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Tuning caged calcium: Photolabile analogues of EGTA with improved optical and chelation properties

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Abstract

The physico-chemical properties of several Ca^{2+}-selective, photolabile chelators are described. These molecules have been developed as part of an effort to produce a caged Ca^{2+} that improved upon the Ca^{2+} chelation properties and light absorption capability of nitrophenyl-EGTA (NP-EGTA). Four dimethoxy-*ortho*-nitrophenyl derivatives of EGTA (called DMNPE-1 through -4), and one analogue of EGTA (DMNPE-5) have been characterized, each of which is bisected upon irradiation. One of these cages has a higher affinity than NP-EGTA: DMNPE-4 has a K_d for Ca^{2+} of 48 nm at pH 7.2 (19 nM at pH 7.4). Furthermore, this cage has a large extinction coefficient of 5120 M^{-1} cm^{-1} at 350 nm (cf. 975 M^{-1} cm^{-1} for NP-EGTA). The other physico-chemical properties of DMNPE-4 are: quantum yield of photolysis of 0.09; bipasic Ca^{2+} release kinetics (70% released with a rate of about 48,000 s^{-1} and 30% at 1.5 s^{-1}) and photoproducts that bind Ca^{2+} with very low affinity (K_d in the range of 2 mM, pH 7.2), hence most of the bound Ca^{2+} is released rapidly and efficiently upon photolysis. Thus, DMNPE-4 has a unique combination of properties that make it an extremely effective Ca^{2+} cage.

Keywords: Caged calcium; Flash photolysis; Divalent cations; Calcium affinity

1. Introduction

The past 20 years has witnessed the concomitant development of two photochemical techniques for studying the dynamic function of living cells: rapid concentration jumps of cellular effectors from biologically inert precursors (caged compounds; reviews [1,2]) and imaging concentrations of small solutes using fluorescent probes [3]. These compounds along with the use of light as a non-invasive tool to gain access to the intracellular environment has revolutionized biophysical studies allowing for the controlled perturbation and monitoring of a number of fundamental cellular processes with high temporal and spatial resolution in living preparations.

The initial, pioneering experiment with caged compounds in living cells was performed by Engels and Schlaeger in 1977, who photoreleased cAMP in rat glioma cells to induce morphological changes [4]. With the subsequent development in caging key intracellular second messengers in 1986–1987 (i.e. nitr-2 (a Ca^{2+} cage [5]), and caged IP_3 [6]), caged compounds became widely used for studying intact cells. Since the introduction of nitr-2, several significant advances in the caging of inorganic calcium ions have been accomplished [7–10]. The reason for this evolution of caged Ca^{2+} technology lies principally in the unique manner of caging the cation, in comparison to all other caged compounds. For organic molecules such cAMP or IP_3, caging is accomplished by blocking the active functionality of the effector by covalent attachment of a photoremovable protecting group, rendering the effector biologically inert. Calcium cannot be caged in like manner, as it does not form cova-

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lent bonds. The basic strategy used to control [Ca^{2+}] is the photochemical modification of the affinity of a known Ca^{2+} chelator, using the caging chemistry originally applied to nucleotides (review [11]). There are several possible high affinity Ca^{2+} chelators available. The tetracarboxylic amines EDTA, EGTA and BAPTA are the most widely used for intracellular complexation of Ca^{2+}. Lehn and Sauvage [12] introduced "*cryptands*", some of which also have reasonably high affinity for Ca^{2+}. However, the most suitable of the cryptands also bind Na^+ and K^+ (viz. the [2.2.1] ligand has $K_d^{Na} = 40\,\mu M$; $K_d^{K} = 0.1\,\mu M$ and $K_d^{Ca} = 0.11\,\mu M$ [12]). Therefore, only the tetracarboxylates show sufficient divalent/monovalent cation selectivity to be considered seriously as potential Ca^{2+} cages, and only EGTA and BAPTA have sufficient calcium/magnesium selectivity to be Ca^{2+}-selective cages.

The compound called nitr-2 was the first photolabile Ca^{2+} chelator synthesized [5]. As a BAPTA-based chelator, its Ca^{2+}-affinity is relatively pH insensitive above 6.5 and it has an extremely low affinity for Mg^{2+}. However, the photochemistry of this cage at $3.5\,s^{-1}$ was too slow to be particularly useful in many biological applications and it had a fairly low pre-photolysis affinity for Ca^{2+} ($K_d = 160\,\mu M$). Subsequently, Tsien et al. [7] introduced several similar molecules with substantially improved physico-chemical properties. Two compounds are significant: (1) nitr-5 has similar chelation properties to nitr-2, however the photochemistry of this cage occurs with a half-time of about 270–410 μs; and (2) nitr-7 possesses a substantially higher affinity for Ca^{2+} ($K_d = 54$ nM), without compromising the speed of photolysis (rates of appearance of the organic photoproducts of nitr-5 and nitr-7 were determined but no rates of Ca^{2+} release have been reported for any of these cages). All three of these cages use the 2-nitropiperonyl chromophore, and so have a large extinction coefficient, effectively absorbing incident light. But the rather modest quantum yield of photolysis, in the range of 0.01–0.05 means that less than optimal use of incident light energy is made. However, the most severe weakness of the nitr series is the extremely small change in affinity produced by irradiation, no more than 54-fold, as the photoproducts have an affinity for Ca^{2+} in the range of 3–6.3 μM, [7]), thus there is little net photorelease of the bound Ca^{2+}.

