Review

Neurobiology with Caged Calcium

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1. Introduction

Cells use a wide variety of chemical messengers for signaling, and ionized calcium (Ca^{2+}) is the single most important information carrier.[1] In this review, I give a brief outline of the wide variety of cells and cellular functions regulated by Ca^{2+} (reviewed in refs 2–5). Using this basic biological context, I tell the history of the development of caged Ca^{2+} molecules from the perspective of the requirements of the experimental application of each photosensitive probe, as they have been used in the context of neuroscience research. Like the development of a series of pharmaceutical drugs designed to tackle a disease, each story is highlighted by epochs. Chemical synthesis of these caged probes has been driven by the needs of experimental biologists to control artificially the processes they study: probes are made, tested, and used to their experimental limit by biologists. The probes are redesigned and improved by chemists according to needs envisioned by biologists, who then apply the second-generation probes to a more demanding series of problems. Thus, just as drug development always has a clear health target in mind, synthesis of photolabile probes such as caged Ca^{2+} follows a similar pattern. This analogy is apt not only with respect to process but also with respect to outcome. Just as pharmaceuticals can often appear promising, we know it is not until they go through an extensive series of trials that can they be used with assurance. Their unwanted side-reactions often manifest themselves late in the game. Likewise, photolabile compounds can superficially appear chemically excellent, but only by extensive biological application can they be rightly called "caged probes".[6]

The term "caged compounds" was coined in 1978 by physiologists to refer to the *functional* encapsulation of a biomolecule (ATP) by use of a photochemical protecting group.[7] Biologists have happily embraced the term since then (reviewed in refs 8–12), as they are not burdened by a quite different alternative meaning of the term. Because "caged" can also mean molecules such as cubane, some chemists have coined different terms, but such attempts at rebranding have not been accepted by the end user biologists (and those scientists working in the field of animal husbandry are oblivious to this dilemma). Thus, throughout this review I will use the term "caged" to mean the functional encapsulation of a chemical messenger in a biologically inert and photosensitive form.[6] Almost all chemical messengers used by cells are organic molecules, so they are caged by covalent modification of a crucial residue (e.g., the γ-phosphate of ATP). Cations such as Ca^{2+} obviously cannot be inactivated in a similar manner, but they can be caged functionally by high affinity coordination.[13,14] Thus, as will be seen, caged Ca^{2+} molecules are uniquely like the "caged compounds" that many chemists might traditionally envisage (except the entire framework is not covalently rigid), in that they are well-defined 3-dimensional structures, as a result of high affinity coordination of Ca^{2+} by photolabile chelators. Chromophore excitation leads to photolysis of a covalent bond, liberating the caged chemical messenger (Figure 1).

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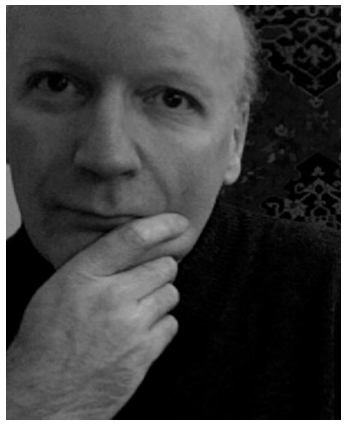

Graham C. R. Ellis-Davies received a Ph.D. in Chemistry from the University of Reading in the U.K. in 1982, under the supervision of Professor Derek Bryce-Smith, one of the fathers of modern organic photochemistry. After postdoctoral work at King's College London, and the Gorleaus Laboratoria, Leiden University, he moved the USA, to work in the Department of Physiology at the University of Pennsylvania with Professor Jack H. Kaplan, FRS, on the development of caged calcium probes and Na,K-ATPase enzymology. During this period (1985−94) he was privileged to work in three laboratories at the Max-Plank Institute: in Frankfurt (Professor Ernst Bamberg), Heidelberg (Professor Wolf Almers), and Gottingen (Professor Erwin Neher). It was these collaborations that inspired the synthesis of some of the caged calcium probes described herein. Since 1994, the development of photochemical probes in the Ellis-Davies laboratory has been supported by the National Institute of General Medical Sciences. This support has also enabled many exciting collaborations with biologists worldwide. Most notably, interactions with Professors Haruo Kasai (University of Tokyo) and Ernst Niggli (University of Bern) have been truly inspiring.

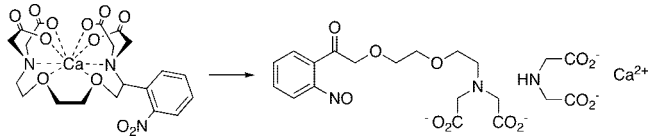

Figure 1. Ca^{2+} uncaging by photolysis of NP-EGTA. Ca^{2+} is efficiently released by photochemical lysis of the chelator backbone, converting a high affinity, tetracarboxylic acid chelator into two low affinity dicarboxylic acid molecules.

2. What Does Ca^{2+} Do inside Cells?

The importance of Ca^{2+} inside cells was firmly established over 100 years ago by a series of key experiments by Ringer.[15–17] During the period 1940−70, various physiologists confirmed these ideas. In particular, direct injection of Ca^{2+} into muscle fibers and nerves cells definitively established that an increase in the intracellular Ca^{2+} concentration (or $[Ca^{2+}]_i$) was the essential event for muscle contraction and nerve impulses.[18] We now know that changes in $[Ca^{2+}]_i$ control a dazzling myriad of cells or cellular functions, including muscle contraction, secretion of neurotransmitters and hormones, gene transcription, synaptic plasticity, fertilization, movement of cells (nonmuscle motility) and wound healing, cell death, gating of ion channels, the activity of kinases and phosphatases, etc.[1–5] Often Ca^{2+} itself does not directly control a target protein, but a Ca-activated calmodulin complex does.[19,20] Here I highlight those processes that have been studied using caged Ca^{2+} photolysis.

2.1. Roles of Ca^{2+} at Nerve Terminals

The whole process of transmission at fast synapses in the CNS is over in less than 10 ms. Action potentials stimulate Ca^{2+} entry through presynaptic channels, the duration of which is extremely brief (greater than hundreds of microseconds) and is restricted to the volume adjacent to the plasma membrane.[21] These rapid transients cause the fusion of nearby synaptic vesicles containing neurotransmitters with the plasma membrane, and release of the neurotransmitter into the synaptic cleft. Ca^{2+} also has many important postsynaptic functions. Ca enters postsynaptic cells through ion channels called NMDA receptors,[22] or it is released from internal stores[23] by inositol-1,4,5-trisphosphate (IP_3). These local changes of Ca^{2+} signals can last for tens to hundreds of milliseconds, causing changes in synaptic strength.[24]

2.2. Secretion in Neuroendocrine cells

Many cell types outside the CNS use Ca^{2+}-driven secretion to release important substances; insulin release by beta cells in the pancreas is the prime example of such a process. Insulin is released by the same type of molecular machinery as glutamate in the CNS, but the temporal duration is somewhat slower.[25]

