Chapter 15

Flash photolysis of caged compounds

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1. Introduction

Photolabile 'caged' compounds are biologically inert precursors of active molecules, which when irradiated with a pulse of light free the active species at its site of action (see Fig. 1). Speed is the principal advantage offered by these probes; photochemical reactions can be very fast, with release of the active species often complete within a millisecond. The method can thus be used to study the kinetics and concentration dependence of reactions at sites that are not otherwise accessible. A number of processes can potentially distort the time course of responses to receptor ligands. These include diffusion of the ligand to the receptor, receptor desensitisation and breakdown and removal of the ligand by tissue enzymes, all of which can be substantial during the onset of a response when application is slow. These problems are by-passed with photolabile probes, which can be pre-equilibrated with the tissue in the inactive form, flash photolysis producing a pulse of agonist at a known concentration on demand.

Photolabile probes can be used to study intracellular pathways, overcoming the barrier formed by the cell membrane to the application of intracellular mediators or modulators. Access of the caged molecule can be by membrane permeabilisation, by microinjection or, most effectively, by perfusion from a patch pipette in whole-cell patch-clamp recording. It is important for quantitative studies that the intracellular concentration of the caged molecule is known. Depending on the size of the caged molecule, the cell and the patch pipette, it can take many minutes for exchange of the cell contents in whole-cell recording to reach equilibrium (Pusch & Neher, 1988).

Two further advantages offered by photolabile probes are the relative ease with which the intensity and the area of the light spot can be varied. By varying the intensity of the activating light, it is possible to vary the extent of photolysis and hence the concentration of the active species released at its site of action. Sufficient intensity for photolysis can be achieved in light spots large enough to encompass an intact tissue, for example a strip of muscle, or a population of cells. On the other hand, light can also be focussed to a small spot, allowing irradiation of a single cell or even part of a cell. Thus it should be possible to apply flash photolysis to study responses to

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localised release of important signalling molecules in specialised regions of the cell, and to compare this with the effects of release imposed throughout the cell.

Studies in the nineteen seventies and early eighties were limited by the availability of only a few 'caged' molecules, such as caged ATP and photoisomerisable, bisquaternary, cholinergic ligands (Lester & Nerbonne, 1982). Although photolabile cyclic nucleotide derivatives were introduced around the same time as caged ATP (Engels & Schlaeger, 1977), it was another six years before their photochemistry was exploited to rapidly activate a biological pathway (Nargeot *et al.*, 1983). Since then there has been an acceleration in the design and development of new caged compounds, with improved photochemical and biological properties, and this has given rise to a growing interest in flash photolysis techniques. A wide range of molecules, specifically designed for flash photolysis studies with biological preparations, is now available and more are under development (Adams & Tsien, 1993). These include photolabile precursors for intracellular second messengers, such as cyclic nucleotides, inositol trisphosphate and calcium, and modulators of intracellular pathways, such as ATP, GTP, GTPγS and caged calcium chelators. There are also caged neurotransmitters such as glutamate, GABA, noradrenaline and serotonin, caged local hormones such as nitric oxide and arachadonic acid, as well as other receptor ligands such as carbachol and phenylephrine. Table 1 lists some of the photochemical properties of probes that are currently available from commercial sources. Methods developed for many more probes have been described (see Walker, 1991). Molecular Probes sell a kit that may be used to synthesise caged phosphate esters and they also offer a custom synthesis service. The available caged compounds may be used for studies on a wide variety of biological systems.

2. The light induced reactions

Light acts as the trigger to initiate a series of reactions. The first step is the absorption by the caged compound of a photon of energy (E),

$$E = h\nu, \tag{1}$$

where h is Planck's constant and ν is the frequency of the light. As a result of absorbing the photon, the caged molecule (M) gains energy and is thereby promoted to an excited state (M^*). This step, which occurs on a time scale of < 1 ns, can be represented as:

$$M + h\nu \rightarrow M^*. \tag{2}$$

M^* is a new chemical species that is highly reactive. After absorbing the photon, M^* can either lose its extra energy and decay back to M or it can proceed to form stable products ($M^* \rightarrow$ products). The reactions leading to the formation of photoproducts are known as the dark reactions, because once M^* has been formed they will procede in the absence of light. The dark reactions are usually relatively slow and vary widely in time course, from microseconds to hundreds of milliseconds, and in efficiency.

Table 1. *Properties and sources of commercially available caged compounds*

Caged compound	Source	Structure	ε ($M^{-1}cm^{-1}\times10^{-3}$)	ϕ	release half time (ms)	*Useful concentration range
Intracellular probes						
ATP	C, M	NPE	17.5 at 260 nm	0.63	7	5-20 mM
	M	DMNPE	5.3 at 354 nm			
ADP	M	NPE	19.6 at 260 nm		8.7	1-20 mM
GTP	C	NPE	~16.9 at 255 nm			1-10 mM
GTP-γ-S	M	NB				1-10 mM
	M	DMNB	4.8 at 355 nm			
	M	NPE	15.4 at 260 nm	0.35	6	
	M	DMNPE	3.6 at 362 nm			
GDP-β-S	M	DMNPE	~16.9 at 260 nm			1-10 mM
cyclic AMP	C, M	NPE	20 at 259 nm			50-500 µM
	M	DMNB	4 at 350 nm		<5	
cyclic GMP	C, M	NPE				50-500 µM
Inositol 1,4,5-trisphosphate	C	NPE	4.2 at 200 nm	0.65	3	1-500 µM
Inorganic phosphate	M	NPE	4.2 at 260 nm	0.54	<0.1	up to 20 mM
Calcium:						
nitr-5	C	see text	see Table 2			0.2 - 10 mM
nitr-7	C					
DM-nitrophen	C					
calcium chelator:						
diazo-2	M					
Extracellular probes						
carbachol	C, M	NPE	5.2 at 262 nm	0.29	0.07	50 -100 µM
	M	CNB	5.2 at 266 nm	0.8	0.04	
adrenaline	M	DMNB				50-500 µM
noradrenaline	M	DMNBOC				50-500 µM
dopamine	M	DMNBOC				50-500 µM
isoprenaline	M	NB				50-500 µM
propranolol	M	NB				100 -500 µM
serotonin	M	DMNBOC				50-500 µM
glutamate	M	DMNB	5.7 at 347 nm			up to 20 mM
MK-801	M	DMNBOC				100-500 µM
aspartate	M	DMNB	6.0 at 347 nm			50-500 µM
GABA	M	DMNB	5.4 at 347 nm			50-500 µM
glycine	M	DMNB	5.7 at 345 nm			50-500 µM
arachidonic acid	M	DMNB				
nitric oxide	M,A, 1	$K_2Ru(NO)Cl_5$	0.56 at 320 nm	0.06	<5	0.5-50 µM