In contrast, DM-nitrophen [8,9] possesses the following physico-chemical properties: an extremely high affinity for Ca^{2+} ($K_d = 5$ nM) which undergoes a 600,000-fold decrease upon irradiation, a good quantum yield of photolysis (0.18), a very rapid Ca^{2+} release rate ($38{,}000\,s^{-1}$, [13]), and a large extinction coefficient ($4300\,M^{-1}\,cm^{-1}$, a similar value to the nitr series). The major drawback of DM-nitrophen for some intracellular studies is its relatively high affinity for Mg ($K_d = 2.5\,\mu M$), since the cage is based on EDTA). Nevertheless, with judicious choice of [Ca^{2+}], [Mg^{2+}], [ATP] and [DM-nitrophen] one can still activate cellular ATPases effectively [14]. In 1994, we introduced our first Ca^{2+}-selective photolabile chelator, called nitrophenyl-EGTA (NP-EGTA, [15]). This cage, like DM-nitrophen, releases Ca^{2+} very quickly ($68{,}000\,s^{-1}$; [13]) and with good quantum efficiency (0.20–0.23). NP-EGTA is, as its name suggests, a chemically modified EGTA, and possesses the same very low affinity for Mg^{2+} as the parent molecule ($K_d^{Mg} = 9$ mM). However, also like its parent, the cage binds Ca^{2+} more weakly ($K_d^{Ca} = 80$ nM) than DM-nitrophen. Furthermore, the use of the unadorned *ortho*-nitrophenyl chromophore means that NP-EGTA makes relatively inefficient use of the incident light for uncaging [16], and also does not undergo two-photon photolysis [17,18].

In this paper, we report the design and properties of a range of derivatives of NP-EGTA (Fig. 1) that are designed to tune and improve the spectral and chelation properties of NP-EGTA, whilst maintaining its divalent cation selectivity and speed of Ca^{2+} uncaging. The synthesis of these cages has already been reported [19]. One of these new Ca^{2+} cages (**dim**ethoxy**nitro**phenyl-**E**GTA-4, DMNPE-4) binds Ca^{2+} with high affinity before photolysis ($K_d = 48$ nM at pH 7.2; 19 nM at pH 7.4), and upon photolysis releases Ca^{2+} with decent quantum yield (ca. 0.09). Laser flash photolysis revealed that 70% of the bound calcium is released with a rate of about $48{,}000\,s^{-1}$ and 30% with a rate of $1.5\,s^{-1}$. This cage uses the 4,5-dimethoxy-2-nitrobenyl chromophore, so it absorbs light much more effectively than NP-EGTA (dimethoxynitrobenyl derivatives of EGTA have extinction

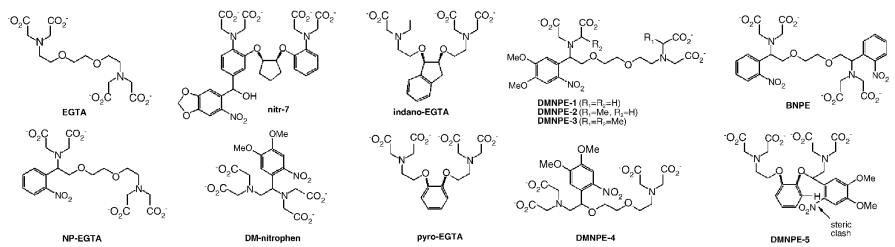

Fig. 1. Structures of Ca^{2+} cages and chelators.

coefficients at 350 nm of 5120 M^{-1} cm^{-1}). Initial testing of the new cage in pancreatic β cells and cardiac myocytes illustrates that this new photolabile Ca^{2+} chelator can be useful for excitation-secretion and excitation-contraction coupling studies [20,21].

2. Methods

2.1. Materials

DMNPE-1 through -5 and BNPE were synthesized according to our published methods [19]. Indano-EGTA and pyro-EGTA were synthesized using our published methods [8,19]. Full synthetic details available on request. Ca-orange-5N was from Molecular Probes, Eugene, OR. $CaCl_2$ (1.00 M stock solution), KCl, KOH (1 M) were of AR grade from BDH, Poole, Dorset, UK. *N*-2-Hyroxyethylpiperizine-*N*′-2-ethanesulphonic acid (Hepes) was from Sigma, St. Louis, MO.

2.2. Measurement of divalent cation affinities

The stability constant (K_a) for the equilibrium binding of a divalent cation (M) by a buffer (B) is defined as:

$$K_a = \frac{[M]_B}{[B]_F\,[M]_F} \quad (1)$$

and the total buffer concentration $[B]_T$ is given by

$$[B]_T = [B]_F + [M]_B \quad (2)$$

combining (1) and (2):

