the amidine and its protonation.

Sensitivity to UV and two-photon photolysis

The high sensitivity of azid-1 to light is in agreement with numerous studies involving polycyclic aromatic azides which show quantum efficiencies approaching unity, particularly for the more extended ring structures [13]. In combination with the high extinction coefficients of the fura-2 stilbene-like chromophore, the light sensitivity (or the product of the extinction coefficient and the quantum yield at the wavelength of irradiation) of azid-1 greatly exceeds that of existing caged calciums by at least an order of magnitude (Table 1). When incorporated into Purkinje cells, azid-1 was able to mimic the action of nitr-7 in producing LTD but with up to tenfold briefer flashes of light. That yet briefer flashes were ineffectual and that more azid-1 was required (2 mM compared to 0.5 mM nitr-7) probably reflects the difference in chelator starting affinity.

Such enhanced photolability permits the use of less expensive flash lamps and lasers, allows more photorelease per flash, and reduces any deleterious effects of the UV light upon the biological sample. In addition, these more favorable optical properties of azid-1 greatly increase its ability to undergo two-photon photolysis compared to conventional nitrobenzyl based caged Ca²⁺ (E.B. Brown, J.B. Shear, S.R.A., R.Y.T., W.W. Webb, unpublished observations). In this emerging and potentially powerful technique [10], two infra-red photons, if absorbed simultaneously, can elicit the same photoreaction as a single UV photon. The high photon density required for such a process confines two-photon photolysis to a volume of a few femtoliters in a tightly focused laser beam of femtosecond infra-red pulses, in comparison with conventional one-photon photolysis where photorelease occurs

throughout the beam. A high two-photon cross section is particularly important for a caged Ca^{2+} as millimolar amounts may have to be photolyzed to overcome the intracellular Ca^{2+} buffer compared to the micromolar or less amounts required for other caged compounds. The maximal two-photon cross section for azid-1 is ~ 1.4 GM (Göppert-Mayer; $1 \text{ GM} = 10^{-50} \text{ cm}^4 \cdot \text{s}/\text{photon}$) at 700 nm, at least 100-fold greater than the value for DM-nitrophen (E.B. Brown, J.B. Shear, S.R.A., R.Y.T., W.W. Webb, unpublished observations). NP-EGTA has negligible cross section under these conditions. The high value for the cross section of azid-1 is not surprising considering the value of 12 GM for Ca^{2+} -bound fura-2 [28], which only differs by the absence of the azide group. The ability to restrict Ca^{2+} elevations to precise subcellular locations will allow more accurate spatial probing of Ca^{2+} -dependent processes such as exocytosis and muscle contraction.

The high extinction coefficient of azid-1 could be a disadvantage in optically thick cells or tissue as inner-filtering could reduce the homogeneity of photorelease through the sample. The conventional caged Ca^{2+} chelators suffer less from this particular problem particularly NP-EGTA which contains an unsubstituted nitrobenzyl group. However, photolyzing sufficient cage to generate the desired increase in Ca^{2+} can then become difficult [29]. For two-photon photolysis, inner-filtering effects are negligible because all caged Ca^{2+} have no absorbance in the infra red.

Comparison of azid-1 with other caged calciums

Azid-1 has several additional advantages over other caged Ca^{2+} currently in use. The over 500-fold decrease in its Ca^{2+} affinity from $0.23 \mu\text{M}$ to $120 \mu\text{M}$ upon photolysis should permit control of the many physiological events that are sensitive to changes in $[\text{Ca}^{2+}]_i$ in this range. In comparison, nitr-5 and nitr-7 permit only modest elevations in Ca^{2+} of a few micromolar allowing only partial or no activation of some Ca^{2+} -sensitive processes, whereas DM-nitrophen and NP-EGTA can produce increases up to millimolar levels (Table 1). Azid-1 binds Ca^{2+} more weakly before photolysis than the other caged Ca^{2+} s, however, so in cells with a typical resting $[\text{Ca}^{2+}]_i$ of 100 nM, less Ca^{2+} will be bound and released upon irradiation. Furthermore, any unphotolyzed Ca^{2+} -free azid-1 will buffer the photoreleased ion (with the expected fast kinetics of BAPTA-based chelators of $> 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and limit the size of the Ca^{2+} elevation. This problem is solved if the light flash is strong enough to photolyze both the Ca^{2+} -free as well as the Ca^{2+} -bound azid-1. Such complete photolysis is aided by the high quantum efficiency of unbound azid-1 and its absorbance at slightly longer wavelengths than the Ca^{2+} -complex. Another answer would be to increase the Ca^{2+} affinity of azid-1 before photolysis by, for example, incorporation of a *cis*-cyclopentane ring into the ethane bridge between the two ether oxygens, as in nitr-7 [5]. This should strengthen the binding by a factor