C: Calbiochem Novabiochem, 3 Heathcoat Building, Highfields Science Park, University Boulevard, Nottingham NG7 2QJ. M: Molecular Probes, Inc., 4849 Pitchford Avenue, Eugene, Oregon 97402-9144 USA. A: Alfa, Johnson Matthey, Orchard Road, Royston, Hertfordshire, SG8 5HE. 1: See *Biophys. J.* **64**, p. A190, Bettache *et al*. (1993).

NB: 2-nitrobenzyl. DMNB: 4,5-dimethoxy-2-nitrobenzyl. NPE: 1(2-nitrophenyl) ethyl. DMNPE: 4,5-dimethoxy-1(2-nitrophenyl) ethyl. CNB: α-carboxy-2-nitrobenzyl. DMNBO:C 4,5-dimethoxy-2-nitrobenzyloxycarbonyl

* Many of these compounds have not been widely tested. The concentration ranges suggested are based on experience with the better tested probes.

The mechanism of photolysis of caged ATP has been studied in detail and is reviewed in McCray & Trentham (1989). One of the stable products is the biologically active molecule. The other products should ideally be biologically inert.

Since only light that is absorbed by the caged molecule can trigger photochemical reactions, the higher the proportion of molecules absorbing the light, the more product will be formed. Absorbance (A) of a caged molecule in solution is determined by

$$A = \varepsilon cl \tag{3}$$

where ε and c are the extinction coefficient and concentration of the molecule respectively, and l is the length of the light path through the solution. Thus for efficient photolysis, ε should be high at wavelengths triggering the photochemical reactions. On the other hand, a high extinction coefficient can lead to poor and uneven photolysis in thick preparations, such as muscles of 100 μm or so diameter or the large neurones (> 200 μm) found in several molluscs, because the molecules at the front surface may absorb much of the light before it reaches the centre of the

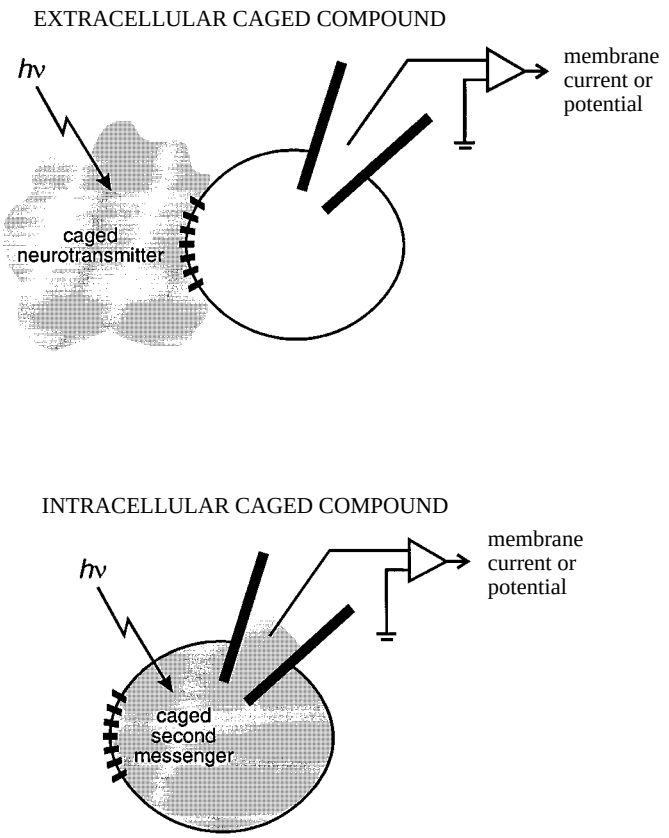


Fig. 1. Cartoon illustrating how flash photolysis of caged compounds can be used to activate biological pathways.

tissue. Photolysis of most of the currently available caged compounds requires wavelengths in the near-UV region of the spectrum, with a peak in efficiency at around 320-360 nm. Wavelengths below 300 nm cause damage to cells and are therefore filtered out of the activating light. Most caged compounds absorb little above 500 nm, so at the wavelengths of normal, incandescent room lighting, most caged compounds are fairly stable. This means that experiments do not need to be carried out in a dark room. It is good practice, however, to minimise exposure of caged compounds to light at all wavelengths. Experimental solutions should be prepared under dimmed light and be kept in light-tight containers or containers wrapped in foil. Most mammalian cells are sufficiently transparent in the near-UV region that they contribute little to the absorbance of the exciting light. Thus photolysis can be efficiently effected in isolated cells and in many thin tissue preparations. Some cells are pigmented, for example cell bodies found in the nervous system of the marine mollusc *Aplysia*. In this case the cells may contribute to absorbance and reduce the amount of effective light reaching the caged molecules (Tsien & Zucker, 1986; Nerbonne & Gurney, 1987). This can interfere with calibration of the amount of active molecule released from a probe, although, if the absorbance properties of the pigment are known, it can be allowed for in the calculations. The influence of cell absorbance and preparation thickness on the photolytic efficiency of a flash are discussed in detail in Landó & Zucker (1989), along with calculations of the volume-average photolysis of nitr-5 in an *Aplysia* neurone, where the cytoplasm has an absorbance coefficient (εc) of 25 cm^{-1} at 360 nm. By comparison rat cerebellar slices have an absorbance coefficient of 10 cm^{-1} at 320 nm, giving 26% attenuation of a flash over 300 μm (Khodakhah & Ogden, 1993).