$$K_a([B]_T - [M]_B) = \frac{[M]_B}{[M]_F} \quad (3)$$

where $[B]_F$ and $[M]_F$ are the free concentrations of buffer and cation; $[M]_B$ the concentration of bound cation; and $[B]_T$ the total concentration of buffer. Eq. (3) is used for the Scatchard-type analysis in Fig. 2 (inset), as tetracarboxylic chelators are known to bind Ca^{2+} in a 1:1 complex. The advantage of this approach in determining the apparent affinity is that it is only dependent on the accurate measurement of $[M]_T$ and $[M]_F$. Estimates of $[Ca^{2+}]_F$ for our high affinity chelators were obtained by fluo-3 emission. Ca^{2+} affinities were measured, as previously [15], in the following way. A solution (3.0 ml) of about 1.0 mM of chelator was used; this concentration easily buffered the micromolar levels of free Ca^{2+} normally present in glassware. The solutions were buffered to the required pH (e.g. pH 7.2 or 7.4) with Hepes and the ionic strength was set with 100 mM KCl (standard for such measurements). Titration with incremental aliquots (3–10 μl) of Ca^{2+} gave data like that shown in Fig. 2, hence the apparent affinity at the relevant pH was obtained, using a K_d of 500 nM for fluo-3 [15]. The Ca^{2+} affinity of the DMNPE-4 photoproduct mixture was determined by photolyzing a solution of the cage (0.50 mM) with an equal amount of Ca^{2+} at pH 7.2. The change in $[Ca^{2+}]_{free}$ was measured with Ca^{2+}-selective electrodes [15], and the extent of photolysis determined by HPLC analysis, in the same way as for the quantum yield determination. Modeling the extent of photolysis reaction and $[Ca^{2+}]$ gives the photoproduct K_d.

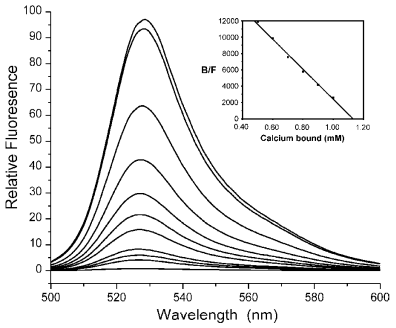

Fig. 2. DMNPE-4 divalent cation affinities. Emission spectra of fluo-3 (10 μM, excitation at 490 nm) resulting from titration at 25 °C of DMNPE-4 (1.16 mM in 40 mM Hepes, 100 mM KCl, pH 7.2). The curves shown result from the addition of $[Ca^{2+}]_{total}$ (in mM) of 0, 0.3, 0.4, 0.5, 0.7, 0.8 0.9, 1.0, 1.1 and 1.2 Scatchard-like analysis of these data is inset. Spectra were measured using a Perkin-Elmer LS-50 (slit widths set at 5 mm).

2.3. Flash photolysis

The photolysis reaction was initiated by a Q-switched ruby laser (Synergie Laser, Boca Raton, FL). The output (694 nm) was frequency doubled by passage through a potassium dihydrogen phosphate (KDP) crystal. The primary 694 nm-output was separated from the secondary output by two dichroic mirrors placed between the KDP crystal and the sample. This configuration produced a 35 ns, 300 mJ pulse of light with a wavelength of 347 nm. For the absorbance measurements, this light was focused by a cylindrical lens onto a 10 mm × 2 mm fluorescence quartz cuvette. The final beam size was approximately 8 mm × 1.5 mm. The absorbance changes were monitored at right angles to the photolysis beam. The light source for the absorbance measurements was an argon laser, model #5500AWC (Ion Laser Technology, Salt Lake City, UT) tuned to produce 100 mW of 457 nm light beam with a diameter of approximately 0.8 mm. The beam was steered along the long axis of the cuvette, perpendicular to the photolysis light path. The beam intensity was attenuated using a compensated attenuator (model #925B, Newport Corporation, Irvine, CA) and one neutral density filter (OD = 2). The detection was via a photomultiplier model #9924B (Thorn EMI, Rockaway, NJ) with a GG400, BG39 and a 450DF20 filter (Omega Optical, Brattleboro, VT) in front. The photocurrent was amplified by a custom built cur-

Table 1
Summary of properties of calcium cages and calcium chelators

	K_d (Ca) (nM)	K_d prods (mM)	Affinity change *X*-fold	K_d (Mg) (mM)	Quantum yield (ϕ)	Extinction coefficient (ε) (M^{-1} cm^{-1})	$\phi \cdot \varepsilon \cdot \Delta K_d$ ($\times 10^4$)	Rate of photolysis (s^{-1})	Rate of Ca release (s^{-1})
EDTA	32			0.005					
EGTA	150			12					
Indano-EGTA	100[a]								
Pyro-EGTA	100[a]								
DM-nitrophen	5 ± 0.2[b]	3	600,000	0.0025	0.18	4,300	46,000	8×10^4	3.8×10^4
NP-EGTA	80 ± 2[c]	1	12,500	9	0.20–0.23	975	240	5×10^5	6.8×10^4
DMNPE-1	125 ± 5[d]	ND			0.10	4,570		5.8×10^4	4.8×10^4
BNPE	46 ± 3[d]	ND			0.23	1,950		5×10^5	ND
DMNPE-2	313[a]								
DMNPE-3	>5000[a]								
DMNPE-4	48 ± 2[e]	2.0	40,000	10*	0.09	5,120	1,800	3.3×10^4	4.8×10^4
DMNPE-5	130 ± 5[d]	ND			0.13	4,620		2.6×10^4	4.6×10^4
BAPTA	110		17						
nitr-5	145	0.0063	54	8.5	0.012–0.035	5,500	1	2.5×10^3	ND
nitr-7	54	0.003	42	5.4	0.011–0.042	5,500	1	2.5×10^3	ND
azid-1	230	0.12	520	8	1.0	33,000	1,700	ND	ND

* 2-Photon uncaging of Ca^{2+} in the presence and absence of 10 mM Mg^{2+} has no effect on the yield (ref. [21]). [a] $n = 1$; [b] $n = 12$; [c] $n = 15$; [d] $n = 3$; [e] $n = 7$.

rent to voltage preamplifier. The bandwidth of the detection system with the preamplifier was at least 5 MHz.