of 3–4 to a K_d of 60–90 nM but would also presumably increase Ca^{2+} binding of the photoproduct by a similar amount resulting in a lower final $[\text{Ca}^{2+}]$ after photolysis. Such modification (although requiring considerable organic synthesis) may be necessary for those applications in which free $[\text{Ca}^{2+}]$ must be kept particularly low before photolysis.

The proposed mechanism for azid-1 photolysis results in the uptake of one proton per Ca^{2+} released. Increasing the pH in the cytoplasm of a cell is therefore a possible side-effect which should be controlled by the addition of pH buffer if the concentration of azid-1 is sufficiently high enough to overcome the strong intrinsic cytoplasmic H^+ buffer. A similar alkalification is also shown by DM-nitrophen and NP-EGTA.

The presence of fluorescent impurities, such as 3-amino-fura-2, in preparations of azid-1 may complicate monitoring of photogenerated Ca^{2+} increases inside cells using fluorescent Ca^{2+} indicators. The impurity, being itself an emission-ratioable indicator like indo-1, could be used to follow Ca^{2+} photorelease, except the high photosensitivity of azid-1 at the required excitation wavelength (about 350 nm) for 3-amino-fura-2 would cause slow release of Ca^{2+} in the absence of a flash. Upon binding Ca^{2+} , 3-amino-fura-2 shifts emission from 490 to 420 nm so release of Ca^{2+} by azid-1 results in fluorescent changes at these wavelengths that could interfere with signals from long wavelength fluorescent indicators such as fluo-3. Further purification of azid-1 following saponification may be necessary to prevent such cross talk.

The metal-binding site of azid-1 is based upon BAPTA or EGTA, (as is nitr-5, nitr-7 and NP-EGTA) and shows the greater than 10^4 selectivity for Ca^{2+} over Mg^{2+} required for producing Ca^{2+} increases without perturbing Mg^{2+} levels under physiological conditions, unlike DM-nitrophen, which acts as a caged Mg^{2+} and a caged Ca^{2+} . Like other BAPTA-based chelators, the Ca^{2+} affinity of azid-1 shows little sensitivity to pH in the physiological range, and fast buffering kinetics (milliseconds), compared to caged Ca^{2+} derived from EDTA (DM-nitrophen) and EGTA (NP-EGTA), which permit more efficient clamping of intracellular levels before and after photolysis. High on-rates for Ca^{2+} binding are important to minimize the generation of transient spikes of high $[\text{Ca}^{2+}]$ lasting a few milliseconds following a flash that leaves sufficient Ca^{2+} -free chelator unphotolyzed to slowly re-bind part of the released Ca^{2+} . Transient Ca^{2+} increases for DM-nitrophen and NP-EGTA have been measured directly using low-affinity fluorescent Ca^{2+} indicators [30,31].

Significance

Rapid increases in levels of intracellular free calcium in the form of transients, oscillations and gradients have been described in many cells in response to a variety of