Efficiency of photolysis is not only determined by ε. It is not sufficient that the caged compound absorbs light well, but each time a photon is absorbed it should be likely to result in formation of the photoproduct. A measure of the effectiveness of an absorbed photon is given by the quantum yield (φ), which is defined as

$$\varphi = \frac{\text{product molecules formed}}{\text{photons absorbed}} . \qquad (4)$$

Thus less light would be required to trigger the release of active molecules from caged compounds with a high extinction coefficient and a high quantum yield. The commercially available probes all have sufficiently high extinction coefficients and quantum yields to permit concentration changes in the physiological range to be produced with flashlamps or lasers.

The proportion of molecules photolysed by a single flash may also be influenced by the lifetime of M^* relative to the flash duration. With some caged molecules, a single flash produces a greater percent conversion than is predicted by the quantum yield. For example, caged cyclic nucleotides have very low quantum yields compared with caged ATP, but photolysis produced by a 1 ms flash is only two-fold greater with caged ATP (Wootton & Trentham, 1989). The discrepancy can be explained if the

excited intermediate formed by irradiating cyclic nucleotides is shorter lived than that formed from caged ATP, with the result that more excitations occur during the flash with the former compounds, thereby amplifying the response.

3. Structure and photochemistry

The currently available caged compounds have the general structure shown in Fig. 2A. Light sensitivity is conferred by the *o*-nitrobenzyl moiety and the simplest caged compound is the *o*-nitrobenzyl ester of the biologically active molecule. Photolysis occurs when irradiation cleaves the precursor at the benzyl carbon to release the active species. Released with the active molecule are a proton and a nitroso by-product, which is usually either an aldehyde or a ketone. These too could have biological effects that must be prevented or controlled as discussed below.

The photochemical properties of caged compounds are modified by varying the nature of the substituents at the benzyl carbon (R_1) and at positions R_2 and R_3 in Fig. 2A. For example, adding methoxy groups at positions R_2 and R_3 causes a red shift of the absorption maximum of caged compounds, thereby improving light absorption in the 300-360 nm range. The rate and efficiency of the photochemical reaction, as well as the biological activity of the precursor and the photolysis by-products, are also influenced. Unfortunately, there is no general rule that can predict how these properties will be affected, because they are also influenced by the nature of the molecule being caged. Thus methoxy groups at positions R_2 and R_3 improve the speed and efficiency of photorelease from caged cyclic nucleotides (Nerbonne, 1986), but slow release from caged ATP and caged phosphate (Wootton & Trentham, 1989). Similarly, a methyl group at R_1 accelerates photorelease from caged carbachol (Milburn et al, 1989) and caged ATP (Kaplan *et al.*, 1978) while making dimethoxy *o*-nitrobenzyl cyclic nucleotides unstable in aqueous solution (Wootton & Trentham, 1989).