2.3. Ca^{2+} Regulated Ion Channels

$[Ca^{2+}]_i$ controls many different ion channels, for example, important potassium channels in many cell types.[26] In cardiac myocytes, the process of Ca^{2+} release from intracellular stores is initiated by a small amount of trigger Ca^{2+} entering the cytoplasm through voltage-gated Ca^{2+} channels in the plasma membrane. This Ca^{2+} signal is amplified by Ca^{2+} binding to RyR on the SR, and these internal Ca^{2+} channels release large quantities of Ca^{2+} required for muscle contraction. Ca^{2+} is also an important cofactor for IP_3-receptors.[24]

2.4. Muscle Contraction

There are three types of muscle cells, skeletal, cardiac, and smooth, and the contraction of all is controlled by fluctuation of $[Ca^{2+}]_i$. The rate of contraction of striated muscles (skeletal and cardiac) is nearly as fast as the process of neurotransmission.[27] At the neuromuscular junction, presynaptic action potentials release acetylcholine using Ca^{2+}-driven vesicle fusion at very fast rates, just like Glu in the CNS; thus, changes in $[Ca^{2+}]_i$ are similar in terms of size and duration.

Postsynaptic Ca^{2+} is even more important in muscles than neurons, as it is essential for triggering muscle contraction. Cell-wide (or global) increases in $[Ca^{2+}]$ last for tens to hundreds of milliseconds. Smooth muscle contracts relatively slowly, so it functions quite differently in the way it connects increases in $[Ca^{2+}]$ and forces development. The signal cascade used in smooth muscle is the same as that in many nonmuscle cells: IP_3-triggered Ca^{2+} release from intracellular Ca^{2+} stores is the major Ca^{2+} source.[28]

2.5. Nonmuscle Motility

Many cells use nonmuscle molecular motors for movement. For example, white blood cells use Ca^{2+}-regulated machinery to seek out their prey. The directionality of growth cones of developing axons and dendrites is also controlled by local and global Ca^{2+} gradients.[29,30] The size and localization of the $[Ca^{2+}]$ signal can direct the growth cone toward or away from its target.[31]

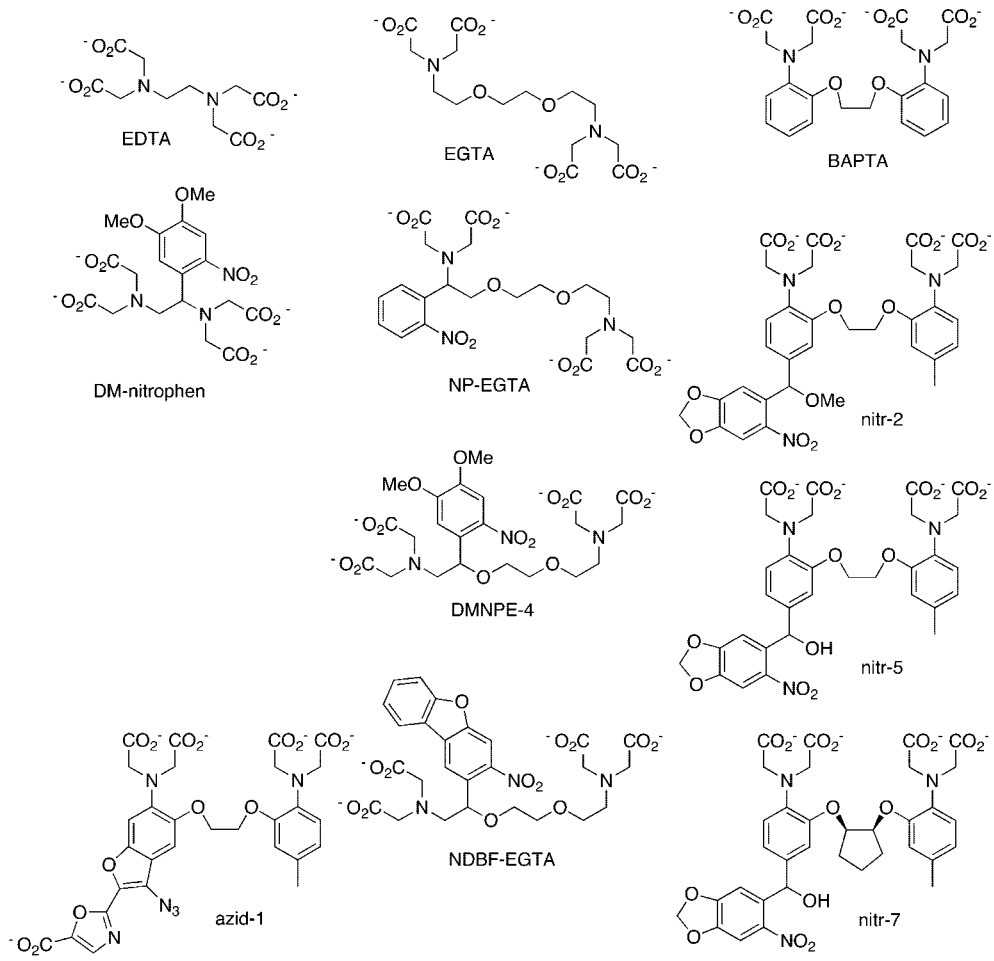

Figure 2. Structures of all the Ca^{2+} cages that have been used in living cells, with their parent chelators (EDTA, EGTA, BAPTA). Possible positive counterions (e.g., protons, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) are omitted from the structures for the sake of simplicity.

2.6. Ca^{2+} Buffering in Cells

The basic sources of Ca^{2+} for cells have already been mentioned above (Ca^{2+} channels and various ligand-gated ion channels), but how do cells terminate their $[Ca^{2+}]$ transients? Ubiquitous Ca^{2+}-ATPases are one means of reducing $[Ca^{2+}]$.[32,33] These integral membrane proteins extrude Ca^{2+} from the cytoplasm, by pumping it back into the store[34] from whence it came, or to the extracellular milieu. Cells can also use a Na^{+}–Ca^{2+} exchanger on the plasma membrane to help this process.[33,35] Calcium binding proteins also buffer the $[Ca^{2+}]_i$ transients during signaling processes to a great extent.[36]

3. Key Biological Applications of Caged Ca^{2+}

Once the importance of Ca^{2+} fluctuations for cell function was well established, the next step for biologists was to obtain a quantitative understanding of how Ca^{2+} worked in cells. At this point in the calcium story, organic chemistry becomes a key component, because without direct measurement and control of $[Ca^{2+}]$, no quantitative understanding of Ca^{2+} function is possible. Chemical synthesis of probes that selectively bind Ca^{2+} with high affinity (K_d in the range 50−250 nM) in the normal intracellular milieu, where other physiological important cations are present in much higher concentrations (e.g., in mammals $[Na^{+}] = 13$ mM, $[K^{+}] = 120$ mM, $[Mg^{2+}] = 1$ mM, but $[Ca^{2+}] = 100$ nM; these values are approximate), has proved to be one of the most important contributions to the field of chemical biology.