stimuli. Fluorescent imaging of this ubiquitous second messenger has also revealed highly localized Ca^{2+} elevations or local 'hot spots' within individual cells. Detailed mechanisms of the generation and control of Ca^{2+} signals and their downstream biochemical effects are still poorly understood, however. One valuable technique used to probe and understand such pathways is the controlled spatial and temporal release of Ca^{2+} by irradiation of photosensitive chelators or caged calciums. Here, we introduce such a photolabile chelator, azid-1, with new and useful properties, by a novel application of the photochemistry of azides to the design of photoreleasable or caged molecules. Azid-1 can selectively produce a photo-induced Ca^{2+} increase to the 10–100 μM range from the typical cellular resting levels of 0.1–0.2 μM in the presence of, and without perturbing, physiological Mg^{2+} concentrations. Such elevated intracellular free calcium is sufficient to activate most Ca^{2+} triggered cellular processes, including muscle contraction and exocytosis of synaptic vesicles in neurons. Azid-1 is considerably more light sensitive than currently available caged calciums which should permit larger increases in Ca^{2+} per flash, decrease any deleterious biological effects of UV irradiation, and allow photorelease of Ca^{2+} by two-photon photolysis in volumes as small as a few femtoliters inside living cells. The technique of two-photon photolysis will enable direct probing of the role of Ca^{2+} with unprecedented spatial resolution within individual cells.

Materials and methods

Chemistry

Chemicals (Aldrich; Milwaukee, WI, USA) and solvents (HPLC-grade, Fisher; Fair Lawn, NJ, USA) were used directly as received unless otherwise noted. Chloroform and dimethylformamide (DMF) were dried over 4 Å molecular sieve. Biochemicals were from Aldrich or Calbiochem (La Jolla, CA, USA).

Proton magnetic resonance spectra (^1H NMR) were recorded on a Gemini 200-MHz spectrometer (Varian; Palo Alto, CA, USA) in CDCl_3 unless otherwise noted, and the chemical shifts are given in δ values relative to tetramethylsilane. Ultra-violet spectra were recorded on a Lambda Array 3840 spectrophotometer (Perkin-Elmer; Norwalk, CT, CA) or a Cary 3E UV-Visible spectrophotometer (Varian; Palo Alto, CA, USA) at 20°C. Electrospray mass spectrometry (5989B, Hewlett-Packard; Palo Alto, CA, USA) of **1b** and its photoproducts was performed by injecting ~1 mM solutions of the triethylammonium salt in $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ and detecting negative ions.

TLC was carried out on precoated silica gel 60F-254 or reverse-phase RP-18, F-254 plates (E Merck, EM Separations; Gibbstown, NJ, USA). For column chromatography, silica gel 60 (230–400 mesh, E Merck) was used. All manipulations of compounds sensitive to near ultraviolet light were performed under an orange safety lamp.

Oxime **4**

3 [12] (1.0 g, 1.36 mmol) dissolved in dioxane (6 ml) and methanol (6 ml) was treated with a solution of hydroxylamine hydrochloride (278 mg, 4 mmol) and sodium acetate (230 mg, 2.8 mmol) dissolved in water (2.8 ml) at room temperature. After gentle warming to 60°C to dissolve any solids, the reaction mixture was kept overnight at room temperature. The product was precipitated by the addition of water (10 ml) and collected by filtration. Recrystallization from 95% ethanol

yielded oxime **4** as white crystals, m.p. 108–110°C. Yield, 0.98 g (95%). ^1H NMR δ 1.16 (t, 12 H, OCH_2CH_3), 2.25 (s, 3H, ArCH_3), 4.05 (q, 4H, OCH_2CH_3), 4.07 (q, 4H, OCH_2CH_3), 4.12 (s, 2H, NCH_2), 4.14 (s, 2H, NCH_2), 4.24 (s, 4H, $\text{OCH}_2\text{CH}_2\text{O}$), 5.00 (s, 2H, benzyl CH_2), 6.39 (s, 1H, H-3), 6.7 (m, 3H, H-3',4',6'), 7.11 (s, 1H, oxime OH), 7.28 (s, 1H, H-6), 7.38 (m, 5H, benzene), 8.45 (s, 1H, $\text{CH}=\text{N}$, syn). ES-MS (positive ion) [$\text{CH}_3\text{CN}:\text{H}_2\text{O}$; 95:5] 752.4 (M+1); calc'd M = 751.3.