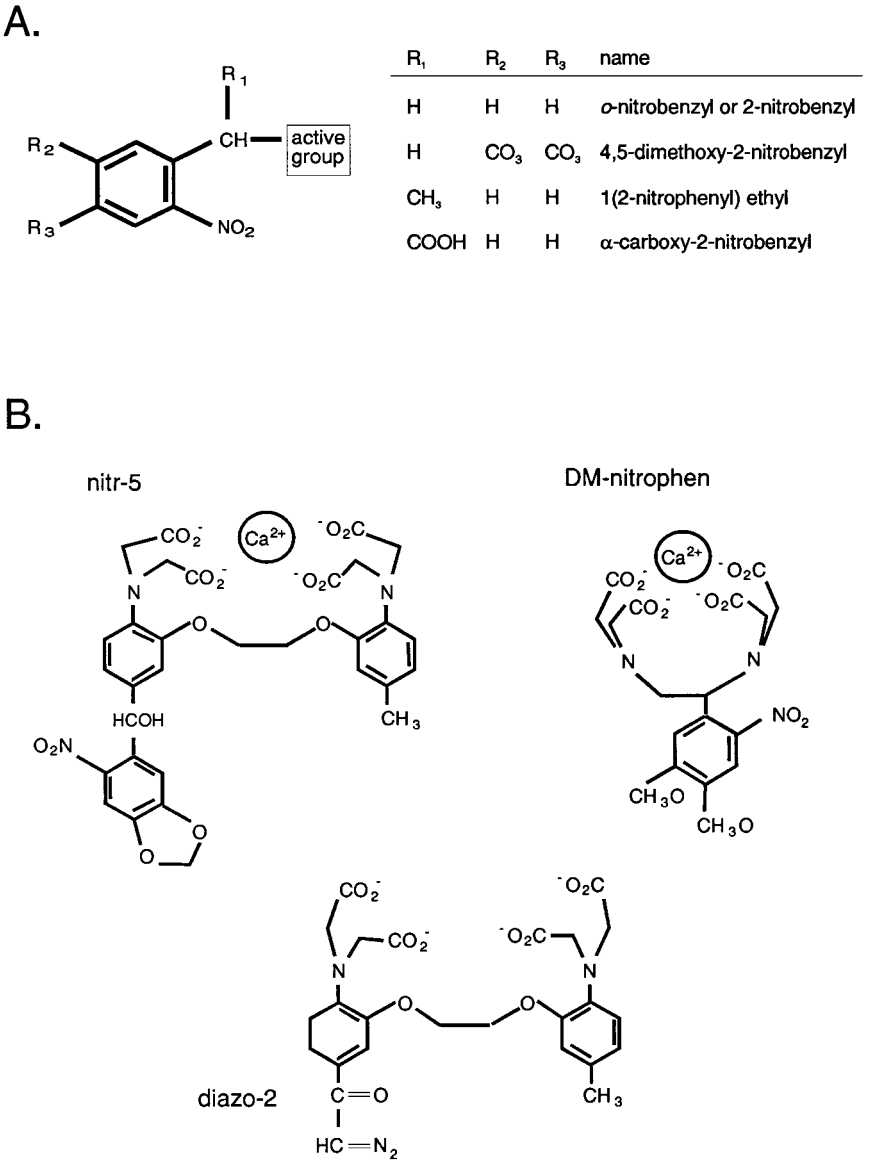
Photolabile cation chelators

Caged compounds can be used to control the concentration of cations inside or outside cells. There are several molecules available that can act either as caged calcium ions (nitr-5, DM-nitrophen) or caged calcium ion chelators (diazo-2). All of these agents incorporate an *o*-nitrobenzyl group (Fig. 2B), the photolysis of which alters the affinity of the molecule toward Ca^{2+}.

The nitr-5 family of compounds (Adams *et al*., 1988), including diazo-2 (Adams *et al.*, 1989), was designed around BAPTA, a Ca^{2+} chelator (K_D=110 nM) with high selectivity for Ca^{2+} over Mg^{2+}. This selectivity is retained in the photolabile probes, both before and after photolysis, and like BAPTA, their Ca^{2+} affinity shows little dependence on pH above pH7. Absorption of a photon by these compounds results in structural rearrangement to a form with altered Ca^{2+} affinity. In the case of nitr-5, photolysis induces a forty-fold loss of affinity for Ca^{2+}. The result is a net release of Ca^{2+} and H_2O is released as a by-product. Diazo-2 gains affinity for Ca^{2+} upon photolysis and therefore reduces free Ca^{2+}. This change is accompanied by the

release of N_2 gas. There is a structurally related molecule, diazo-3, which can provide a suitable control for experiments with the BAPTA-derived molecules. It has similar photochemical properties but has little affinity for Ca^{2+} before or after photolysis, so the photochemical reaction does not produce a change in Ca^{2+} concentration.

DM-nitrophen (Kaplan & Ellis-Davies, 1988) is derived from the chelator EDTA. It binds Ca^{2+} ions tightly (K_D = 5 nM), but also has a significant affinity for Mg^{2+} (K_D



R_1	R_2	R_3	name
H	H	H	*o*-nitrobenzyl or 2-nitrobenzyl
H	CO_3	CO_3	4,5-dimethoxy-2-nitrobenzyl
CH_3	H	H	1(2-nitrophenyl) ethyl
COOH	H	H	α-carboxy-2-nitrobenzyl

Fig. 2. (A) The general structure of caged compounds with a list of commonly used analogues, showing the variation in constituents at positions R_1, R_2 and R_3. (B) Structures of caged Ca^{2+} (nitr-5 and DM-nitrophen) and a caged Ca^{2+} chelator (diazo-2).

= 2.5 μM). When irradiated, the DM-nitrophen molecule is cleaved to yield two photoproducts, both with negligible affinity for Ca^{2+}. The change of affinity is large (K_D > 3 mM after photolysis), enabling millimolar changes in Ca^{2+} concentration to be produced.

DM-nitrophen and nitr-5 each have distinct advantages and disadvantages for use as Ca^{2+} donors. Nitr-5, but not DM-nitrophen at present, can be loaded into cells non-invasively using the membrane permeant acetoxymethyl ester form. Once this has entered cells, it is cleaved by intracellular enzymes to release free nitr-5, enabling responses to a rapid rise in intracellular Ca^{2+} to be studied in unperturbed cells. Both molecules release Ca^{2+} rapidly when irradiated, with submillisecond time constants. Following release, the free Ca^{2+} re-equilibrates with unphotolysed chelator. In the case of nitr-5, re-equilibration is rapid (around 2 μs) and faster than the rate of photolysis, whereas released Ca^{2+} re-equilibrates slowly with unphotolysed DM-nitrophen. The consequences of this are discussed in detail by Zucker (1993). Essentially it means that Ca^{2+} released by photolysis of nitr-5 reaches a steady level in an essentially step-like fashion, whereas partial photolysis of DM-nitrophen produces a pulse of Ca^{2+} that peaks in less than 1 ms, but then declines to a steady level over the next several milliseconds. Thus DM-nitrophen can be used to generate spikes of intracellular Ca^{2+}.

Nitr-5 and DM-nitrophen also differ in their affinities for Ca^{2+} and Mg^{2+}, the dependence of these affinities on pH, and the light-induced change in affinity. The low selectivity of DM-nitrophen for Ca^{2+} over Mg^{2+} means that at the millimolar concentrations of Mg^{2+} present inside cells, a significant fraction of DM-nitrophen would be present as the Mg^{2+} complex, and photolysis would release a mixture of Ca^{2+} and Mg^{2+}. Although this is a disadvantage for studying Ca^{2+} actions in intact cells, it means that DM-nitrophen can be exploited to selectively release Mg^{2+} (O'Rourke *et al.*, 1992). DM-nitrophen also has the disadvantage of a high pH sensitivity.