2.4. Fluorescence measurements

These were carried out by focusing the photolysis laser onto the stage of a Nikon diaphot epifluorescence microscope equipped with a custom built rotating trough assembly. For these experiments, a home-made 12 μl fused quartz trough (5 mm × 3 mm × 0.8 mm) was constructed using ESCO S1-UV fused silica (Esco Products, Oakridge, NJ) and mounted on the stage assembly. The optical components included: a 75 W xenon light source (Nikon); a Zeiss Neofluar objective (6.3× magnification, N.A. = 0.2); excitation filter 488DF20; dichroic 505 DRLP; and a emission filter 570DF20 (Omega Optical) and GG400 filter placed before photomultiplier model #9828B (Thorn EMI, Rockaway, NJ). The data in all experiments were digitized and stored on a digital oscilloscope, model #9304 (Le Croy Corp., Chestnut Ridge, NY). The data were digitized at a maximum rate of 50 MHz. Both types of kinetic studies were carried out at pH 7.2 (60 mM Hepes) with 100 mM KCl.

2.5. Quantum yields

The quantum yield of photolysis of the new dimethoxy cages was determined by comparison to that of MNI-glutamate that has previously been determined to have a quantum yield of photolysis of 0.085 [22]. The time-course of disappearance of the caged compounds was followed by HPLC analysis of the reaction mixture. The amount of unphotolyzed DMNPE-X and MNI-glutamate remaining in the reaction mixture was normalized for each HPLC analysis by comparison to a photochemically inert internal standard, inosine (100 μM). The filtered output (300–400 nm) of 500 W high pressure Hg arc lamp was used to photolyze solutions of caged Ca^{2+} (0.2 mM) in buffer (40 mM Hepes, 100 mM KCl, pH 7.2) in a cuvette with a 1.0 mm pathlength. The quantum yield is not wavelength dependent over this range. We use 350 nm for comparison (Table 1) as this is useful wavelength for two reasons: most microscope objectives are opaque below 330 nm, and the output lasers most often used is about 350 nm (frequency doubled ruby laser is 347 nm, and Kr–Ar lasers are in the 351–364 nm range. Kr–Ar lasers are the standard UV laser for all confocal microscopes.) It should be noted that DMNPE-4 and MNI-glu have similar absorption spectra over the 300–400 nm range. Each cage was photolyzed 3 (DMNPE-1/5) or 4 (DMNPE-4) times, with and without saturating [Ca^{2+}] added. HPLC was performed using a 4 mm × 250 mm Hamilton PRP-1 reverse-phase HPLC column and a Beckman System Gold 168 pump delivering 0.8 ml/min. Pump A delivered 100% H_2O/0.1% TFA and pump B 100% acetonitrile/0.1% TFA. The components were separated by a linear gradient over 30 min from 8 to 28% buffer B. A Beckman 128 photodiode array detector monitored the eluant. Each sample was analyzed seven times (in the case DMNPE-4) or four times (DMNPE-1/5).

3. Results and discussion

3.1. Optical tuning of nitrophenyl-EGTA

The *ortho*-nitrophenyl chromophore [23] has proved extremely useful for caging an extremely wide variety

of biologically important molecules since its initial use [4]. However, its fairly small extinction coefficient means that it does not make efficient use of the incident light (cf. [16]), giving rise to the possibility of cellular photodamage when large amounts of near-UV light are required to produce high photolysis levels. A caged Ca^{2+} which absorbs light more efficiently and at a longer (less phototoxic) wavelength is clearly more desirable.

We have extended the synthetic route used for the construction of NP-EGTA to Ca^{2+} cages bearing dimethoxy substituents at the four and five-positions of the *ortho*-nitrophenyl chromophore (DMNPE-1 through -3; [19]). Furthermore, these two new synthetic routes to photolabile EGTA analogues also produced optically tuned nitrophenyl-EGTAs (DMNPE-4 and -5; [19]). The UV–vis absorption spectrum of DMNPE-4 is compared to that of NP-EGTA in Fig. 3. It can be seen that the dimethoxy substituents impart two important improvements to the optical properties of the cage: their electron donating capacity increases the extinction coefficient and shifts the peak of absorption spectrum to the red, compared to NP-EGTA. DMNPE-4 has an extinction coefficient of 5,120 M^{-1} cm^{-1} at 350 nm. Thus, since DMNPE-4 makes much more efficient use of the incident light than NP-EGTA, less light is required to release the same amount of Ca^{2+}. This five-fold increase in extinction coefficient compared to NP-EGTA is vital for 2-photon photolysis of caged Ca^{2+}. Brown and Webb [17] have shown that the unadorned *ortho*-nitrophenyl chromophore (NP-EGTA) has a negligible 2-photon cross-section, whereas the dimethoxynitrophenyl chromophore (DM-nitrophen) has a larger 2-photon cross-section, and so can undergo 2-photon photolysis more effectively (see [24] for 2-photon photolysis of DM-nitrophen in cardiac myocytes). We have already used 2PP of the DMNPE-4:Ca^{2+} complex to produce "artificial Ca^{2+} sparks" in cardiac myocytes where the cellular free [Mg^{2+}] is around 1 mM [21].