The chemical synthesis of effective fluorescent Ca^{2+} dyes in the period from 1980−1988[37,38] prestaged the development and application of caged Ca^{2+} probes. These two pieces of chemical technology, in conjunction with other technologies such as patch clamp,[39] laser-scanning confocal microscopy,[40] microfluorimetry, genetic manipulation of proteins, etc., all combined to enable physiologists to start to obtain a detailed molecular picture of Ca^{2+} signaling cascades in many different cells.[41]

In 1980, the first effective fluorescent Ca^{2+} dye (quin2; ref 42) was introduced. High affinity Ca^{2+} chelators such as EDTA and EGTA (Figure 2) had been studied for many years, but the Ca^{2+} affinity of these molecules is very sensitive to changes of pH in the physiological range (6.5−7.5). Adding two aromatic rings to the backbone of EGTA to create BAPTA[42] (Figure 2) removed this problem, while maintaining the high Ca^{2+} and low Mg^{2+} affinity of EGTA. Some additional modifications produced the fluorescent Ca^{2+} dye quin2. This dye started the "calcium imaging revolution", but since it was not very bright and near-UV illumination was required, it was far from ideal as a probe. The synthesis of fura-2 and fluo-3 solved each of these problems, and occurred during the same period as the development of the first Ca^{2+} cages.[37]

In parallel to the development of these fluorescent Ca^{2+} dyes, several other important advances in technology occurred: (1) the patch clamp technique became widely practiced; (2) laser-scanning confocal microscopy was invented; and (3) DNA-based sequencing of proteins was

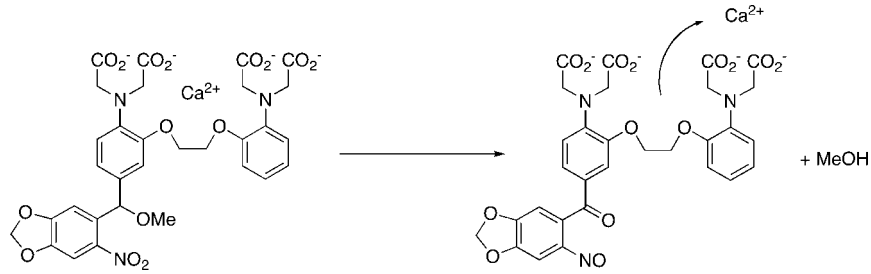

Figure 3. Photochemistry of nitr-2. Irradiation lowers the buffering capacity of the chelator for Ca^{2+} by creation of a conjugated electron-withdrawing group on one aromatic ring.

developed, followed by the ability to do site-directed mutagenesis of any protein.[41] Crucially, all these techniques were commercialized, along with summer courses to teach them to young (and sometimes old) scientists. Importantly for Ca^{2+} uncaging, flash lamps and ratiometric Ca^{2+} microfluorimetry using fura-2 were also commercialized. Thus, by 1993, one could *simultaneously* uncage Ca^{2+} and measure membrane capacitance along with [Ca^{2+}] with *one* piece of commercial software.

3.1. Initial Applications of Caged Ca^{2+}

The first caged Ca^{2+} probes were developed independently by two groups during the period of 1986−1988.[43–45] Two of these probes (nitr-5 and DM-nitrophen, Figure 2) worked quite well and so became commercially available. This enabled many laboratories easy access to the "first generation" Ca^{2+} cages. Thus, the strengths and weaknesses of nitr-5 and DM-nitrophen became apparent, giving impetus to the development of "second generation" probes.

3.1.1. Photorelease of Ca^{2+} in Aplysia

The first published experiments with a Ca^{2+} cage highlighted almost all the strengths and weaknesses of caged Ca^{2+} biology and chemistry. During the period of development of fura-2 and fluo-3, Tsien and co-workers also synthesized caged Ca^{2+} probes, nitr-1 through nitr-7 (ref 44; I will refer to the collective members of this group as nitr-X). In 1986, the first of these probes was revealed. Injection of nitr-2 into Aplysia neurons, under two-electrode voltage clamp conditions, allowed Tsien and Zucker to estimate the relationship between photoreleased [Ca^{2+}] in the postsynaptic cell and the evoked K^+ channel current.[43] Even though the quantum yield of photolysis (ϕ) of nitr-2 was low[44] (0.01, not 0.1 as stated in the first paper[43]) and the rate of photolysis was slow (5 s^{-1}), large K^+ currents could be evoked by flash (4 ms pulse) or "gentle" (1 s) photolysis. The photoreaction of nitr-2 (Figure 3) utilized the same nitrobenzyl photochemistry of caged ATP.[7] Photolysis eliminated methanol, creating a benzylic carbonyl functionality, and reduced the pK_a of the conjugated N atom. This chemistry changed the affinity of nitr-2 from 160 nM to about 8 μM; thus, some of the bound Ca^{2+} was released. The kinetically slow and photochemically inefficient release of Ca^{2+} by nitr-2 was seen as a potential problem for future neurophysiological experiments,[43] so improved caged Ca^{2+} compounds were made.[44]

3.1.2. Neurotransmitter Secretion Is Controlled by Presynaptic [Ca^{2+}] per se

Since the formulation of the "calcium hypothesis" of neurotransmitter release,[46] and its basic confirmation,[18] many details of the mechanism remained to be understood. For example, the quantitative relationship between presynaptic [Ca^{2+}] and postsynaptic current, the nature of the cooperativity of Ca^{2+} binding to the secretory machinery, the effects of cellular diversity on rates of secretion, the exact nature of presynaptic processing of synaptic vesicles, the specificity of the amino acid residues that control or regulate secretion, the role of membrane potential in neurotransmitter release, etc.[47] The development of good caged Ca^{2+} probes has enabled neurophysiologists to address these and other basic question of synaptic and endocrine physiology.

Photorelease of Ca^{2+} from nitr-5 in presynaptic terminals, while monitoring postsynaptic currents, enabled Zucker and Haydon[48] to answer definitively a raging controversy of the 1980s in the area of synaptic physiology.[49] It was accepted that presynaptic membrane depolarization is required for voltage-gated Ca^{2+} opening and neurotransmitter release, but did the change in membrane potential itself also modulate transmission, as well as [Ca^{2+}]? Photolysis of nitr-5 loaded into the presynaptic cell through a sharp glass electrode showed that only [Ca^{2+}] affected the size of the postsynaptic current. Removal of external Ca^{2+} and varying the presynaptic membrane potential from −120 to + 40 mV had no effect on postsynaptic currents.[48] These simple but elegant caged Ca^{2+} experiments are exemplary of the power of uncaging technology to address fundamental biological questions. Since uncaging bypasses the normal upstream source of the biological messenger (in this case Ca^{2+}), from its downstream effects, complex signaling cascades are dramatically simplified, permitting their parsing into controllable units.