Nitrile **5**

A solution of **4** (0.98 g, 1.31 mmol) in chloroform (2 ml) was added in one portion to a suspension of phosgene iminium chloride (0.36 g, 2.2 mmol) in chloroform (5 ml). After 10 min reflux, during which HCl fumes were evolved, the resulting solution was evaporated to dryness, and the residue boiled with methanol (50 ml). After cooling in ice, **5** was collected by filtration and washed with several portions of cold methanol. Yield 0.83 g (87%). M.p. 145–146°C. ^1H NMR δ 1.16 (t, 6H, OCH_2CH_3), 1.17 (t, 6H, OCH_2CH_3), 2.26 (s, 3H, ArCH_3), 4.06 (q, 4H, OCH_2CH_3), 4.07 (q, 4H, OCH_2CH_3), 4.11 (s, 8H, NCH_2), 4.20 (s, 4H, $\text{OCH}_2\text{CH}_2\text{O}$), 5.12 (s, 2H, benzyl CH_2), 6.31 (s, 1H, H-3), 6.6–6.8 (m, 3H, H-3',4',6'), 7.00 (s, 1H, H-6), 7.39 (m, 5H, benzene). ES-MS (positive ion) [$\text{CH}_3\text{CN}:\text{H}_2\text{O}$; 95:5] 734.4 (M+1); calc'd M = 733.3.

Salicylnitrile **6**

5 (300 mg, 0.41 mmol) was catalytically hydrogenated at room temperature and pressure with 300 mg of 5% Pd/C in ethyl acetate:acetic acid (25 ml, 1:2 v/v). Uptake was complete in 30 min; the reaction mixture was filtered and evaporated to dryness to yield the product, **6**. Recrystallization from ethanol gave a pale yellow solid, m.p. 139–141°C. Yield 226 mg (86%). ^1H NMR δ 1.17 (t, 6H, OCH_2CH_3), 1.19 (t, 6H, OCH_2CH_3), 1.55 (br s, ~2H, OH and DOH), 2.26 (s, 3H, ArCH_3), 4.08 (q, 4H, OCH_2CH_3), 4.09 (q, 4H, OCH_2CH_3), 4.12 (s, 4H, NCH_2), 4.18 (s, 4H, NCH_2), 4.21 (s, 4H, $\text{OCH}_2\text{CH}_2\text{O}$), 6.27 (s, 1H, H-3), 6.6–6.8 (m, 3H, H-3',4',6'), 6.90 (s, 1H, H-6). ES-MS (positive ion) [$\text{CH}_3\text{CN}:\text{H}_2\text{O}$; 95:5] 644.3 (M+1); calc'd M = 643.3.

3-Amino-2-oxazolylbenzofuran **7**

6 (100 mg, 0.155 mmol), ethyl 2-(chloromethyl)oxazole-5-carboxylate (30 mg, 0.16 mmol; prepared as described [12]) and anhydrous potassium carbonate (30 mg, 0.22 mmol) were heated in dry DMF (0.25 ml) at 130°C for 1 h under an argon atmosphere. The cooled reaction mixture was diluted with water (5 ml), acidified with acetic acid and extracted (3 x 5 ml) with ethyl acetate. After drying over sodium sulfate, the extract was evaporated to dryness to yield crude **7**, that was further purified by silica gel chromatography eluting with ethyl acetate/hexane. The resulting yellow solid was triturated with isopropyl ether and filtered. Yield 71 mg (61%). M.p. 83–85°C. ^1H NMR δ 1.16 (t, 6H, OCH_2CH_3), 1.21 (t, 6H, OCH_2CH_3), 1.40 (t, 3H, oxazole OCH_2CH_3), 2.26 (s, 3H, ArCH_3), 4.06 (q, 4H, OCH_2CH_3), 4.12 (q, 4H, OCH_2CH_3), 4.15 (s, 4H, NCH_2), 4.20 (s, 4H, NCH_2), 4.3 (dt, 4H, $\text{OCH}_2\text{CH}_2\text{O}$), 4.41 (q, 2H, oxazole OCH_2CH_3), 5.09 (s, 2H, NH_2), 6.67–6.81 (m, 3H, H-3',4',6'), 6.88 (s, 1H, H-7), 7.14 (s, 1H, H-4), 7.85 (s, 1H, oxazole). ES-MS (positive ion) [$\text{CH}_3\text{CN}:\text{H}_2\text{O}$; 95:5] 797.3 (M+1); calc'd M = 796.3.