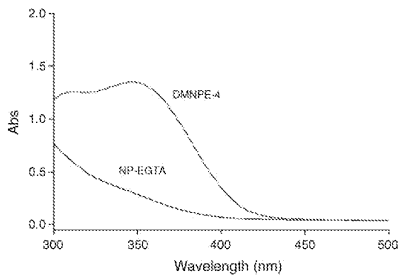

Fig. 3. DMNPE-4 and NP-EGTA UV–vis absorption spectra.

3.2. Development of a high affinity nitrophenyl-EGTA

The resting intracellular [Ca^{2+}] in the 100–300 nM range. Therefore, one might design a Ca^{2+} cage with a K_d value in this range so as not to perturb the resting Ca^{2+} concentration in these cells [7]. However, a more detailed consideration of the practical consequence of this reveals that a Ca^{2+} cage with a K_d value >100 nM is quite ineffective for releasing *large* quantities Ca^{2+} that are necessary for studying biological processes such as neurotransmission. This is because only about 50% of such a chelator can be loaded before photolysis and if less than 50% photolysis is achieved, *then most of the photolytically released* Ca^{2+} *will rebind to unloaded, unphotolysed chelator* [11]. Therefore, the target affinity of a Ca^{2+} cage before photolysis is in the 5–20 nM range, as 90% loading of such a chelator can be achieved. Photolysis of a small percentage of the total [cage Ca^{2+}] will then liberate the bound cation. We have tested the physico-chemical properties of four compounds, representing different approaches to increasing the affinity of EGTA for Ca^{2+}.

3.2.1. Approach 1

The first strategy tested for increasing the affinity of NP-EGTA was based upon the strategy used to increase the Ca^{2+} affinity of nitr-7 over nitr-5 ([7]; see Table 1). The Tsien group found that establishing a fixed *cis* configuration between the ethereal oxgyens of the BAPTA chelator with a cyclopentyl ring produced a 2.7-fold increase in affinity.

Two non-photolabile EGTA analogues were synthesized to test the effectiveness of applying a *cis* steric constraint to the central ethereal oxygens to EGTA-based chelators as a means of increasing the Ca^{2+} affinity compared to that of EGTA. Such *cis* constraint can be introduced not only by using a cyclopentyl ring (like nitr-7) but also using a benzene ring in place of the two central carbons atoms of EGTA. The EGTA analogue we synthesized using a cyclopentyl ring, in analogy to nitr-7, "indano-EGTA", had an affinity for Ca^{2+} of 100 nM at pH 7.2. The di-substituted benzene derivative (actually a pyrocatcheto-based Ca^{2+} chelator, "pyro-EGTA", Fig. 1), also had a K_d^{Ca} of 100 nM at pH 7.2. EGTA itself has a K_d^{Ca} of 150 nM at pH 7.2, thus both implementations of Approach 1 did increase the affinity of EGTA slightly. Encouraged by these results, we synthesized a photolabile derivative of "pyro-EGTA", anticipating that this molecule would have a higher affinity for Ca^{2+} than NP-EGTA (K_d^{Ca} = 80 nM at pH 7.2). However, the presence of the caging chromophore in the photolabile version of pro-EGTA (DMNPE-5, Fig. 1) increased the K_d of the chelator for Ca^{2+} from 100 to 130 nM. A possible explanation for this decrease in Ca^{2+} affinity is illustrated in the structure of DMNPE-5 in Fig. 1. The *peri* hydrogen on the aromatic ring adjacent to the caging group could clash with the nitro substituent, disturbing slightly the Ca^{2+} coordination sphere. Such interactions are not present in pyro-EGTA (there is no pendant cage chromophore), or in nitr-7; but they are inherent to our strategy for producing a photosensitive EGTA using

Approach 1, therefore further development along this line was not pursued.

3.2.2. Approach 2

A second approach to a higher affinity NP-EGTA was again based upon results obtained by manipulation of the Ca^{2+} affinity of BAPTA-based chelators. Clarke et al. [25] found that substitution of methyl groups between nitrogen and the carboxylate moieties of BAPTA which decreased the pK_a^H of the nitrogen atoms involved in Ca^{2+} coordination enhanced Ca^{2+} affinity at neutral pH. In applying the same logic DMNPE-2, and DMNPE-3 were synthesized along with the "parent" dimethoxynitrophenyl-EGTA (DMNPE-1, see Fig. 1). However, we found that the methyl substituents dramatically *decreased* the affinity of the molecules for Ca^{2+} compared to the parent chelator (K_d^{Ca} DMNPE-1, 125 nM versus DMNPE-2, 330 nM and DMNPE-3, >5 μM; see Table 1). This resulted from the increased pK_a's of the nitrogens with their basicity, caused a large reduction in their apparent affinity for Ca^{2+} at physiological pH. Analysis of the pH dependence of the Ca^{2+} affinity of DMNPE-1 in the 7.2–7.8 pH range indicated that this chelator has a $pK_a^{Ca} = 9.3$ ($K_a = 2.0 \times 10^9\ M^{-1}$, or $K_d = 5.0 \times 10^{-10}$ M) and pK_a^{H}'s = 6.3 and 9.9. These should be compared to corresponding pK_a^H values of 10.9, 9.0 and 9.6 for EGTA [26]; and 10.3, 7.4 and 9.6 for NP-EGTA. These data indicate that the dimethoxy substituents of DMNPE-1 dramatically reduce the *absolute* Ca^{2+} affinity of the chelator that was not offset by the reduction in pK_a^H.

