A new caged Ca\textsuperscript{2+}, azid-1, is far more photosensitive than nitrobenzyl-based chelators

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Background: Photolabile chelators that release Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} affinity, selectivity for Ca\textsuperscript{2+} over Mg\textsuperscript{2+} 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 Ca\textsuperscript{2+} indicator, fura-2, has been modified with an azide substituent on the benzofuran 3-position. Azid-1 binds Ca\textsuperscript{2+} with a dissociation constant (K\textsubscript{d}) of \( \approx 230 \) nM, which changes to 120 \( \mu \)M after photolysis with ultraviolet light (330–380 nm). Mg\textsuperscript{2+} binding is weak (8–9 mM K\textsubscript{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 \( \mathrm{N}_3^- \) 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 Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} photolytically released from azid-1 could replace the normal depolarization-induced Ca\textsuperscript{2+} transient in triggering synaptic plasticity.

Conclusions: Azid-1 promises to be a useful tool for generating highly controlled spatial and temporal increases of Ca\textsuperscript{2+} in studies of the many Ca\textsuperscript{2+}-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 Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}], by photolysis of caged calcium chelators has become a standard technique in physiology and cell biology [1–4]. A high affinity photolabile Ca\textsuperscript{2+} chelator is introduced into the cell as the predominant Ca\textsuperscript{2+} buffer (at concentrations of a few millimolar) and irradiated with ultra-violet (UV) light to produce a weaker binding photoproduc, thus releasing Ca\textsuperscript{2+}. The starting affinity and the selectivity of the chelator for Ca\textsuperscript{2+}, degree of loading with Ca\textsuperscript{2+}, change in affinity for Ca\textsuperscript{2+} upon photolysis, and the photosensitivity of the caged Ca\textsuperscript{2+} govern the magnitude of the elevation in Ca\textsuperscript{2+} attainable per flash of UV light. These properties of the currently available caged Ca\textsuperscript{2+} chelators, nitr-5 [5], nitr-7 [5], dimethoxy (DM)–nitrophen [6], and nitrophosphyl (NP)–EGTA [7] are summarized in Table 1.

All the currently available Ca\textsuperscript{2+} chelators exploit the two-nitrobenzyl photochemistry [2,8,9] used by most caged compounds to either electronically (e.g. the nitro series of caged Ca\textsuperscript{2+}) or sterically (DM–nitrophen and NP–EGTA) reduce the binding of Ca\textsuperscript{2+} to the chelator. This leads to a limit on the overall light sensitivity of such chelators as these groups have low absorban (\( \approx 5000 \) M\textsuperscript{-1} cm\textsuperscript{-1}) and modest quantum efficiencies (0.20 or less). Low photosensitivities limit the magnitude and duration of the Ca\textsuperscript{2+} jump attainable, particularly because unphotolyzed Ca\textsuperscript{2+}-free chelator can rebind the photoreleased Ca\textsuperscript{2+}. 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 photorelesable compounds [10], a technique, in which two infra-red photons replace a single ultra-violet photon, that promises far greater spatial...
Table 1

Properties of nitr-5, nitr-7, DM-nitroph en, NP-EGTA and azid-1.\(^a\)

<table>
<thead>
<tr>
<th>Photolabile chelator</th>
<th>(K_d) for Ca(^{2+}) ((\mu)M) before hv</th>
<th>(K_d) for Ca(^{2+}) ((\mu)M) after hv</th>
<th>(K_d) for Mg(^{2+}) (mM) before hv</th>
<th>(K_d) for Mg(^{2+}) (mM) after hv</th>
<th>Quantum yield(^b)</th>
<th>Extinction coefficient (\varepsilon) (M(^{-1}) cm(^{-1}))</th>
<th>Light sensitivity(^c) (rQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitr-5(^e)</td>
<td>0.145</td>
<td>6.3</td>
<td>8.5</td>
<td>8</td>
<td>0.035</td>
<td>5500</td>
<td>190</td>
</tr>
<tr>
<td>nitr-7(^f)</td>
<td>0.054</td>
<td>3.0</td>
<td>5.4</td>
<td>5</td>
<td>0.042</td>
<td>5500</td>
<td>231</td>
</tr>
<tr>
<td>DM-nitroph en(^g)</td>
<td>0.005</td>
<td>3000</td>
<td>0.005</td>
<td>3</td>
<td>0.18</td>
<td>4330</td>
<td>780</td>
</tr>
<tr>
<td>NP-EGTA(^h)</td>
<td>0.080</td>
<td>1000</td>
<td>9.0</td>
<td>-9</td>
<td>0.23</td>
<td>974</td>
<td>224</td>
</tr>
<tr>
<td>azid-1</td>
<td>0.23</td>
<td>120</td>
<td>7.6</td>
<td>-9</td>
<td>1</td>
<td>33,000</td>
<td>33,000</td>
</tr>
</tbody>
</table>