The development[44] of nitr-5 and nitr-7 was a significant improvement upon nitr-2. Both nitr-5 and nitr-7 photolyzed with a rate of about 2,500 s^{-1} (Table 1), and although this rate is significantly slower than Ca^{2+} entry at presynaptic terminals, it is a substantial improvement on nitr-2. Further, all the nitr-X probes have affinities that are pH insensitive in the physiological range and show low affinities for Mg^{2+} (several millimolar), both highly desirable (but not essential) properties for caged Ca^{2+} probes. However, these molecules are less than ideal caged Ca^{2+} probes because their release of Ca^{2+} is chemically and photochemically very inefficient.

Photochemical efficiency is defined by the product of the ϕ and the molecular extinction coefficient (ϵ), $\phi\epsilon$. The nitr cages have $\phi\epsilon$ = ca. 500, slightly larger than that for NPE-ATP. Since light can be phototoxic to cells, it is desirable to have much higher $\phi\epsilon$ for some experiments; however, this value (500) does not impose an absolute constraint on how much Ca^{2+} can be uncaged, as more light can be used, if it is tolerated by the cells under study.

Chemical inefficiency is a more serious deficiency, as this does impose an upper bound on the total amount of Ca^{2+}

Table 1[a]

	K_d(Ca),[b] nM	K_d prods, mM	affinity change, *x*-fold	K_d(Mg),[b] mM	quantum yield of photolysis	extinction coefficient, M^{-1} cm^{-1}	2PCS at 720−730 nm, GM	rate of photolysis, s^{-1}	rate of Ca release, s^{-1}
EDTA	32			0.005					
EGTA	150			12					
DM-nitrophen	5	3	600,000	0.0025	0.18[c]	4,300	0.01−0.04	8×10^4	3.8×10^4
NP-EGTA	80	1	12,500	9	0.23	975	0.001	5×10^5	6.8×10^4
DMNPE-4	48	2	41,700	10	0.09	5,120	0.01−0.04	3.3×10^4	4.5×10^4
NDBF−EGTA	100	2	20,000	15	0.7	18,400	0.6	2.6×10^4	2.0×10^4
BAPTA	110		17						
nitr-5	145	0.0063	54	8.5	0.012	5,500	ND	2.5×10^3	ND[d]
nitr-7	54	0.003	42	5.4	0.011	5,500	ND	2.5×10^3	ND[d]
azid-1	230	0.12	520	8	1.0	33,000	1	ND	500[e]

[a] Note and abbreviations: ND, not determined; 2PCS, 2-photon cross section. [b] Affinities measured at pH 7.2 and 250 mOsm. [c] Quantum yield of Ca release. [d] Probably equal to the rate of photolysis. [e] Lower limit of the measurement method.

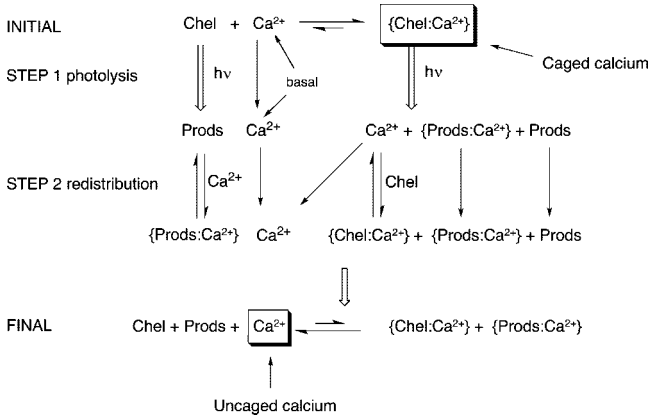

Figure 4. Scheme illustrating the basic changes involved in Ca^{2+} uncaging by photolysis of a high affinity Ca^{2+} chelator. In the initial state, the equilibrium should lie to the right-hand side, favoring "caged Ca^{2+}" (top line), and after photolysis, net release of Ca^{2+} is predicated on more than all the free, unloaded cage ("Chel" top line) being photolyzed, so driving the equilibrium to the left-hand side in the final state. Note that the photoproducts must have some finite affinity for Ca^{2+} (Table 1) and so will chelate photoreleased Ca^{2+}, reducing the overall chemical yield of uncaged Ca^{2+}.

per mole that can be uncaged. This limit for the nitr compounds stems both from the modest changes in Ca^{2+} affinity arising from photolysis (ΔK_d is about 40-fold) and from the relatively low affinity before photolysis[44]. All Ca^{2+}-triggered cellular processes have a threshold for activation (i.e., the $[Ca^{2+}]$ at which they initiate). This value is somewhat variable between cell types, having a range of about 300 nM to 1 μM.[14] Since only bound Ca^{2+} can be uncaged, the K_d before photolysis sets the percentage loading of the photolabile chelator before the unbound or free $[Ca^{2+}]$ reaches the threshold. If the Ca^{2+} cage has $K_d = 140$ nM (nitr-5), then only 40% of the chelator complexes Ca^{2+}, leaving 60% free chelator.[14] *Net* release of Ca^{2+} under these circumstances requires photolysis of >60% total cage; otherwise, uncaged Ca^{2+} will be recomplexed by unloaded, unphotolyzed cage (Figure 4). The modest change in affinity (ΔK_d) has an even more profound effect on the chemical efficiency of Ca^{2+} uncaging from nitr-X photolabile chelators. Since the photoproducts of uncaging of nitr-X compounds have affinities for Ca^{2+} in the range of 1−10 μM (Table), most of the Ca^{2+} remains complexed after photolysis.

3.2. Toward a Quantitative Model of Ca^{2+}-Triggered Secretion

In contrast to the chemistry of the nitr series of caged Ca^{2+} probes (Figure 3), the backbone of DM-nitrophen is cleaved; this destroys the high affinity Ca^{2+} coordination sphere of the chelator (see example of NP-EGTA in Figure 1). This fragmentation approach to Ca^{2+} release inevitably produced much larger changes in ΔK_d than the modest 40-fold changes of nitr-5 (Table 1). Before photolysis, the K_d DM-nitrophen for Ca^{2+} is 5 nM, and the photoproducts have an average affinity of about 3 mM; thus, the $\Delta K_d = 600{,}000$-fold at pH 7.2. The ϕ for Ca^{2+} release is 0.18, and the rate of Ca^{2+} release is 38,000 s^{-1}. Thus, the photochemical and chemical deficiencies of the nitr probes were essentially solved by the introduction of DM-nitrophen. However, since DM-nitrophen was based on EDTA, it shares similar Mg^{2+} and proton affinities with those of the parent chelator.[45,50] These "deficiencies" have proved surprisingly unimportant for many physiological studies in secretory cells such as melanorophs, chromaffin, CHO, pancreatic beta, PC12, RBL, retinal bipolar, mast, acinar, the calyx of Held, etc.[25,51]

3.2.1. Initial Applications of DM-nitrophen: Ca^{2+} Uncaging in Axons from Crustacea

Zucker and co-workers revisited the issue of the relationship between membrane depolarization and Ca^{2+} entry in the crayfish motor axon,[52,53] by injecting DM-nitrophen and fura-2 with and without added Ca^{2+}. Uncaging Ca^{2+} in the presynaptic terminal with external Ca^{2+} channel blockers ($[Co^{2+}] = 13.5$ mM/$[Mg^{2+}] = 30$ mM or [EGTA] = 2 mM) allowed the following conclusion to be drawn: "the normal spike-evoked secretion of neurotransmitter is not affected directly by presynaptic voltage but is triggered exclusively by an increase in $[Ca^{2+}]_i$ near transmitter release sites."[52] The same laboratory also used DM-nitrophen to study transmitter release in the squid giant axon.[54] However, these classic neurophysiological preparations proved difficult to work with when seeking a quantitative model of Ca^{2+}-controlled secretion, as the intracellular spaces are not easily dialyzed to homogeneity (pressure injection of high concentrations of probes was required); therefore, Zucker and other neurophysiologists turned to more tractable preparations such as chromaffin cells and melanotrophs. Uncaging of Ca^{2+} from the DM-nitrophen:Ca^{2+} complex was measured using ratiometric Ca^{2+} microfluorimetry in these neuroendocrine

cells, and it initiated the development of the general Ca^{2+}-trigger secretion model we know today.