The favourable properties of DM-nitrophen, as compared with nitr-5, are its higher Ca^{2+} affinity and much greater change in affinity when irradiated. At resting cellular levels of Ca^{2+}, a greater proportion of DM-nitrophen would be bound to Ca^{2+} and larger changes in Ca^{2+} concentration could be produced by photolysis. The Ca^{2+} affinities of nitr-5 before (K_D = 145 nM) and after (K_D = 6 μM) photolysis are in the physiological range of Ca^{2+} concentrations, so nitr-5 will affect cell Ca^{2+} buffering.

Table 2. *Properties of commercially available photolabile Ca^{2+} chelators*

	K_D for Ca^{2+} binding (μM)				K_D for Mg^{2+} binding (mM)	
	pre photolysis	*post photolysis*	photolysis rate (s^{-1})	quantum yield	*pre photolysis*	*post photolysis*
nitr-5	0.145	6	4000	0.03-0.1	8.5	8
nitr-7	0.048	~2	556	~0.1	6	
DM-nitrophen	0.005	2000	3000	0.18	0.005	3
diazo-2	2.2	0.073	>2000			

In whole-cell, patch-clamp experiments, nitr-5 is usually the dominant Ca^{2+} buffer in the cell. Provided the extent of photolysis is known (see below), the free Ca^{2+} concentration before and after a flash can be calculated using the programme given in Gurney (1991). Landó and Zucker (1989) also discuss calculations of free Ca^{2+} produced in cells by photolysis of nitr-5, which incorporate the Ca^{2+} buffering capacity of cytoplasm.

Relevant properties of the photolabile Ca^{2+} probes are listed in Table 2 for comparison.

4. Sources of error in the use of caged compounds

The main sources of error in flash photolysis experiments with caged compounds are:

1. the unphotolysed caged compound itself has pharmacological activity in the tissue;
2. the by-products of photolysis are biologically active;
3. the flash itself triggers a response in the absence of a caged compound;
4. release of the active molecule limits the time course of the response.

Ideally, the caged compound and its photolysis products other than the one of biological interest should be inert, and flashes in the 300-400 nm wavelength range should cause no measurable response in the absence of the probe. This has usually been found to be the case, although it is important to test the effects of the 'caged' precursor, photolysis by-products and light in the biological system being studied.

Effects of the precursor

As already noted, nitr-5 can alter the Ca^{2+} buffering capacity of cells, both before and after photolysis, and this property is frequently exploited to control the intracellular Ca^{2+} concentration. Other caged compounds are also known to influence cellular activity prior to photolysis. For example, the nitrophenylethyl analogue of caged ATP was found to bind to the same site on the Na/K ATPase as free ATP, albeit with lower affinity (Forbush, 1984). It has also been found to bind to myosin (Dantzig *et al.*, 1989) and to cause partial blockade of ATP-sensitive K^+ channels in cardiac (Nichols et al, 1990) and smooth (Gurney, 1993) muscle. Nitrobenzyl acetate is a photolabile proton donor, which in some cells is cleaved by intracellular enzymes to lower pH without photolysis (Spray *et al.*, 1984). The nitrobenzyl ester of cyclic AMP spontaneously generates cyclic AMP inside cells in a similar way (Korth & Engels, 1979), whereas the dimethoxy nitrobenzyl ester, which for a number of reasons is the preferred analogue for photolysis experiments, is more stable (Nerbonne *et al.*, 1984). We have also found differences in the basal activity of the nitrophenylethyl and the dimethoxy nitrobenzyl derivatives of cyclic GMP in vascular muscle (Gurney, 1993). An early version of caged carbachol, the nitrophenylethyl derivative, was found to be active at both nicotinic and muscarinic acetylcholine receptors in the absence of photolysis (Walker *et al.*, 1986). This activity was subsequently eliminated by

introducing a carboxylate group into the benzyl carbon to make N-(α-carboxy-2-nitrobenzyl) carbachol (Milburn *et al.*, 1989). Three isomeric forms of caged IP3, resulting from esterification of each of the three phosphate groups, have been isolated and they each have different properties. The 4-phosphate ester is inactive before photolysis, but IP_3 caged on the 1-phosphate induces intracellular Ca^{2+} release in smooth muscle while the 5-phosphate ester inhibits IP_3 3-kinase (Walker *et al*., 1989). Commercial sources of caged IP_3 contain a mixture of the 4- and 5-phosphate esters.

Effects of the photolysis by-products

As indicated earlier, photolysis of most caged compounds results in the release of a proton and a nitroso by-product as well as the biologically active molecule. Changes in pH caused by proton release can be suppressed by strongly buffering the experimental solution. The nitroso by-products present a more difficult problem, since they are reactive towards sulphydryl groups on proteins. Reactivity can be reduced by derivatising the benzyl carbon (McCray et al, 1980) with, for example, a methyl group, as in the nitrophenyl ethyl derivatives ($R_1 = CH_3$ in Fig. 2A). Even so, the nitrosoacetophenone produced alongside photolysis of nitrophenylethyl ATP inactivates the Na/K ATPase of erythrocyte ghosts (Kaplan et al, 1978). Such effects can, however, be minimised by adding a hydrophilic thiol such as glutathione or dithiothreitol to the experimental solution, since these agents protect sulphhydryl and amino groups on cell proteins by inactivating the nitrosoketone photoproduct (Kaplan *et al.*, 1978). Fortunately, most receptor ligands and intracellular messengers work in the micromolar concentration range, so effective concentrations of the biologically interesting molecule can be produced with relatively small amounts of the by-product. The photoproducts are only likely to cause a problem when high concentrations of a caged compound are photolysed, for example, when millimolar concentrations of ATP are needed to activate muscle contraction.