\(^a\)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 ± 2°C, except for DM–nitroph en and NP–EGTA which have values that are pH sensitive in this range so they were determined at pH 7.2. \(^b\)The quantum yield is the efficiency of photolysis of the Ca\(^{2+}\)-bound chelator. \(^c\)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. \(^d\)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. \(^e\)From [5]; \(^f\)from [6]; \(^g\)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 Ca\(^{2+}\):Mg\(^{2+}\) selectivity, pH insensitivity and fast buffering kinetics of the BAPTA (1,2-bis(2-aminoethoxy)ethane-N\(^2\),N\(^4\),N\(^6\),N\(^8\)-tetraacetic acid)-based nitr series of chelators, the large change in Ca\(^{2+}\) affinity of DM–nitroph en 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 [Ca\(^{2+}\)], resting levels to provide maximal buffering power and therefore control of pre-illumination Ca\(^{2+}\) levels. After photolysis, final [Ca\(^{2+}\)] 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 fulfill most of these criteria. Azid-1, the 3-azido derivative of fura-2 [12], has the high Ca\(^{2+}\):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 M\(^{-1}\) 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 amines. 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 salicylaldehyde (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 salicylaldehyde 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

[Diagram of Ca\(^{2+}\) complex with azid-1 (1a) leading to the formation of an amidinium product (2a) and photorelease of Ca\(^{2+}\).]
binding Ca\textsuperscript{2+} (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 Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+}-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 Ca\textsuperscript{2+} when photolyzed. Azid-1 was therefore investigated further as a potential caged Ca\textsuperscript{2+}.

Spectral properties, Ca\textsuperscript{2+} binding, photolysis and quantum yield of azid-1

The absorbance spectra of azid-1 with and without Ca\textsuperscript{2+} 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]. Ca\textsuperscript{2+}-free azid-1 absorbed maximally at 372 nm with an extinction coefficient of 27,000 M\textsuperscript{-1} cm\textsuperscript{-1}, compared to values of 362 nm and 27,000 M\textsuperscript{-1} cm\textsuperscript{-1} for fura-2 [12]. In the presence of Ca\textsuperscript{2+}, the spectrum of azid-1 shifted 30 nm to shorter wavelengths giving a maximal absorbance of 33,000 M\textsuperscript{-1} cm\textsuperscript{-1} at 342 nm compared to 33,000 M\textsuperscript{-1} cm\textsuperscript{-1} at 335 nm for fura-2. The Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+}. The slight weakening of Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+}, 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 Ca\textsuperscript{2+} produced a shift to the blue (to about 350 nm) and a large decrease in absorbance. A Ca\textsuperscript{2+} titration of the photoproduct revealed, unexpectedly, that the Ca\textsuperscript{2+} affinity was reduced over 500-fold to about 120 μM (Figure 3d). Photolysis of azid-1 in Ca\textsuperscript{2+} resulted in a similar spectrum indicating the same photoproduct was formed with and without Ca\textsuperscript{2+} (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 Ca\textsuperscript{2+} respectively. The value of >1 (i.e. more than 1 product molecule formed per photon
Figure 3