Azid-1 pentaethyl ester **8**

7 (20 mg, 26.4 μmol) was dissolved in cold glacial acetic acid (300 μl) and added dropwise with stirring to a solution of nitrosyl hydrogensulfate (20 mg, 157 μmol , Lancaster Synthesis; Windham, NH, USA) in concentrated sulfuric acid (200 μl) at 0°C. After 20 min, the reaction mixture was added dropwise to a ice-cold saturated aqueous solution of sodium azide (20 ml) with vigorous stirring (CARE; hydrazoic acid generated; carry out in well-ventilated fumehood). After neutralization with solid sodium bicarbonate, the mixture was diluted with water and extracted (3 x 20 ml) with ethyl acetate. Drying over sodium sulfate and evaporation to dryness yielded crude **8** which was further purified by silica gel column chromatography eluting with ethyl acetate/hexane followed by trituration with ethanol. Yield of pale yellow solid, 13.5 mg (66 %). M.p. 114–116°C. ^1H NMR δ 1.16 (t, 6H, OCH_2CH_3), 1.18 (t, 6H, OCH_2CH_3), 1.42 (t, 3H, oxazole OCH_2CH_3), 2.27 (s, 3H, ArCH_3),

4.05 (q, 8H, OCH₂CH₃), 4.12 (s, 4H, NCH₂), 4.22 (s, 4H, NCH₂), 4.32 (s, 4H, OCH₂CH₂O), 4.43 (q, 2H, oxazole OCH₂CH₃), 6.70–6.80 (m, 3H, H–3',4',6'), 6.95 (s, 1H, H–7), 7.13 (s, 1H, H–4), 7.91 (s, 1H, oxazole). ES-MS (positive ion) [CH₃CN:H₂O; 95:5] 823.2 (M+1); calc'd M = 822.3.

Azid-1 pentapotassium salt 1b

Azid-1 pentaethyl ester **8** (6.71 mg, 8.15 μmol) was saponified to the penta-anion by dissolution in dioxane (0.2 ml) and methanol (0.2 ml), followed by addition of aqueous 1M potassium hydroxide (79 μl, 79 μmol) and keeping at room temperature overnight. The organic solvents were evaporated under a stream of N₂ at room temperature and the resulting solution neutralized to pH~7 with a solution of an appropriate acid such as MOPS or HCl. Alternatively the free acid can be precipitated by the addition of dilute hydrochloric acid to pH 2, cooling on ice and collecting the resulting pale yellow precipitate by centrifugation. Addition of the calculated amount (i.e. 5 equivalents) of aqueous KOH generates the pentapotassium salt. Solutions were kept frozen between use and only handled under orange safety light or subdued lighting. ¹NMR D₂O δ 2.25 (s, 3H, ArCH₃), 3.72 (s, 4H, 2'-NCH₂), 3.85 (s, 4H, 6-NCH₂), 4.40 (s br, 4H, OCH₂CH₂O), 6.77 (s, 2H, H–3',4'), 6.95 (s, 1H, H–6'), 6.99 (s, 1H, H–7), 7.32 (s, 1H, H–4), 7.64 (s, 1H, oxazole).

¹NMR of photolysis product

Azid-1 pentaethyl ester (5 mg, 6 μmol) was saponified as described above and adjusted to pH 7 with dilute hydrochloric acid. The solution was evaporated to dryness and then repeatedly (two to three times) redissolved in D₂O (0.5 ml) and re-evaporated. Photolysis was accomplished by irradiation for 5 min with a Spectroline TC-365A UV transilluminator (Spectronics Corp; Westbury, NY, CA). ¹NMR D₂O δ 2.21 (s, 3H, ArCH₃), 3.77 (s, 4H, 2'-NCH₂), 4.08 (s br, 4H, OCH₂CH₂O), 4.27 (s, 4H, 6-NCH₂), 5.91 (s, 1H, H–7), 6.65 (dd, 2H, H–3',4'), 6.91 (s, 1H, H–6'), 7.25 (s, 1H, H–4), 7.45 (s, 1H, oxazole).

