Flash Photolysis of Caged Compounds: Casting Light on Physiological Processes

Stefano Giovannardi, Luca Landò, and Antonio Peres

Photorelease of bioactive molecules from inactive precursors is a very powerful tool for the study of the molecular mechanisms underlying physiological processes as diverse as ionic channel modulation, exocytosis, phototransduction, ligand-receptor interaction, and cross-bridge activity. A brief account of the methodology, available compounds, and fields of application is presented here.

A caged compound is a biologically relevant molecule rendered inactive by a link to a chemical group (the “cage”) through a photolabile bond. Typically, the active molecule can be released by breaking the bond with a pulse of intense light in the near ultraviolet (UV; 350–360 nm) range. Special systems generating very brief and intense flashes are often used for this purpose, and thus the technique is also called “flash photolysis.”

The characteristics of this approach are advantageous because a particular intra- or extracellular region of the preparation can be preloaded with an exactly determined amount of substance in the inactive form; subsequently, it can be activated at a very precise time. In this way, delays...
due to diffusion of substances into the preparation and spatial and temporal inhomogeneities may be minimized. Therefore, kinetics studies of ligand-receptor interaction can be performed as well as characterization of Ca$^{2+}$-dependent currents or determination of Ca$^{2+}$ cooperativity in neurosecretion.

A number of other secondary, but often very useful, advantages may be offered by the use of caging molecules: in certain cases, for instance, the caging group protects the agonist against metabolic transformation before it reaches its target [inositol 1,4,5-trisphosphate (IP$_3$)]; in others, the cage renders the substance membrane permeable (adenosine 3',5'-cyclic monophosphate, luciferin). Finally, problems related to desensitization of the receptors are also avoided.

**Variety of caged compounds**

Initially, photorelease of caged compounds concerned primarily molecules normally produced or released in cells and tissues, such as ATP or neurotransmitters. Now, the number of endogenous compounds has been significantly increased: the neurotransmitter list includes glycine, γ-aminobutyric acid, glutamic and aspartic acids, serotonin, dopamine, epinephrine, and many others; second messengers include cyclic nucleotides, cyclic ADP-ribose, as well as arachidonic acid and photoactivatable nitric oxide donors; in addition to Ca$^{2+}$, the concentration of H$^+$ can be made to increase on photolysis. The variety of the commercially available compounds has also been greatly widened to include exogenous substances such as chelators, ionophores (both for divalent and monovalent cations), enzyme inhibitors or activators (serine-protease inhibitor, protein kinase C activator), fluorescent dyes, and others. Finally, a researcher can also create his own particular caged molecule using commercially available caging kits (6).

**Applications in physiology**

*Manipulation of intracellular Ca$^{2+}$.* Control of the cytosolic Ca$^{2+}$ level is of primary importance for the study of a wide variety of cellular events, from ion channel regulation to secretory vesicle fusion, muscle fiber contraction, and regulation of biochemical pathways. Researchers familiar with electrophysiological techniques used to keep low levels of intracellular Ca$^{2+}$ concentration by introducing rather high quantities of Ca$^{2+}$ chelating agents, such as ethylene glycol-bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA) or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N''-tetraacetic acid (BAPTA), in their whole cell pipettes. We may define this sort of method as a “passive approach” because the desired free Ca$^{2+}$ level, in this case, would stay constant in the presence of any sort of perturbation. In cells in which voltage-dependent Ca$^{2+}$ channels are present on the membrane, their controlled opening, caused by voltage-clamp steps, may be conveniently used to produce entry of Ca$^{2+}$ from the outside.

Use of caged compounds, active on the cellular Ca$^{2+}$ homeostasis, represents a much more versatile possibility of actively controlling the cytosolic Ca$^{2+}$ level.

Three categories of caged compounds have been used to control intracellular Ca$^{2+}$ in single cells: 1) photosensitive Ca$^{2+}$ chelators, such as DM-Nitrophen, nitrophenyl-EGTA (NP-EGTA), and diazo 2 (1, 5, 13); 2) calcium ionophores, derived from the well-known A-23187; and 3) second messengers, such as caged IP$_3$ and caged cyclic ADP-ribose.

DM-Nitrophen and NP-EGTA have structures deriving from EDTA and from EGTA, respectively (Fig. 1), with the addition of groups that influence the affinity of the molecule for Ca$^{2+}$. When the photolabile bond is broken, the structural change causes a decrease in the affinity of the chelator for Ca$^{2+}$ with consequent release of the ion. In the case of DM-Nitrophen, the dissociation constant for Ca$^{2+}$ increases from 5.0 × 10$^{-9}$ to 3.0 × 10$^{-3}$ M, whereas, for NP-EGTA, the increase is from 80 × 10$^{-9}$ to 1 × 10$^{-3}$ M. Diazoo
2 works in the opposite way: its affinity for Ca\textsuperscript{2+}, in fact, is relatively low (2.2 \times 10^{-6} M) before photolysis, and it increases by a factor of 30 after photolysis. This last compound may then be used to rapidly dampen high Ca\textsuperscript{2+} levels produced by other stimulants.