3.2.2. Photolysis of DM-nitrophen in Melanotrophs and Chromaffin Cells

In 1991−1993, two groups independently published studies of the secretory activity of neuroendocrine cells stimulated by rapid UV photolysis of DM-nitrophen that was quantified by ratiometric $[Ca^{2+}]_i$ measurements and monitored by whole-cell capacitance measurements. When vesicles containing hormones or transmitters fuse with the plasma membrane, the vesicle membrane is absorbed into the membrane of the cell, increasing its surface area. This change can be quantified by measuring changes in cell capacitance in the whole-cell patch-clamp mode. The release of vesicle contents can often also be detected directly using amperometry.[55,56] Spherical cells are especially amenable to capacitance measurements, as their simple shape allows exquisite space-clamp. Thomas and Almers found that uncaging of Ca^{2+} in the range of 50−300 μM inside melanotrophs (pituitary cells) elicited changes in membrane capacitance that had three distinct temporal phases.[57] The most rapid phase had a rate of vesicle fusion of ca. 17,000 s^{-1}, followed by two slower rates of 7,000 and 500 s^{-1} (refs 58 and 59). The corresponding slower steps were also resolved by Neher and Zucker in their similar study of Ca^{2+}-driven changes in membrane capacitance of chromaffin cells.[60] In these studies (and those described below), the fluorescent Ca^{2+} dyes were carefully calibrated in situ, before and after photolysis, so precise measurements of Ca^{2+} could be made. Thomas et al. showed that the affinity for Ca^{2+} of the rapid step was about 27 μM and that three Ca^{2+} ions were bound by the sensor for fusion.[59] In 1994, a study of chromaffin cells at comparable resolution yielded similar results, except that it appeared that four Ca^{2+} ions bound to the sensor with an affinity in the range of 7−21 μM.[61] The range of $[Ca^{2+}]$ uncaged in this work was systematically varied over an extremely wide range (0.6−600 μM) that could only be accomplished using DM-nitrophen at a concentration of 10 mM. Even at such high concentration, full photolysis of nitr-5 would only release relatively modest amounts of Ca^{2+} due to its inherent chemical inefficiency of Ca^{2+} uncaging.

3.2.3. Photolysis of DM-nitrophen in Retinal Bipolar Neurons

The expertise gathered from these studies of chromaffin cells enabled Neher and colleagues to use exactly the same methods to study secretory rates in neurons.[62] In contrast to most neurons in the mammalian CNS, gold fish retina have bipolar neurons that are well suited to capacitance measurements. Bipolar cells appeared to be quite different from neuroendocrine cells, having only one phase of secretion. The K_d of the Ca^{2+} sensor was extremely high, being 194 μM, and maximal rates of vesicle fusion were about 1,200,000 s^{-1}. The conclusion was that these "properties ... allow the neuron to fulfill are the requirements of fast neuronal signalling."[62]

3.2.4. Photolysis of DM-nitrophen in Pancreatic β-Cells, CHO Cells, and PC12 Cells, and Gonadotrophs

Following the pioneering applications of DM-nitrophen in melanotrophs and chromaffin cells outlined above, the secretory activity of several other neuroendocrine cells was quantified using time-resolved whole-cell capacitance measurements.[25]Photolysis of caged Ca^{2+} inside pancreatic β-cells,[63,64] acinar cells,[65] CHO cells,[66] PC12 cells,[67] and gonadotrophs[68] elicited vesicle fusion with temporal profiles (i.e., fast-medium-slow or fast-slow) similar to those of melanotrophs and chromaffin cells, while the exact details of the kinetic steps were found to depend on the cell type.[25] It should be noted that in some cases amperometric measurements of release of the contents of secretory vesicles have been used in parallel with capacitance measurements.[65,69] Capacitance is a measure of the net change in membrane surface area (i.e., the balance of exocytosis and endocytosis), whereas amperometry is a "true measure" of secretion.[70]

3.2.5. Photolysis of DM-nitrophen in the Calyx of Held

Since the Ca^{2+} hypothesis of neurotransmitter secretion[46] was advanced by Sir Bernard Katz in 1965, a "Holy Grail" experiment for synaptic physiologists was to define the quantitative relationship between the concentration of Ca^{2+} experienced locally by individual synaptic vesicles and the postsynaptic response evoked by secretion of the neurotransmitter from such a vesicle.[47] Well-studied synapses from the mammalian CNS such as pyramidal neurons are too small and delicate for such detailed study. One specialized synapse has proved sufficiently large and robust for detailed examination with double patch clamp techniques, namely the calyx of Held.[71] The calyx of Held is an excitatory glutamatergic synapse arising from globular bushy cells in the anterior ventral cochlear nucleus onto a principal cell in the medial nucleus of the trapezoid body. This synapse has a diameter of >10 μm and is amenable to whole-cell patch clamp recording from pre- and postsynaptic cells *in situ* (i.e., in acutely isolate brain slices that preserve intact the complex architecture of neuronal cells in the mammalian CNS).