3.2.3. Approach 3

The substitution of one *o*-nitrophenyl chromophore onto a carbon atom next to a nitrogen on the EGTA backbone gives NP-EGTA and results in an increase in the apparent affinity from 150 to 80 nM at pH 7.2, because the electron withdrawing capacity of the aromatic substituent decreases the basicity of the adjacent nitrogen atom. It was reasoned from this that substitution of a second electron withdrawing substituent adjacent to the *other* nitrogen atom would increase the affinity even more. This indeed proved to be the case. The Ca^{2+} affinity of bis-nitrophenyl-EGTA (BNPE) was 46 nM at pH 7.2.

3.2.4. Approach 4

In DMNPE-4, the caging chromophore was moved from the carbon *alpha* to the amino functionality to a carbon adjacent to an ethereal oxygen, thus providing effective insulation from deleterious effects of the dimethoxy substituent on the apparent Ca^{2+} affinity seen for DMNPE-1, whilst maintaining the optical tuning of this cage. DMNPE-4 has an affinity for Ca^{2+} of 48 nM at pH 7.2 and 19 nM at pH 7.4 (cf. 80 and 36 nM for NP-EGTA and 150 and 60 nM for EGTA). Clearly the positioning of the caging chromophore at this point introduces very favorable steric constraints into the Ca^{2+} binding pocket of the EGTA chelator.

Photolysis of a solution of DMNPE-4 (0.5 mM) with Ca^{2+} allowed us to obtain an estimate of the K_d for Ca^{2+} of the product mixture. We found that 50% photolysis when $[Ca^{2+}]_{total}$ of 0.55 mM was present changed the $[Ca^{2+}]$ from 50.1 to 186 μM, and 75% photolysis when $[Ca^{2+}]_{total}$ of 0.47 mM was present changed the $[Ca^{2+}]$ from 0.58 to 66 μM. Thus, the photoproducts from DMNPE-4 have an affinity for Ca^{2+} of about 2 mM at pH 7.2. Therefore, photolysis of DMNPE-4 produces about a 40,000-fold change in the apparent affinity for Ca^{2+} at pH 7.2. This value is similar to NP-EGTA and DM-nitrophen, and is consistent with chelator fragmentation as shown in Fig. 6.

3.3. Light absorption and use

The quantum yield of photolysis of DMNPE-4 was measured by comparing the time course of photolysis using continuous radiation of a solution containing approximately equal concentrations of MNI-glutamate and DMNPE-4 having the same optical density at 350 nm (total optical density 0.4). Inosine, which is transparent at this wavelength, was used as an internal standard. We found that the MNI-glutamate was photolyzed at the same rate as DMNPE-4, implying the quantum yield of uncaging of DMNPE-4 is about 0.09, in the presence of saturating concentrations of Ca^{2+}. The value was found in the absence of any added Ca^{2+} was 0.06. The quantum yields of photolysis of DMNPE-1 and DMNPE-5 were measured in the same way, and were found to be about 0.10 and 0.13, respectively.

Another mode of de-excitation of aromatic chromophores is by fluorescence emission. We found that, in keeping with all other nitrobenzyl chromophores, DMNPE-4 is not fluorescent.

The product of the quantum yield (ϕ) and extinction coefficient (ε) is often used as a measure of the effectiveness of a particular caged compound and/or caging chromophore [10,26]. This comparison is meaningful when each bond broken yields a molecule in solution (e.g. [26], though such comparisons really ought be made at wavelengths of light that are actually used in biological experiments in microscopes. However, in the case of caged calcium, each bond broken by photolysis does *not* yield a free calcium ion [11]. So, in Table 1 we provide a comparison that we believe gives users a better representation of the efficiency of Ca^{2+} uncaging. Since the efficiency of Ca^{2+} release is also based upon the affinity change produced by photolysis, column 8 of Table 1 shows the product of quantum yield, extinction coefficient *and affinity change* (ΔK_d, *X*-fold). When this additional fundamental property of calcium cages is considered, DM-nitrophen is the most efficient in releasing Ca^{2+}, while azid-1 and DMNPE-4 are much less efficient. However, if physiological $[Mg^{2+}]_{free}$ are required, then a highly Ca^{2+}-selective caged must used (viz. azid-1 or DMNPE-4), as DM-nitrophen has quite a high affinity for Mg^{2+}. Finally, we would like to note that this analysis suggests that azid-1 really ought to find good use in 2-photon uncaging experiments, as it has by far the largest 2-photon cross-section of any Ca^{2+} cage.