Effects of the photolysis by-products are not always easy to control for. Parallel experiments could be performed using a structurally related caged compound that undergoes similar photochemical reactions with the same efficiency, but does not release the molecule of interest. For example, caged inorganic phosphate could be used as a control for caged ATP or GTP, as could diazo-3 be used as a control for diazo-2 or nitr-5. Another approach is to add excess of the biologically active molecule to the caged compound solution to pre-activate the pathway of interest prior to photolysis. Since under these conditions the same photoproducts will be generated, any remaining response to photolysis is probably due to the by-products of the photochemical reaction. These kinds of experiment would also control for biological effects of the intermediate, *M**, which, although it is highly reactive, is sufficiently short-lived to have minimal effect.

Direct effects of the activating light

The application of caged compounds is clearly complicated in tissues that show sensitivity to the activating light. There are only a few biological tissues, such as

photoreceptors, that are expected to respond to light at 300-400 nm. Thus in most tissues studied so far, the light pulses used to photolyse caged compounds have not been found to have effects unless the caged compound was present. Surprisingly, though, vascular smooth muscle is sensitive to near-UV light (Furchgott *et al.*, 1955), such that flashes of the intensity required to photolyse caged compounds can cause the muscle to relax and can alter the activity of membrane channels (Gurney, 1993). The relaxation results from light-induced activation of the cytosolic guanylyl cyclase (Karlsson *et al*., 1984; Wigilius *et al.*, 1990) and it can be blocked by inhibitors of the enzyme, such as haemoglobin. The relaxant effect of light is only observed when the muscle has been precontracted by exposure to a vasoconstrictor or elevated extracellular K^+ concentrations. In common with other agents that stimulate guanylyl cyclase in vascular muscle, light has no effect on basal tension, even although intracellular cyclic GMP concentrations would be raised. This highlights the need to test for light-induced effects thoroughly; it does not follow that because light does not appear to produce a response that it has not altered the biochemistry of the cell in a way that could interfere with the response of interest. Nitric oxide, an activator of the cytosolic guanylyl cyclase is increasingly being shown to have widespread biological effects (Moncada *et al.*, 1991), suggesting that this enzyme could be important in a number of tissues. Thus potential effects of light should be seriously considered.

The Xenon flashlamps used for photolysis produce a wide spectrum of light, including long wavelengths that could potentially heat the experimental preparation and induce a response. These wavelengths have rarely been found to cause problems, probably because there is usually a sufficient depth of fluid covering the preparation for these wavelengths to be absorbed and dissipated before they reach the preparation. These wavelengths can be filtered out of the activating light if they do cause a problem.

Slow photolysis

Although the potential speed offered by photolysis is one of the main reasons for developing caged compounds, it should not be assumed that photorelease is always fast. The rates of the photochemical reactions vary enormously among different caged compounds, and among different analogues of the same caged compound. For example, ATP is released from nitrophenylethyl ATP with a time constant of 12 ms, compared with 55 ms from the dimethoxy nitrophenylethyl analogue at 20°C and pH 7 (Wootton & Trentham, 1989). A new analogue of caged ATP, a benzoin ester, was recently described that photolyses more rapidly, with a time constant less than 10 μs (Trentham *et al.*, 1992). The currently available forms of caged glutamate (Corrie *et al.*, 1993) and caged phenylephrine (Walker & Trentham, 1988) photolyse slowly, with time constants of around 50 and 200 ms respectively, although promising new analogues that photolyse more rapidly are being developed (reviewed in Adams & Tsien, 1993). The slow release from these compounds is due to slow dark reactions, which appear to involve multiple steps. Such slow release may be acceptable for some experiments. However, when the principal interest is in the kinetics of a

biological process, then it is important to ensure that photorelease from the probe proceeds with sufficient speed that it will not limit the time course of the response.

5. Equipment

An intense light source, with output in the near UV, is the only specialised equipment needed for experiments with caged compounds. This usually takes the form of a laser or a xenon flashlamp, both of which can provide high intensity UV light concentrated into a brief pulse. Lasers commonly used include the frequency-doubled ruby laser, which produces an intense, 200 mJ pulse at 347 nm in 50 ns, and a cheaper, lower energy nitrogen laser (200 μJ at 337 nm), with which sufficient intensity can be achieved by focussing through a microscope objective. Lasers have the advantage of producing very brief (ns) pulses of monochromatic light at sufficient intensity to cause photolysis. Xenon flashlamps produce a broad spectrum, from 250 to 1500 nm, the pulse usually lasting around 1 ms. Flashlamps are less expensive than UV lasers, easier to maintain and are somewhat easier to incorporate into a microelectrode setup. They function by discharging a high-voltage capacitor across a short-arc flash tube that is filled with xenon gas. A 12 kV trigger pulse ionises the gas, which then emits light as it conducts. The intensity of the light emitted is determined by the energy discharged through the lamp upon ignition, i.e. the charge on the capacitor. This can be adjusted to vary the light intensity and extent of photolysis. The arc is generated between the two electrodes in the bulb, which are separated by a few mm. Thus the arc essentially forms a point source, the light from which is collected and focussed with quartz lenses as illustrated in Fig. 3A, or with an elliptical mirror (Fig. 3B). The overall energy output is about 3-fold higher when an elliptical mirror is used, although the light is focussed into a larger diameter spot (8-10 mm compared with ~3 mm). This configuration is well suited for use with larger preparations, for example intact muscle strips. The energy density is about 60-70 % of that produced using the more usual configuration with a short focal length (25 mm). Flashlamps designed for photolysis experiments can be obtained from Hi-Tech Scientific Ltd., Salisbury, the U.K. distributers for Dr. Rapp, Optoelektronik, Hamburg, Germany. See Rapp and Guth (1988) for a technical description of these lamps. A flashlamp is also available from Chadwick Helmuth, El Monte, California (Strobex model 238). However, it is not supplied with a lamp housing or focussing optics, which can be obtained from an optical supplies company.