Photolysis of the DM-nitrophen:Ca^{2+} complex rapidly released Ca^{2+} to varying concentrations (2−25 μM), depending on the irradiation power, throughout the presynaptic terminal. $[Ca^{2+}]_i$ was quantified in the same way as in chromaffin cells and correlated with the evoked postsynaptic currents.[72] Since this nerve terminal is unusually large, with several active zones, many vesicles fuse asynchronously, so the absolute release rate was calculated by modeling and deconvolution of the total current. These experiments showed that each synaptic vesicle experiences on average a very rapid pulse (width HMHW = 0.4 ms) of Ca^{2+} in less than 1 ms after the action potential, with a size in the range of 10−25 μM. Similar to chromaffin cells, the secretory complex bound 4−5 Ca^{2+} ions. Interestingly, the Ca^{2+} affinity of this mammalian central synapse was much higher than that of the gold fish retina bipolar neuron and the cochlea hair cell.[73] Subsequent elegant studies on the same synapse using DM-nitrophen and capacitance measurements,[74,75] or with added exogenous buffers, have confirmed and extended these basic findings.[76] (Laser photolysis of NP-EGTA[77] was reported at the same time as the work with DM-nitrophen.[72])

All of the experiments described so far with DM-nitrophen have involved dialysis of the cytosol with "Mg^{2+} free" solutions (but judicious choice of [DM-nitrophen], $[Ca^{2+}]$,

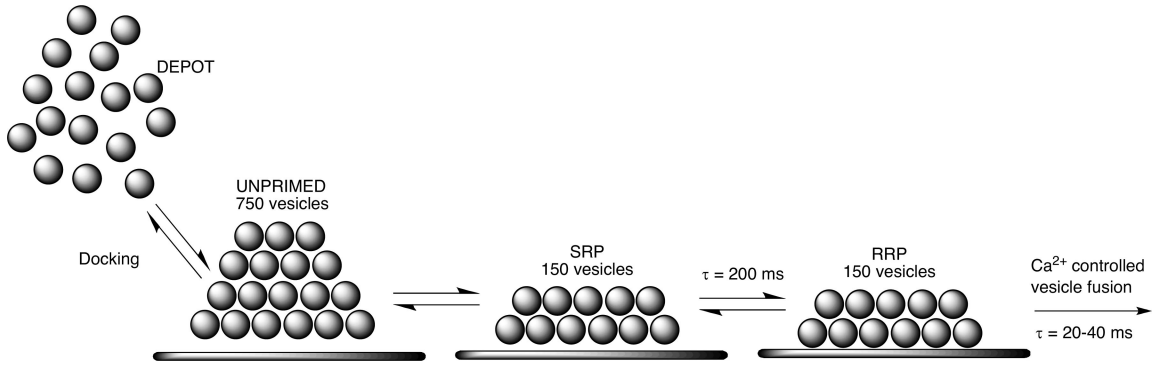

Figure 5. Scheme of the basic steps involved in Ca^{2+} regulated secretion of synaptic vesicles in neuroendocrine cells.

$[Mg^{2+}]$, and [ATP] permitted reasonable amounts of Mg^{2+}:ATP to be present inside the chromaffin cells[78] or calyx of Held[72,74,75] without compromising Ca^{2+} uncaging). Clearly, this situation is "nonphysiological", as the concentration of free Mg^{2+} inside cells is estimated to be 1−2 mM[79]and the total Mg^{2+} to be as much as 30 mM.[80] Since the volume of the patch pipet is much larger than that of any cell, much of this Mg^{2+} is washed away during whole-cell equilibration. Photolysis of the nitr-5:Ca^{2+} complex in the presence of "normal $[Mg^{2+}]$" permits elevation of $[Ca^{2+}]_i$ to modest levels of 1−2 μM at best; therefore, studies requiring much larger $[Ca^{2+}]$ jumps and physiological $[Mg^{2+}/Mg^{2+}ATP]$ required a new caged Ca^{2+} probe.

3.3. Dissecting the Secretory Machinery Using Caged Ca^{2+}

The Ca^{2+}-activated secretory machinery has over 100 proteins and so is very complex.[81] However, a core set proteins have been defined as a minimum for membrane fusion (the cell's plasma membrane and the vesicles' membrane become contiguous during exocytosis[82]). This core is "supplemented" in reality by a host of other proteins that regulate secretion in many ways.[83,84] Mg^{2+} ions and $Mg^{2+}ATP$ are essential for the proper function of many of these enzymes, as well as a multitude of other cellular functions. Thus, intracellular uncaging of Ca^{2+} in the absence of normal quantities of Mg^{2+} and $Mg^{2+}ATP$ is less than ideal. Furthermore, dialyzing a cell with DM-nitrophen 85% loaded with Ca^{2+} caused the $[Ca^{2+}]$ to increase dramatically to suprathreshold levels for tens of seconds[85] because the "zero Mg^{2+}" solution of the patch pipet, upon whole-cell break-in, "hits a wall" of free Mg^{2+} inside the cell, which rapidly displaces measurable amounts of Ca^{2+} from the cage. Such loading transients could slightly perturb quiescent secretory machinery, so development of a photochemically and chemically efficient and cation selective photolabile Ca^{2+} chelator was considered highly desirable. Calculations suggest that under typical intracellular conditions (viz. 100 nM Ca^{2+}, 1 mM Mg^{2+}, 5 mM $Mg^{2+}ATP$, pH 7.2) DM-nitrophen would be 97% loaded with magnesium, thus making the AM-ester of DM-nitrophen difficult to use.[86] NP-EGTA was developed in 1994 as an efficient yet cation selective photolabile chelator to permit Ca^{2+} uncaging under normal physiological conditions.[87]

3.3.1. Development and Testing of NP-EGTA

NP-EGTA is a photolabile derivative of EGTA[87] (Figures 1 and 2). This caged Ca^{2+} probe was the second *ortho*-nitrophenyl derivative of EGTA synthesized. Unlike the first,[88] which had a low affinity for Ca^{2+} (ca. 25 μM), NP-EGTA has K_d = 80 nM (cf. EGTA 150 nM at pH 7.2). Clearly, the position of the chromophore is crucial for the integrity of the high-affinity Ca^{2+} coordination sphere. Exhaustive photolysis of NP-EGTA produces photoproducts with an average K_d of about 1.0 mM, a 12500-fold decrease (nitr-5 changes 54-fold). Ca^{2+} is released with a rate[89] of 68,000 s^{-1}, and with moderate photochemical efficiency ($\phi\epsilon$ = 224, cf. nitr-5 rate = 2,500, $\phi\epsilon$ = 66). Importantly, $[Mg^{2+}]$ of 1 mM has no effect on these physicochemical properties. Comparisons of the tension transients of chemical skinned rabbit skeletal muscle fibers evoked by liberation of Ca^{2+} from either NP-EGTA:Ca^{2+} (with $[Mg^{2+}]$ = 1 mM) or DM-nitrophen:Ca^{2+} (with $[Mg^{2+}]$ = 64 μM) were identical and rapid (half-times of ca. 40 ms). Since four Ca^{2+} ions must bind rapidly to troponin C for maximal tension, this initial test of NP-EGTA was satisfactory evidence of its viability as an efficient caged Ca^{2+} probe. Subsequent experiments with NP-EGTA have proved this to be the case. Photolysis of NP-EGTA has been used in studies of ion channel gating,[90–92] cell motility,[31,93] muscle contraction,[27,94–97] cell division,[98] and secretory cells[25,47,83,84] where physiological concentrations of Mg^{2+} are required. In particular is the last area of study that has seen the most extensive application of this calcium cage. The cage has also proved to uniquely useful for photorelease of Ca^{2+} in intact cells by loading with the AM ester technique.