Table 2
Rates of photolysis of DMNPE-1, -4, and -5

	Amplitudes and rates with Ca^{2+}				Amplitudes and rates without Ca^{2+}			
	a_1	k_1 ($\times 10^4$ s^{-1})	a_2	k_2 ($\times 10^4$ s^{-1})	a_1	k_1 ($\times 10^4$ s^{-1})	a_2	k_2 ($\times 10^4$ s^{-1})
DMNPE-1	437 ± 70	10.8 ± 1.9	356 ± 50	1.6 ± 0.2 (n = 26)	174 ± 38	7.7 ± 2.7	432 ± 27	1.5 ± 0.1 (n = 17)
DMNPE-4	155 ± 51	9.2 ± 4.1	281 ± 38	1.5 ± 0.2 (n = 41)	170 ± 46	8.8 ± 3.0	190 ± 54	1.3 ± 0.3 (n = 18)
DMNPE-5	139 ± 29	9.1 ± 3.2	369 ± 25	1.3 ± 0.2 (n = 9)	260 ± 37	17.7 ± 7.3	348 ± 40	1.5 ± 0.3 (n = 22)

The rates of decay of the *aci*-nitro transient intermediates where measured as described in Section 2 (values are the mean ± S.D.). Representative time-dependent absorption changes for the DMNPE-4:Ca^{2+} complex are shown in Fig. 4.

The extinction coefficient of DMNPE-4 is 5,120 M^{-1} cm^{-1}. This value is similar to other dimethoxynitrophenyl cages [7,9]. For practical purposes optical densities (OD) of the cage in a cell should be less than 20%, or inhomogeneous uncaging across the cell will result from inner filtering effects. For example, if [cage] = 2 mM, then the extinction coefficient of the cage must be less than 10,000 M^{-1} cm^{-1} for 100 μm pathlength. In chromaffin cells (ca. 20 μm diameter) Neher and co-workers have often used [DM-nitrophen] = 10 mM [14,27–29]. This solution has an OD of ca. 0.09 (DMNPE-4 would have a similar value). If 10 mM azid-1 was used, the OD would rise to 0.66. Because of inner filtering effect by azid-1 a graded photolysis of the cage would result, and consequently, an uneven release of Ca^{2+} across the cell, Thus, for most 1-photon uncaging experiments, the moderate extinction coefficient of the dimethoxynitro cage is advantageous.

3.4. Rates of photochemical reaction and Ca^{2+} release

The primary photochemical process of electronically excited *ortho*-nitrotoluenes (viz. all the cages described here) is benzylic proton abstraction [30]. It is this reaction that is responsible for the de-excitation of the chromophore via π-electron reorganization to the ground state *aci*-nitro intermediate. If the benzylic carbon bears a hetro atom, then these highly energetic intermediates relax by bond rupture, and concomitant release of the caged substrate (reviewed in refs. [31–33]).

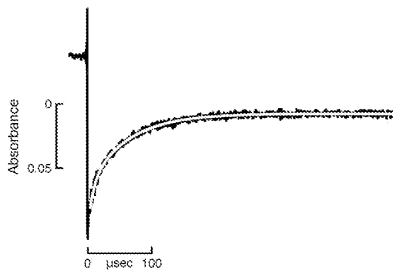

Fig. 4. *Aci*-nitro transient intermediates. A single pulse from a frequency-doubled ruby laser (347 nm, 180 mJ) was used to photolyze a solution of cage (2.0 mM DMNPE-4) and of Ca^{2+} (2.1 mM) at pH 7.2 (60 mM Hepes) with 100 mM KCl at 25 °C. The absorption transients were monitored at 457 nm. The declining phase of the signal was fit to a double exponential expression plus a constant, and the resultant values were used to calculate the solid curves overlaid on the trace.

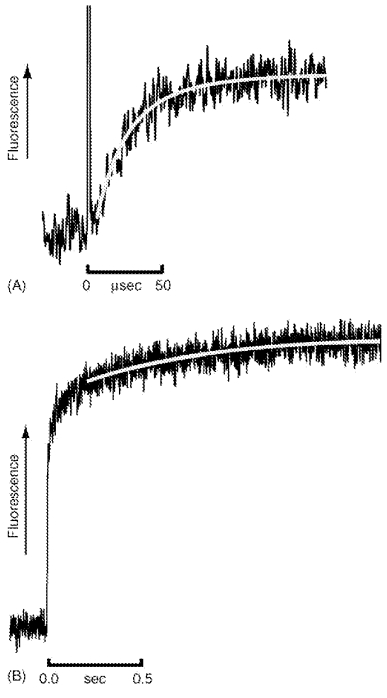

Fig. 5. Time-dependent increase in fluorescence of Ca-orange-5N upon photolysis of DMNPE-4:Ca^{2+}. A single pulse from a frequency-doubled ruby laser (347 nm, 180 mJ) was used to photolyze a solution of DMNPE-4 (2.0 mM) in the presence of Ca^{2+} (2.1 mM) at pH 7.2 (60 mM Hepes) with 100 mM KCl at 25 °C. The fluorescence signal from Ca-orange-5N (50 μM) was monitored: excitation 488 ± 10 nm, emission 570 ± 10 nm. Two time scales show the fast (A) and slow (B) release of Ca^{2+} from the DMNPE-4:Ca^{2+} complex.