Filters are placed in the light path to narrow the spectrum and remove wavelengths <300 nm. One that is often used is the Schott UG 11 bandpass filter, which shows peak transmission (~80%) at 320 nm, but also has a smaller transmission peak in the near infrared region of the spectrum, which can be a problem. For this reason, the Hoya U350 is sometimes preferred. After filtering, the total output of the lamp between 300 and 400 nm can be 200 mJ. The Rapp lamp photolyses 50% of caged ATP molecules when fired directly through a UG11 filter from 4 cm, equivalent to an

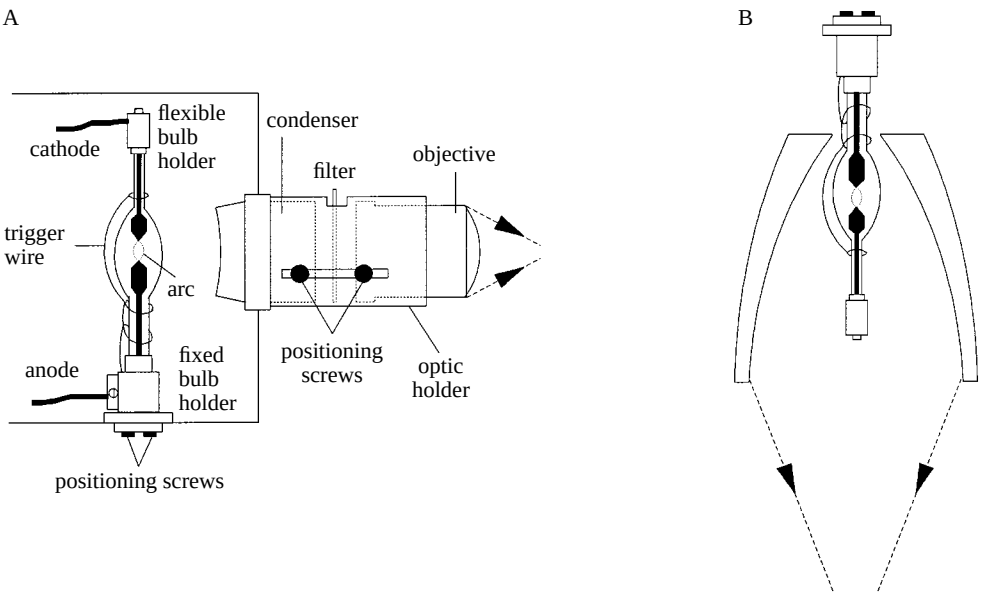


Fig. 3. (A) Diagram of the optical arrangement inside the lamp housing of a standard Rapp flashlamp, illustrating the use of a quartz condensor and objective to focus the light spot. From Gurney (1991). (B) An ellipsoid mirror arrangement to focus the light.

energy density of about 400 mJ cm^{-2}. In order to increase the intensity of effective light reaching a preparation, and hence the extent of photolysis caused by a single flash, we frequently use broader spectrum, cut-off filters that only remove wavelengths <300 nm.

Several problems are encountered when incorporating a flashlamp into an electrophysiology set up. One is the well documented photoelectric effect, which occurs when light hits a metalic surface, causing electrons to be ejected and current to flow. Thus any metal in the light path, such as AgCl electrodes, will introduce an artifactual current when the light is flashed. This can be prevented by covering the electrodes and keeping them out of the light path. Currents could also be introduced by stray light hitting electrodes and input connector pins, although we have never found this to be a problem. Bigger problems are presented by the discharge current (2,000 A) and the 12 kV pulse that triggers the flash. These can generate an enormous electrical artefact, which can saturate amplifiers and they are not easily shielded. The recharging of the capacitors is also apparent sometimes as a ripple in the current record immediately following a flash. In addition, there is an audible thumping noise when the lamp discharges, which is associated with movement of the lamp in its housing. This produces mechanical artefacts and can sometimes result in loss of microelectrode impalement or a membrane-pipette seal. To minimise these effects it is desirable to place the lamp as far away from the electrodes as possible, and preferably outside the Faraday cage. This can be done if the light is directed onto a preparation through a light guide (Fig. 4). Fibre optics do not transmit enough UV

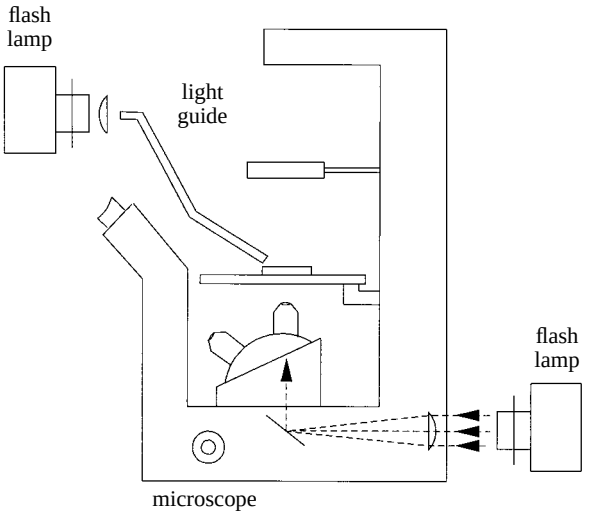


Fig. 4. Light can be focused directly onto a preparation, or directed through a liquid light guide or the optical path of a microscope. From Gurney (1993).

light to be useful, unless they are made of very expensive quartz. Liquid light guides transmit well from 270 to 720 nm, although the efficiency of transmission depends on the length and diameter of the light guide. With a 1 m guide, maximum transmittance is about 80%, but it falls off fairly steeply below 400 nm, such that at 300 nm only 40% is transmitted. The loss of light in this region is unacceptably high if the light guide is used in combination with a UG11 filter. We prefer to remove short wavelengths by placing a borosilicate glass coverslip, about 0.1 mm thick (no. 0 thickness; BDH, Poole, Dorset), at the input to the light guide. The coverslip cuts off sharply below 320 nm, but transmits greater than 90% at longer wavelengths.