(a) Absorbance spectra of unphotolyzed azid-1 as a function of free [Ca$^{2+}$]. The titration was performed at 22°C by using 10 ml of 100 mM KCl, 10 mM K-MOPS, 10 mM K$_2$H$_2$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 K$_2$Ca$^{2+}$EGTA and 11 µM azid-1, readjusting the pH to 7.14, and recording the spectrum which was then in 9 mM K$_2$H$_2$EGTA. Subsequent iterations to reach n mM K$_2$Ca$^{2+}$EGTA, (10-n) mM K$_2$H$_2$EGTA, n = 2–10, were performed by discarding 10.0(11-n) ml and replacing it with equal volumes of the 10 mM K$_2$Ca$^{2+}$EGTA, 11 µM azid-1 stock. After n = 10 had had been reached to give a free Ca$^{2+}$ between 10$^{-5}$ and 10$^{-6}$ 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 [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 Tris$_2$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 x 10$^{-6}$ einstein cm$^{-2}$ s$^{-1}$ from the Spectrolite 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 ± 2°C. (c) Absorbance spectra during photolysis of azid-1 in the absence of Ca$^{2+}$. The method was as described in (a) 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 x 10$^{-6}$ einstein cm$^{-2}$ s$^{-1}$ from the Spectrolite 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 [Ca$^{2+}$]. The photoproduct was produced by irradiating a 4 µM solution of azid-1 in 100 mM KCl (Ultrax grade; J.T. Baker Chem Co., Phillipsburg, NJ), 10 mM K-MOPS, pH 7.30, using the Spectrolite lamp at 365 nm until completion. The titration was performed by the stepwise addition of concentrated CaCl$_2$ solution to attain a total [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 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 and 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 pentahydrate 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 H-nuclear magnetic resonance (NMR) by photolyzing 5 mM azid-1 at pH 7 in D\(_2\)O 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 (N-CH\(_3\)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 aldehyde or nitride 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, M = CH\(_3\)), a truncated model of azid-1 in which the photochemically irrelevant bis(carboxymethyl)aminophenoxymethoxy 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-(benzylxoy)nitrobenzene. Before photolysis, the major peak had a molecular ion of 430.2, corresponding to the single 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 photoproduc 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 vinyllogous N-hydroxyamidine 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-methoxyamidine 15 which hydrolyzes to 21, consistent with the mass spectra.
Flash photolysis kinetics in vitro
For use as a caged Ca\(^{2+}\) in many biological systems, azid-1 should release Ca\(^{2+}\) rapidly (within a millisecond) following a light flash. The proposed mechanism for generating decreased Ca\(^{2+}\) binding involves dark reactions which could be rate-limiting. To determine the rate of Ca\(^{2+}\) release following flash photolysis, we used the fluorescent Ca\(^{2+}\) indicator fluo-3 which permits monitoring of the Ca\(^{2+}\) concentration at wavelengths that do not photolyze azid-1. The kinetics of Ca\(^{2+}\) binding to fluo-3 have been estimated to be >10\(^{8}\) M\(^{-1}\)s\(^{-1}\) [15] and therefore should not limit the detection of Ca\(^{2+}\) 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 Ca\(^{2+}\), fluo-3 fluorescence increased to a new level with kinetics that were faster than the time-resolution of the instrument indicating the release of Ca\(^{2+}\) from photolyzed azid-1 is complete within 2 ms (data not shown). Control experiments omitting azid-1 and/or Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 presynaptic 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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} is sufficient to replace climbing fiber activity and depolarization, and the elevation of [Ca\textsuperscript{2+}]\textsubscript{i} is the only essential function of photolysis 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\textsuperscript{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\textsuperscript{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
photoremoveable 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-azidothiophenyl)pyridine and 2-azidothiophenylpyridine 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 imino-cyclohexadienyl 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 vinyllogous N-OH amide (14) or a vinyllogous N-H amide (16) which would protonate (2 and 18 respectively) at neutral pH with expected pK₅ > 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 photoprotect 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 amine 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\textsuperscript{2+} as millimolar amounts may have to be photolyzed to overcome the intracellular Ca\textsuperscript{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 (Güppert-Mayer; 1 GM = 10\textsuperscript{-50} cm\textsuperscript{4} s/photons) 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\textsuperscript{2+}-bound fura-2 [28], which only differs by the absence of the azide group. The ability to restrict Ca\textsuperscript{2+} elevations to precise subcellular locations will allow more accurate spatial probing of Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} can then become difficult [29]. For two-photon photolysis, inner-filtering effects are negligible because all caged Ca\textsuperscript{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\textsuperscript{2+} currently in use. The over 500-fold decrease in its Ca\textsuperscript{2+} affinity from 0.23 μM to 120 μM upon photolysis should permit control of the many physiological events that are sensitive to changes in [Ca\textsuperscript{2+}] in this range. In comparison, nitr-5 and nitr-7 permit only modest elevations in Ca\textsuperscript{2+} of a few micromolar allowing only partial or no activation of some Ca\textsuperscript{2+}-sensitive processes, whereas DM-nitrophen and NP-EGTA can produce increases up to millimolar levels (Table 1). Azid-1 binds Ca\textsuperscript{2+} more weakly before photolysis than the other caged Ca\textsuperscript{2+}, however, so in cells with a typical resting [Ca\textsuperscript{2+}], of 100 nM, less Ca\textsuperscript{2+} will be bound and released upon irradiation. Furthermore, any unphotolyzed Ca\textsuperscript{2+}-free azid-1 will buffer the photoreleased ion (with the expected fast kinetics of BAPTA-based chelators of >10\textsuperscript{9} M\textsuperscript{-1} s\textsuperscript{-1} and limit the size of the Ca\textsuperscript{2+} elevation. This problem is solved if the light flash is strong enough to photolyze both the Ca\textsuperscript{2+}-free as well as the Ca\textsuperscript{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\textsuperscript{2+}-complex. Another answer would be to increase the Ca\textsuperscript{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\textsubscript{d} of 60–90 nM but would also presumably increase Ca\textsuperscript{2+} binding of the photoproduct by a similar amount resulting in a lower final [Ca\textsuperscript{2+}] after photolysis. Such modification (although requiring considerable organic synthesis) may be necessary for those applications in which free [Ca\textsuperscript{2+}] must be kept particularly low before photolysis.