3.3.2. Studies of Chromaffin Cells Using NP-EGTA

Knowledge of the basic mechanism of calcium driven secretion, derived from studies with DM-nitrophen, provided the foundation for the next level of analysis of the kinetics of the secretory machinery using photolysis of NP-EGTA in chromaffin cells and the calyx of Held.[51,77] Numerous recent studies in chromaffin cells[51,83,84,99–138,132,139–145] have been comprehensively reviewed recently.[83,84] Vesicles are pictured to exist in four pools: (1) readily releasable; (2) slowly releasable; (3) unprimed; and (4) depot (Figure 5). Increases in intracellular calcium concentration directly trigger vesicle fusion with the plasma membrane from the readily releasable pool (RRP), while the other three pools are upstream of, and feed into, this step. Ca^{2+} uncaging from NP-EGTA has revealed several proteins and biochemical reactions that modulate the flow of vesicles toward full fusion. For example, Rab3 regulates exit from the depot pool, whereas Munc13, Munc18, Syntaxin, Snapin, Complexin, and RIM seem to regulate priming. PKA, SNAP-25, and SNAP-23 control the stability of the RRP.[84] Such detailed molecule studies of secretion are tractable in chromaffin cells,

as overexpression techniques of target proteins in primary cultured cells are now feasible.[146] Some cells have been isolated from transgenic mice.[112]

3.3.3. Astrocyte−Neuron Interactions Studied with NP-EGTA

Though the whole-cell patch clamp technique revolutionized physiology, the approach is not without its downside. In fact, its very strength also turns out to be a serious weakness. Open contact with the cell's cytosol provides electrical and chemical control of the internal milieu, at the expense of washing out all of the soluble factors inside the cell. Using light to control and monitor cell chemistry is an attractive alternative to the patch clamp electrode, especially when Ca^{2+} is the object of inquiry. Caged Ca^{2+} probes can be loaded through the cell membrane, along with fluorescent Ca^{2+} dyes, using the AM ester technique.[37]

In contrast to cultured neurons, acutely isolated brain slices preserve much of the complex 3-dimensional architecture of the mammalian brain. In such intact tissue, each astrocyte has contact with over 100,000 synapses.[147] Thus, there is a growing consensus concerning the importance of the secretion of gliotransmitters (ATP and glutamate) in the modulation neurotransmission and the activity of neural circuits.[148] Astrocytes are also widely recognized to supply nutrients to neurons from the blood stream (so-called neurovascular coupling[149]). Photolysis of AM-ester loaded NP-EGTA in astrocytes has been used to provide unique insights into both these processes. Thus, photolytic release of Ca^{2+} while monitoring the coincident currents in adjacent patch-clamped pyramidal neurons revealed significant synchrony between elevations in astrocytic [Ca^{2+}] and neuronal currents.[150] These currents were blocked by NMDA receptor antagonists. Ca^{2+} uncaging in intact astrocytes in retina caused either dilation or constriction of adjacent arterioles, depending on the presence of two arachidonic acid metabolites (EET and 20-HETE).[151]

All the experiments discussed so far have used near-UV light sources for uncaging. Flash lamps were used for most of the studies in chromaffin cells, whereas shuttered CW Ar−Kr lasers or pulsed lasers (either frequency doubled ruby or frequency tripled YAG) were used in most of the studies on other cells. Over the past 10 years, mode-locked Ti: sapphire lasers have become commercially available, making nonlinear excitation techniques practical for biologists. These light sources have been widely used for imaging.[152] A few laboratories have also used 2-photon excitation for uncaging.

4. Two-Photon Uncaging of Ca^{2+} in Living Cells

In the 1920s, the English scientist Paul Dirac formulated the quantum mechanical description of the scattering and refraction of light.[153] He described scattering as a *two-photon* process involving the destruction and creation of a *pair of photons*, giving rise to what became known as the "Dirac dispersion theory". In 1931 Maria Gopert-Mayer used this theory to describe other two-photon processes, such as the simultaneous emission or absorption of two photons. Her Ph.D. thesis provided the basis of two-photon excitation spectroscopy and two-photon imaging as we know it today.[152] The latter technique is useful for modern neurobiology, as the IR light (700−1000 nm) used for 2-photon imaging is scattered less by brain tissue than the visible light used for confocal microscopy. The second significant advantage of 2-photon imaging over confocal microscopy is that the excited singlet state is only created in a small volume due to the nonlinear nature of the 2-photon absorption process. The minute size of the excitation volume (0.5−1 μm in x/y and 1−2 μm in z, at the diffraction limit) obviates the need for the pinhole that is required for confocal microscopy. When used for uncaging, 2-photon excitation can produce "focal puffs" of photoreleased signaling molecules, if the rate of release is much faster than the rate of diffusion of the excited stated out of the focal volume.[6,154]

Niggli and co-workers have two-photon uncaging of Ca^{2+} in acutely isolated cardiac myocytes to mimic the elemental events involved in Ca^{2+}-induced Ca^{2+}-release in these cells.[155–158] DM-nitrophen was used for all these experiments as the calcium cage and was dialyzed into individual myocytes via a patch pipet. These studies took advantage of the very high affinity of DM-nitrophen for Ca^{2+} (Table 1), since the chelator was loaded to 99% occupancy with Ca^{2+} without stimulating cellular responses. This situation permitted efficient, highly localized uncaging of Ca^{2+}, without rebinding of uncaged Ca^{2+} to unloaded, unphotolyzed chelator (Figure 4). In order to accomplish this, no Mg^{2+} was included in the dialysis solution. Since Mg^{2+} is essential for the long-term health of cells, as well as many regulatory processes, these experimental conditions are somewhat unsatisfactory and have stimulated the development of *Ca^{2+}-selective cages* that will undergo efficient two-excitation. Three such probes have been reported.[159–161]

The first of these to be made was azid-1, a photosensitive derivative of fura-2 designed initially for photocross-linking the Ca^{2+} dye to cellular proteins. Illumination of this probe preserves the BAPTA coordination sphere but not the fluorescence properties of the fura dye, as an amidoxime photoproduct is generated.[159] This new electron withdrawing substituent reduces the molecule's affinity for Ca^{2+} more efficiently than any of the nitr Ca^{2+} cages. Combined with its superior photochemical properties (Table 1), this makes azid-1 more (photo)chemically efficient than any nitr cage at releasing Ca^{2+}. The rate of Ca^{2+} release by azid-1 was not precisely measured but has a value of about 500 s^{-1}. DMNPE-4 was the second 2P calcium cage made.[160] This molecule attempted to combine the choicest properties of DM-nitrophen and NP-EGTA in one photosensitive chelator. This ideal was not completely achievable, as EGTA has a lower affinity for Ca^{2+} than EDTA (Table 1); nevertheless, DMNPE-4 releases Ca^{2+} rapidly and efficiently enough for focal 2P uncaging of Ca^{2+} in the presence 10 mM Mg^{2+} in living cells.[162] Finally, a new generic caging chromophore for 2P photolysis was introduced in 2006, called NDBF (nitrodibenzofuran). The Ca^{2+} cage made with this chromophore, NDBF-EGTA, is almost as photosensitive as azid-1 (Table 1) but is much more *chemically* efficient at releasing the caged Ca^{2+}, due to the large change in affinity upon irradiation. Also, NDBF-EGTA releases Ca^{2+} at a rate of 20,000 s^{-1}, making diffraction-limited 2P uncaging of Ca^{2+} feasible. Thus, two-photon photolysis in cardiac myocytes, loaded via a patch pipet, induced localized CICR.[161] No neuronal studies using 2P excitation of azid-1, DMNPE-4, or NDBF-EGTA have been reported. However, we have found that 2P photolysis of DMNPE-4, loaded into astrocytes in a living mouse using the AM ester technique, can produce cell-wide Ca^{2+} signals, as reported by fluo-4 fluorescence (Figure 6, G. Ellis-Davies, unpublished data). Similar reports using DM-nitrophen AM in brain slices have appeared,[163]