The light from a flashlamp can also be directed onto cells *via* the optical path of a microscope, most easily through the epifluorescence port (Fig. 4). In theory this should be the optimal configuration because the light is focussed into an extremely small spot. It is important, however, to use a microscope that transmits well in the near UV; most microscopes do not. In addition the objective should have a high numerical aperture to let as much light through as possible. The simplest arrangement is to place the flashlamp condensor directly in front of the epifluorescence port and focus it using a 200 mm quartz lens onto a dichroic mirror, which is selected to reflect maximally below 500 nm. The dichroic mirror is placed in the light path below the objective, and set at a 45% angle so that short wavelengths are reflected up through the objective. With a Nikon fluor 40×, n.a. 0.85 lens in an Olympus IMT 2 microscope, we can photolyse >5% of caged ATP with a single flash. That particular microscope is limited by a glass lens in the light path, which we have not yet been able to replace. Using a similar microcope with improved optics should permit

greater photolysis. The main difficulty with this configuration is calibrating the amount of photolysis, because the irradiated spot is so small (~400 μm diam).

6. Calibration

For the photosensitive probes to provide useful biological information, it is important that the concentration change resulting from photolysis can be determined accurately. This is usually done by flashing a drop of caged compound solution and measuring the concentration of the active molecule generated or the amount of caged compound destroyed. I find this the hardest part of the experiment, because to ensure that all of the drop is irradiated during photolysis, it must be smaller than the light spot that is focussed on to it. It is a particular problem when the light flash is focussed through microscope optics. Since the droplets are very small they can evaporate quickly, particularly when flashed. To prevent this the droplets can be covered with a thin film of mineral oil. After flashing, the droplets are diluted to provide a sufficient volume for analysis. Depending on the properties of the caged compound and the released species, photolysis can be measured from absorbance changes of the solution or by high performance liquid chromatography (HPLC). The volume of the flashed droplets can be small. To measure photolysis through a microscope, we inject <0.2 μl of test solution into a blob of mineral oil and then retrieve it after photolysis. These volumes are difficult to measure accurately, so it is useful to add a marker molecule to the solution, whose concentration is known and does not change with photolysis. For example, for calibrating the photolysis of caged ATP the marker could be adenosine monophosphate, because it has a different retention time on HPLC to ATP and caged ATP.

The photolysis of a caged compound can also be monitored by measuring the pH change that accompanies the release of the biologically active molecule. This method is particularly well suited to calibrating photolysis in the small volumes of solution flashed through a microscope objective. Several indicators are available for the direct measurement of pH changes. Khodakhah & Ogden (1993) used the fluorescent indicator, BCECF (2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein), with micro-spectrofluorimetry, to calibrate the photolysis of caged ATP and IP_3. They measured 52% photolysis of caged ATP by a single, full-power flash (~100 mJ) at 300-350 nm in 1 ms. The pH change could alternatively be measured using the absorbance indicators phenol red or bromophenol blue. In theory, the changes in Ca^{2+} concentration produced by photolysis of caged calcium or caged Ca^{2+} chelators can be simultaneously measured with Ca^{2+} indicators, either in test droplets of solution or by incorporating the indicator into a cell along with the caged compound. Fluorescent Ca^{2+} indicators may be used to monitor changes in cell Ca^{2+}. However, their use is inadvisable when quantitative measurements of Ca^{2+} concentration are needed. Recent experiments have shown that the presence of caged Ca^{2+} compounds in a mixture with fluorescent Ca^{2+} indicators changes the fluorescent properties of the indicators (Zucker, 1992). The excitation spectra of the indicators fura-2 and fluo-3

were distorted by the presence of photolabile chelators, due to differential absorbance of the excitation light and Ca^{2+}-dependent fluorescence by the photolabile chelators. It may be possible to exploit the inherent fluorescence of nitr-5 to monitor the Ca^{2+} concentration changes produced by photolysis. The absorbance of caged Ca^{2+} molecules can also be monitored to calibrate their photolysis (Gurney, 1991), since the absorbance spectra change markedly upon photolysis.

It is not always necessary to calibrate different caged compounds separately. If the quantum yield of the caged compound is known, then caged ATP (φ=0.63) may be used as a 'standard' to calibrate its photolysis in a particular experimental setup. As noted earlier, the extent of photolysis produced by a flash depends on the number of excitations during the flash as well as φ. Nevertheless, in practice it is found that with most caged compounds, the amount of flash-induced photolysis relative to that of caged ATP is equal to the ratio of their quantum yields (Walker *et al.*, 1989). Caged cyclic nucleotides appear to be the exceptions (Wootton & Trentham, 1989).

7. Further reading

A number of reviews of caged compounds have appeared in the last few years, which cover their chemistry and applications (Nerbonne, 1986; Gurney & Lester, 1987; Kaplan & Somlyo, 1989; McCray & Trentham, 1989; Kao & Adams, 1992; Gurney, 1993; Adams & Tsien, 1993; Corrie & Trentham, 1993) including several chapters in the 1990 volume of Annual Review of Physiology. In addition there are a number of technical papers outlining the methodology (Gurney, 1991; 1993), including methods for synthesising caged compounds and measuring their photolysis (Walker, 1991; Walker *et al.*, 1989).

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