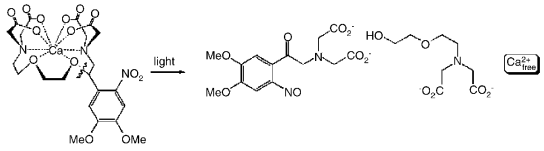

Fig. 6. Chemical reaction for DMNPE-4 photofragmentation.

Two types of time-dependent measurements were made to estimate the rates of the photochemical reaction and Ca^{2+} release from the dimethoxy-NPE Ca^{2+} cages. First, flash photolysis of *o*-nitrobenzyl compounds produces transient intermediates (or *aci*-nitro species, see [32] for a complete review of the reaction mechanism) that absorb at 450 nm. A single pulse from a frequency-doubled ruby laser (180 mJ at 347 nm) generated such *aci*-nitro species, the rate of disappearance of which was probed with the 457 nm line of an argon laser. DMNPE-1, -4 and -5 each exhibited *aci*-nitro signals that were fitted to a double exponential equation. These data are show in Table 2. At pH 7.2 the decay rates for DMNPE-4 were 92,000 and 15,000 s^{-1} in the presence of Ca^{2+} and 87,000 and 13,000 s^{-1} in the absence of Ca^{2+} (representative decay trace is illustrated in Fig. 4). We believe that the simplest interpretation of these data is that the chelator is cleaved by a mechanism that initially divides along two parallel paths (giving two *aci*-nitro intermediates) that finally converge to give the same photoproducts (cf. [13]). Secondly, laser flash photolysis (180 mJ at 347 nm) of each cage (2 mM) in the presence of saturating Ca^{2+} (2.1 mM) at physiological pH elicited the time-dependent increase in fluorescence of the indicator Ca-orange-5N (50 μM) shown in Fig. 5A, which could be fitted with a monoexponential rate constant of in the range of 48,000–53,000 s^{-1}. These data are very similar to that of DM-nitrophen [13], the other dimethoxy Ca^{2+} cage of which we have made a detailed photochemical study, and suggest that chelator fragmentation is the rate-limiting step in the Ca^{2+} release process. Uncaging of Ca^{2+} from DMNPE-4 produced a signal from the indicator that also had an additional very slow component, comprising about 30% of the overall fluorescence change, having a rate of 1.5 s^{-1} (Fig. 5B). There is no corresponding slow *aci*-nitro transient signal, and so the origins of this very slow release are not clear.

It has been reported that DM-nitrophen undergoes partial photodecarboxylation [32], in parallel to simple chelator scission. We could detect almost no CO_2 signals from flash photolysis of DMNPE-4 using FT–IR measurements (Wirz, Boudelbous, and Ellis-Davies, unpublished results). This datum, in conjunction with the low Ca^{2+} affinity of the DMNPE-4 photoproducts, suggest the vast majority of the chelator undergoes the fragmention reaction shown in Fig. 6.

We can discern no overall pattern in the effect of addition of the electron donating dimethoxy (DM) functionalityies on *ortho*-nitrobenzyl photochemistry. Consideration of our own work on DMNPE-1 and NP-EGTA and that of Wootton and Trentham [34] indicates that the presence of dimethoxys slow uncaging by 1 order magnitude for amines and phosphates [e.g. 1-(DM-nitrophenyl)-ethyl caged ATP]. But a systematic investigation of the effect of aromatic substitution of the rate of *aci*-nitro decay by Muralidharan and Nerbonne [35] showed the opposite trend. However, when we compare our kinetic data for uncaging protected phenols (i.e. that of DMNPE-5) and alcohols (i.e. DMNPE-4), with that of Muralidharan and Nerbonne [35] and Corrie and Trentham [1], respectively, we find that the presence of dimethoxys *speeds up* the rate of *aci*-nitro decay by 1000-fold. We must conclude that predicting photolysis rates of novel caged compounds based on their structural similarity with caged compound with known physico-chemical properties is unreliable and thus requires that the rate of uncaging of each new compound synthesized must be measured.

4. Conclusion

Light damage to living tissue is a serious limitation for many optical probes used in cell physiology. Therefore, the development of an optically tuned, Ca^{2+}-selective cage (viz. DMNPE-4) that requires less energy to give large changes in $[Ca^{2+}]_{free}$ is a useful advance of caged Ca^{2+} technology. Its improved properties will make experiments on living cells more feasible and easier principally because of extended cell viability during photolysis. Furthermore, photobleaching of other optical probes that are excited with a similar wavelength to that of uncaging light (e.g. fura-2) are also be minimized with DMNPE-4 for the same reason.

DMNPE-4 is a calcium cage that has a similar sized extinction coefficient to the widely used (and commercially available) DM-nitrophen and nitr-5. However, unlike the former, it is a Ca^{2+}-selective cage, and unlike the later, it has a higher affinity before photolysis and a lower affinity after photolysis. In addition, it has a much larger extinction coefficient than our original Ca^{2+}-selective cage, NP-EGTA. Thus, it has a unique combination of physico-chemical properties, so that photolysis of the DMPNE-4:Ca^{2+} complex releases large quantities of Ca^{2+}, with sufficient speed, to activate synaptic vesicle exocytosis in PC12 cells [36,37] and to study Ca^{2+} nucleolar buffering via 2-photon uncaging [38].

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