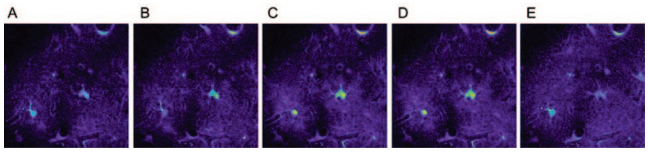

Figure 6. Example of 2-photon uncaging of Ca^{2+} in an astrocyte in a living mouse. DMNPE-4 and fluo-4 were coloaded as their AM esters into astrocytes in the cortex of the living anesthetized animal. Uncaging was accomplished using a mode-lock Ti:sapphire laser (at 720 nm), and imaging used a second laser (at 860 nm). The lasers were independently controlled by two sets of *x*/*y* galvanometers using a Prairie Technologies Ultima scan head. Uncaging was effected in the cell in the center of the image between frames A and B. Frames B, C, and D are three successive 3 s scans after uncaging, while frame E is 70 s after frame A. The increase in $[Ca^{2+}]$ detected by fluo-4 is represented in the pseudocolor display.

even though theory would predict[86] that inside intact cells DM-nitrophen will be mostly caged Mg^{2+}, due to the high $[Mg^{2+}]$.[80]

5. Summary: Comparison of the Chemical Properties of Ca^{2+} Cages

The properties of all the Ca^{2+} cages that have been used in living cells are summarized in Table 1. There is no "ideal" cage, as there are many properties of these photolabile chelators that must be considered: (1) chemical yield of Ca^{2+}; (2) photochemical yield; (3) effectiveness of light absorption (the extinction coefficient and 2-photon cross section); (4) rate of Ca^{2+} release; and (5) pH sensitivity of cation binding and cation binding selectivity.

From the point of view of chemical yield of Ca^{2+}, photolysis of DM-nitrophen is the most efficient cage, as this molecule has the highest affinity before photolysis and the lowest after. Thus, calculations suggest that DM-nitrophen may release an order of magnitude more Ca^{2+} than any other cage.[14] In practice, concentration jumps of as much as 600 μM free Ca^{2+} in living neurons have been reported.[61] After the extreme efficiency of DM-nitrophen, the other cages may be roughly grouped in two, according to the mode of affinity change (cf. Figures 1 and 3). Cutting the chelator backbone renders NP-EGTA, DMNPE-4, and NDBF-EGTA considerably more chemically efficient than nitr-X or azid-1.

Azid-1 is strikingly efficient photochemically, having a quantum yield of photolysis of unity. Such efficiency is especially attractive for a cage designed for 2P uncaging, as it has the potential to deliver large jumps in Ca^{2+} concentrations in the 2P focal volume that could mimic the Ca^{2+} microdomains arising from Ca^{2+} channel openning.[164] NDBF-EGTA is almost as photochemically efficient as azid-1, having a quantum yield of 0.7. NDBF-EGTA and azid-1 are about 5−10 times more photochemically efficient than the other EG(D)TA based cages and 70−100 times better than the nitr cages.

In terms of light absorption, azid-1 is, again, the best chromophore. Since it is based on fura-2, it has a large extinction coefficient, which is about 1.8 times larger than that of NDBF. The other cages use the traditional nitrobenzyl or dimethoxynitrobenzyl chromophore, which absorb light 5−34 times less efficiently than azid-1. For studies of typically small cells in the CNS such as pyramidal neurons or astrocytes, a large extinction coefficient is potentially useful. But for larger preparations, it is a potential problem. Since caged compounds are normally used for uniform, cellwide elevations in $[Ca^{2+}]$, a large path length of photolysis could lead to the generation of Ca^{2+} gradients due to inner filtering of the photolysis beam. The potential of 2P uncaging of Ca^{2+} in neurons has yet to be exploited, but recent experience with caged glutamate[154] suggests that the combined properties of the new Ca^{2+} cage NDBF-EGTA are highly desirable.

Ca^{2+} signaling at central synapses is very fast, having $[Ca^{2+}]$ jumps of less than 1 ms.[72] Thus, Ca^{2+} release rates of approximately an order magnitude faster than this rate are desirable, so that the speed of Ca^{2+} release is not rate limiting. DM-nitrophen, NP-EGTA, DMNPE-4, and NDBF-EGTA all satisfy this criterion easily, but nitr cages and azid-1 could be regarded as "on the edge" of the rate limit. This release is also important when one seeks to use 2P uncaging to mimic Ca^{2+} microdomains, as the half-time for diffusion of Ca^{2+} from the middle of the 2P focal volume is 0.15 ms.[165] Thus, slow Ca^{2+} uncaging would produce a "mist" of Ca^{2+}, rather than the desired sharp puncta.[6]

The selectivity of cation binding is clearly a vital property for any Ca^{2+} cage. All the cages described in this review start with chelators that effectively discriminate for group 2 alkaline earth metals over group 1 alkali metals. Since intracellular $[K^+ + Na^+]$ is >100 mM compared to 100 nM for Ca^{2+}, this selectivity is the *sine qua non* for Ca^{2+} cages and rules out the use of crown ether chelators. Since intracellular $[Mg^{2+}]$ is about 1 mM,[79,80] a Ca^{2+}/Mg^{2+} selectivity of 10^5 is required for a truly Ca^{2+} selective cage. For this reason, all but one of the cages in Table 1 use either EGTA or BAPTA chelators. Finally, the pH sensitivity of the affinity for Ca^{2+} has been touted as an important property for Ca^{2+} cages.[44] Thus, BAPTA-based probes should be preferred to EG(D)TA-based probes. However, it is evident that the proton binding of EG(D)TA-based probes has not prevented their wide application, so the importance of this final criterion seems only superficially important. Indeed, in the case of DM-nitrophen, this very "weakness" has been used to advantage.[166]

In summary, all the Ca^{2+} cages listed in Table 1 have been used effectively for many types of physiological experiments in neurons, astrocytes, etc. This is especially true of the three commercially available probes DM-nitrophen, nitr-5, and NP-EGTA. Even though these probes all have "flaws", each has been used successfully in carefully designed experiments, showing the importance of interdisciplinary collaboration between the realms of organic chemistry, photophysics, and physiology.

6. Note Added in Proof

I have filed a PCT patent application for uncaging using the NBDF chromophore.

7. Acknowledgments

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