The proposed mechanism for azid-1 photolysis results in the uptake of one proton per Ca\textsuperscript{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\textsuperscript{+} buffer. A similar alkalification is also shown by DM-nitrophen and NP-EGTA.

The presence of fluorescent impurities, such as 3-aminofura-2, in preparations of azid-1 may complicate monitoring of photogenerated Ca\textsuperscript{2+} increases inside cells using fluorescent Ca\textsuperscript{2+} indicators. The impurity, being itself an emission-ratioable indicator like indo-1, could be used to follow Ca\textsuperscript{2+} photorelease, except the high photosensitivity of azid-1 at the required excitation wavelength (about 350 nm) for 3-aminofura-2 would cause slow release of Ca\textsuperscript{2+} in the absence of a flash. Upon binding Ca\textsuperscript{2+}, 3-aminofura-2 shifts emission from 490 to 420 nm so release of Ca\textsuperscript{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\textsuperscript{4} selectivity for Ca\textsuperscript{2+} over Mg\textsuperscript{2+} required for producing Ca\textsuperscript{2+} increases without perturbing Mg\textsuperscript{2+} levels under physiological conditions, unlike DM-nitrophen, which acts as a caged Mg\textsuperscript{2+} and a caged Ca\textsuperscript{2+}. Like other BAPTA-based chelators, the Ca\textsuperscript{2+} affinity of azid-1 shows little sensitivity to pH in the physiological range, and fast buffering kinetics (milliseconds), compared to caged Ca\textsuperscript{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\textsuperscript{2+} binding are important to minimize the generation of transient spikes of high [Ca\textsuperscript{2+}] lasting a few milliseconds following a flash that leaves sufficient Ca\textsuperscript{2+}-free chelator unphotolyzed to slowly re-bind part of the released Ca\textsuperscript{2+}. Transient Ca\textsuperscript{2+} increases for DM-nitrophen and NP-EGTA have been measured directly using low-affinity fluorescent Ca\textsuperscript{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 Ca²⁺ elevations or local ‘hot spots’ within individual cells. Detailed mechanisms of the generation and control of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Mg²⁺ concentrations. Such elevated intracellular free calcium is sufficient to activate most Ca²⁺ triggered cellular processes, including muscle contraction and exocytosis of synaptic vesicles in neurones. Azid-1 is considerably more light sensitive than currently available caged calciums which should permit larger increases in Ca²⁺ per flash, decrease any deleterious biological effects of UV irradiation, and allow photorelease of Ca²⁺ 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 Ca²⁺ 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 sieves. 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 CDCl₃ 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. Electrospay mass spectrometry (5999B/Hewlett-Packard; Palo Alto, CA, USA) of 1b and its photoproducts was performed by injecting ~1 mM solutions of the triethylammonium salt in CH₃CN/H₂O 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 (96%). 1H NMR δ 1.16 (t, 2H, OCH₂CH₃), 2.25 (s, 3H, ArCH₃), 4.05 (q, 4H, OCH₂CH₂), 4.07 (q, 2H, OCH₂CH₂), 4.12 (s, 2H, NCH₂), 4.14 (s, 2H, NCH₂), 4.24 (s, 4H, OCH₂CH₂), 5.00 (s, 2H, benzyl CH₂), 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, CH = = syn). ES-MS (positive ion) [CH₃CNH₂O; 98.5] 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, OCH₂CH₃), 1.17 (t, 6H, OCH₂CH₃), 2.26 (s, 3H, ArCH₃), 4.06 (q, 4H, OCH₂CH₂), 4.07 (q, 4H, OCH₂CH₂), 4.11 (s, 8H, NCH₂), 4.20 (s, 4H, OCH₂CH₂), 5.12 (s, 2H, benzyl CH₂) 6.31 (s, 1H, H–3), 6.6–6.6 (m, 3H, H–3’,4’,6’), 7.00 (s, 1H, H–6), 7.39 (m, 5H, benzene). ES-MS (positive ion) [CH₃CNH₂O; 98.5] 734.4 (M+1); calc'd M = 733.5.