Calcium and magnesium affinities

Ca²⁺-binding constants for azid-1 before photolysis were determined by monitoring UV-visible spectra during titration of EGTA buffers to varying free Ca²⁺ levels by the reciprocal dilution method [5]. The dissociation constants of Ca-EGTA were calculated as previously described [32]. Photolyzed azid-1 was titrated by adding aliquots of standard solutions of CaCl₂ to a nominally Ca²⁺-free solution in which the Ca²⁺ contamination present initially in the solution before titration was negligible (less than a few micromolar). Hill plots of the resulting spectral changes against -log[Ca²⁺] gave the dissociation constants as x intercepts with gradients of 1.01 ± 0.01 and 0.96 ± 0.01 for azid-1 and the photoproduct respectively, indicating a 1:1 complex of the chelator and Ca²⁺ is formed.

Free [Mg²⁺] was likewise controlled by Mg/EGTA buffers, assuming an apparent dissociation constant for Mg-EGTA complex (including its monoprotated form) of 6 mM at pH 7.60 in 0.1 M ionic strength [32].

Quantum efficiency of photolysis

The photolysis quantum efficiencies of azid-1 was determined by alternately irradiating a buffered solution of the substrate containing zero (1 mM EDTA) or saturating Ca²⁺ with a known intensity of near-UV light and recording the resulting absorbance spectrum in a spectrophotometer as described previously [5]. A B-100 Mercury lamp (Spectronics Corp; Westbury, NY, CA) was used as a source of 365 nm light and its intensity (~1–2 × 10⁻⁸ einsteins cm⁻² s⁻¹) measured each experimental day by actinometry using 6 mM potassium ferrioxalate [33].

Flash photolysis

Flash photolysis of azid-1 and the concomitant monitoring of free Ca²⁺ concentration with fluo-3, a fluorescent Ca²⁺-indicator, were performed using methodology and instrumentation described previously [34] except a flash-lamp was used for photorelease. Briefly, output from a 150 W Xe arc lamp was passed through a grating monochromator to

yield the 490 nm light used to probe the fluo-3 indicator. The fluo-3 intensity from droplets (~50–100 μm diameter) of buffered aqueous solutions of part Ca²⁺-loaded azid-1 and fluo-3 (0.65 mM azid-1, 0.5 mM CaCl₂, 0.1 mM fluo-3, 25 mM KPO₄ pH 7.2) prepared using a glass microinjection pipet, under mineral oil on a IM-35 inverted fluorescence microscope stage (Zeiss; Thornwood, NY, USA) was recorded by a photomultiplier and processed by a DM3100 fluorimeter (Spex; Edison, NJ, USA). Photolyses were performed by triggering a Strobex 238 xenon flash lamp (Chadwick Helmuth; El Monte, CA, USA) through a UV UG-1 bandpass filter (Rolyn Optics; Covina, CA, USA) placed in the excitation path of the Zeiss fluorescence microscope. A custom dichroic mirror (DR 505LP UV-enhanced, Omega; Battleboro, VT, USA) was placed in the microscope epifluorescence filter cube to reflect both UV and 490 nm light while retaining transmission at wavelengths > 510 nm, where fluo-3 emits strongly. The time resolution of this apparatus was limited by the 2 ms acquisition time of the fluorimeter.

Biological tests

Electrophysiological recordings from Purkinje cells in cerebellar brain slices freshly prepared from young adult rats were performed as described previously [16]. Photolytic illumination was provided by a 200 W DC mercury arc lamp with an electromechanical shutter, focused through the epi-illumination pathway of an Axioplan microscope (Zeiss; Thornwood, NY, USA) with a 10 × water-immersion objective. Photolytic flashes were 100 ms at 1 Hz for 30 s. Inner-filtering (from azid-1 and some tissue opacity to UV) is unlikely to occur at the site of LTD induction in the dendrites with a total thickness of a few μm in these sagittal slices. It is possible this may slightly limit the extent of photolysis in the cell body with a diameter of 20–40 μm. The intracellular pipette solution contained: 130 mM K⁺CH₃SO₃⁻, 10 mM KCl, 10 mM K HEPES, 4.6 mM MgCl₂, 4 mM Na ATP, 1 mM Na GTP, 1 mM EGTA, 16 mM sucrose, and 2 mM azid-1 at pH 7.35 and 295 mOsmol.

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