The efficacy of these kinds of substances can be monitored by measuring the cytosolic Ca\textsuperscript{2+} concentration. This cannot be done using fura 2 together with the caged compounds because fura 2 requires excitation wavelengths that would cause significant photolysis of the caged substance (typically 340 and 380 nm). It is therefore necessary to make use of different Ca\textsuperscript{2+} indicators whose excitation wavelength(s) is in the visible range. One of the most used indicators for this purpose has been fluo 3 (9). This substance has a maximum absorption at 490 nm and increases its 520-nm emission as a function of free Ca\textsuperscript{2+}; however, fluo 3 does not exhibit a spectral shift like fura 2, and this prevents ratiometric determinations. Figure 2 shows an example of Ca\textsuperscript{2+} changes induced by graded stimulation with caged IP\textsubscript{3}.

Recently, however, new long-wavelength Ca\textsuperscript{2+} indicators have been synthesized, such as fura red and calcium green (6), which allow use of a ratio procedure for the quantization of Ca\textsuperscript{2+} levels. These characteristics make them suitable for use in flash photolysis experiments.

**Ligand-receptor interactions.** Fast kinetic studies of ligand-receptor interaction are made possible by the instantaneous photolysis of the ligand. Often the evaluation of the rate constants of these reactions has been hampered by diffusion problems. These problems can be overcome by letting the inactive caged ligand diffuse as long as necessary to reach a uniform concentration in the proximity of the receptor; then the response induced by the instantaneous photolytic flash will more reliably reflect the kinetics of the transduction process. Rate constants in the order of 10^{-6} s have been evaluated in this way (10).

These advantages apply also to intracellular receptors, such as the ryanodine receptors, that can be activated by releasing Ca\textsuperscript{2+} from photosensitive chelators (7).

**Regulation of ionic channels.** Modulation of membrane ionic channels by cytosolic second messengers is another field for which photolysis is particularly well suited. The use of photosensitive Ca\textsuperscript{2+} chelators finds application for the study of the numerous Ca\textsuperscript{2+}-activated channels (8); IP\textsubscript{3} and cyclic nucleotide-gated channels may also be investigated in this way. Figure 3 shows an example of K\textsuperscript{+} channel activation after photorelease of Ca\textsuperscript{2+} from a caged chelator.

Photolysis can also be used to transform a normally active molecule into an inactive one. An example is given by the Ca\textsuperscript{2+} channel blocker nifedipine: in this case, the photolytic flash may relieve the inhibition of the Ca\textsuperscript{2+} channels, offering a new way of investigating the gating process (4).

**Secretion.** Ca\textsuperscript{2+} triggers exocytosis of secretory vesicles in both nerve endings and endocrine cells; manipulation of the Ca\textsuperscript{2+} concentration by photolysis of Ca\textsuperscript{2+} chelators allows the study of the secretory mechanisms with very high time resolution, especially if coupled with membrane capacitance measurements (12).

**Muscle contraction.** Muscle contraction and relaxation were among the first physiological processes studied by flash photolysis (3). For these kinds of studies, caged ATP is often used (15). Repeated flashes can be delivered to the same preparation, and the amount of released ATP can be calibrated against the energy of flash, allowing dose-response experiments. In this way, the shortening induced by hydrolysis of a single ATP molecule can be estimated (15). The steps of the relaxation process have also been investigated by either photolysis of caged ATP or photoactivation of the Ca\textsuperscript{2+} chelator diazo 2.

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**FIGURE 2.** Ca\textsuperscript{2+} responses induced by variable amounts of photoreleased inositol 1,4,5-trisphosphate (IP\textsubscript{3}). IP\textsubscript{3}-releasing flashes appear as interruptions in the fluorescent trace and are indicated by arrowheads. A: a barely detectable Ca\textsuperscript{2+} increase is caused by a 50-ms flash. B: doubling the flash duration makes the Ca\textsuperscript{2+} level slowly increase toward the generation of a Ca\textsuperscript{2+} spike. C and D: successive doubling of the flash duration elicits Ca\textsuperscript{2+} transients having a larger peak and a shorter lag. In D, a slowly declining plateau also appears, which is terminated by a fast descent to the basal level. [Reprinted from Ref. 11, with kind permission of Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.]
A UV laser is the best choice because it can generate a powerful and collimated light emission. The output beam can be brought to the excitation path of an epifluorescence microscope, allowing further focusing on the biological preparation. Coupling of such a system with a confocal microscope permits uncaging of the desired compound in a very small volume (“focal uncaging,” Ref. 14).

The main drawback of the UV laser is its cost; recently, however, a low-cost UV laser suitable for flash photolysis of caged compounds has been announced (2), which should render this technique affordable to many laboratories.

Conclusions

Photolysis of caged compounds may be combined with other optical and electrophysiological techniques to study a wide variety of physiological processes at the cellular level. In particular, whole cell patch-clamp and confocal microfluorometry represent techniques that can be profitably used to monitor cellular responses to phototactivation of the caged molecule.

The perspective of the availability of low-cost, high-efficiency light sources, together with the continuously increasing number of commercial compounds and the possibility of creating a personally tailored caged substance, would allow a large number of laboratories to utilize this technique.

With the help of this powerful technique, much progress will be gained in understanding the elementary events at the base of many physiological processes.

References