Sacilyl nitrile 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. 139–144°C. Yield 226 mg (86%). 1H NMR δ 1.17 (t, 6H, OCH₂CH₃), 1.19 (t, 6H, OCH₂CH₃), 1.55 (br s, ~2H, OH and DOH), 2.26 (s, 3H, ArCH₃), 4.08 (q, 4H, OCH₂CH₂), 4.09 (q, 4H, OCH₂CH₂), 4.12 (s, 4H, NCH₂), 4.18 (s, 4H, NCH₂), 4.21 (s, 4H, OCH₂CH₂), 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) [CH₃CNH₂O; 98.5] 644.3 (M+1); calc'd M = 643.5.

3-Amino-2-oxazolylbenzofuran 7

6 (100 mg, 0.155 mmol), ethyl 2-(chloromethyl)oxazoline-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, at 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 (81%). M.p. 85–86°C. 1H NMR δ 8.16 (t, 6H, OCH₂CH₃), 1.21 (t, 6H, OCH₂CH₃), 1.40 (t, 3H, oxazole OCH₂CH₃), 2.26 (s, 3H, ArCH₃), 4.06 (q, 4H, OCH₂CH₂), 4.12 (q, 4H, OCH₂CH₂), 4.15 (s, 4H, NCH₂), 4.20 (s, 4H, NCH₂), 4.3 (dt, 4H, OCH₂CH₂), 4.41 (q, 2H, oxazole OCH₂CH₃), 5.09 (s, 2H, NH₂), 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) [CH₃CNH₂O; 98.5] 797.3 (M+1); calc'd M = 796.3.

Azid-1 pentanoyl 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 hydrosulphate (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; hydrazic 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 (68%). M.p. 114–116°C. 1H NMR δ 8.16 (t, 6H, OCH₂CH₃), 1.18 (t, 6H, OCH₂CH₃), 1.42 (t, 3H, oxazole OCH₂CH₃), 2.27 (s, 3H, ArCH₃),
Azid-1 pentapotassium salt

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 Spectrolite TC-365A UV transilluminator (Spectronics Corp; Westbury, NY, CA). ¹NMR D₂O 0.225 (s, 3H, ArCH₃), 3.72 (s, 4H, 2'NCH₂), 3.85 (s, 4H, 6-NCH₃), 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 Spectrolite TC-365A UV transilluminator (Spectronics Corp; Westbury, NY, CA). ¹NMR D₂O 0.225 (s, 3H, ArCH₃), 3.72 (s, 4H, 2'NCH₂), 3.85 (s, 4H, 6-NCH₃), 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).

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 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 intersects 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 monoprotonated 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 alternate irradiating a buffered solution of the substrate compound 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 500-W mercury lamp (Spectronics Corp; Westbury, NY, CA) was used as a source of 365 nm light and its intensity (~1.2 × 10⁻⁴ einstein cm⁻² s⁻¹) measured each experimental day by actinometry using 6 mM potassium ferroxalate [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 15-35° inverted fluorescence microscope stage (Zeiss; Thornwood, NY, USA) was recorded by a photomultiplier and processed by a DM3100 fluorimeter (Spex; Edison, NJ, USA). Photolysis were performed by triggering a Strobotek 238 xenon flash lamp (Chadwick Helmut, 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 DG mercury arc lamp with an electromechanical shutter, focused through the epirillumination pathway of an Axioskop 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 dentrites with a total thickness of a few μm in these sagittal slices. It is possible this may slightly limit the extent ofphotolysis in the cell body with a diameter of 20–40 μm. The intra-cellular pipette solution contained: 130 mM KCl, 4 mM MgCl₂, 4 mM NaATP, 1 mM NaGTP, 1 mM EGTA, 16 mM sucrose, and 2 mM azid-1 at pH 7.35 and 295 mMosm.

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