Caged Intracellular Messengers and the Inositol Phosphate Signaling Pathway

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1. Introduction

The development of caged intracellular messenger compounds offers an elegant new approach to quantitative, time-resolved studies of intracellular signaling pathways. Several reviews of caged compounds have recently appeared (Kaplan and Somlyo, 1989; McCray and Trentham, 1989; Homsher and Miller, 1990; Somlyo and Somlyo, 1990), which may be consulted for details of their synthesis, properties, and use. The present chapter concentrates on practical aspects of the use of caged compounds, derived from work in the author's laboratory using caged inositol trisphosphate to study signaling in *Xenopus* oocytes.

1.1. Utility of Caged Compounds for Studying Intracellular Messenger Systems

The receptor sites for inositol 1,4,5-trisphosphate ($InsP_3$) and other second messengers are intracellular. Thus, in order to study their actions, it is necessary to have a means of introducing these compounds into the cytoplasm. Ideally, this should permit a rapid, precise, and spatially homogeneous control of the intracellular concentration.

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Several approaches are currently in use for intracellular introduction of second messengers. One favored for biochemical studies is to permeabilize the cell membrane by various procedures, so that low-molecular-weight compounds applied in the external solution may gain access to the cell interior (*see* chapter by Taylor et al.).When used with small cells, this method gives good control of the intracellular concentration of applied second messenger and, with appropriate mixing devices, allows time resolution of the order of tens of milliseconds (e.g., Meyer and Stryer, 1990). Significant disadvantages include the possible loss of important constituents from the cytoplasm, the inability to localize messengers to particular parts of the cells, and disruption of the electrical properties of the plasma membrane.

For studies on intact single cells, a simple approach is to inject messenger compounds through an intracellular micropipet, by means of iontophoresis or pressure ejection (e.g., Sumikawa et al., 1989). This allows rapid (millisecond) applications and, with pressure injection, the total amount of compound injected may be measured. However, the resulting intracellular concentration is difficult to estimate and is likely to be spatially inhomogeneous since the compound diffuses away from the pipet tip. A related method of introducing messenger compounds is to allow them to diffuse into the cell from a whole-cell, patch-clamp recording pipet (e.g., Changya et al., 1989), which serves both for electrical recording and intracellular perfusion. Disadvantages of this technique are that the slow rate of access by diffusion from the pipet limits measurements of the time-course of responses, and metabolism of the intracellular messenger may raise further doubts as to its concentration (and even chemical identity) at the functional intracellular receptor sites.

The recent development and commercial availability of caged intracellular messenger compounds provide a new approach that circumvents many of the limitations inherent in the techniques described above. Caged compounds are inactive precursors of intracellular messenger that can be introduced into cells and allowed, at leisure, to diffuse throughout the cytoplasm. Illumination with near UV light is then used to liberate the active messenger compounds. Major advantages of this technique include the following:

1. Rapid (millisecond or shorter) jumps in intracellular concentration can be achieved;
2. The resulting intracellular concentration of second messenger can be precisely regulated; and
3. Manipulation of the photolysis light allows messenger molecules to be liberated uniformly throughout the cell or at any desired location.

A clear example illustrating the improvements available by using caged compounds as compared to previously available techniques is provided by experiments on hepatocytes perfused by a whole-cell pipet (Ogden et al., 1990). Inclusion of 10 μM InsP_3 in the pipet solution produced no consistent responses, whereas photorelease of a few hundred nanomoles of InsP_3 from caged InsP_3 evoked clear signals.

1.1.1. Properties of Caged Compounds

The term "caged" was coined by Kaplan et al. (1978) to describe ATP that was rendered inaccessible to the Na^+:K^+ pump by covalently linking its terminal phosphate to a photolabile nitrobenzyl group. Although this nomenclature should strictly refer only to clathrates, its use in the sense introduced by Kaplan et al. is now widespread and gives a clear idea of the principle involved.

A wide variety of caged compounds are presently available from suppliers, including Calbiochem (LaJolla, CA) and Molecular Probes (Eugene, OR), and the list will undoubtedly grow. Caged intracellular messengers include caged Ca^{2+} (Kaplan and Ellis-Davies, 1988; Adams et al., 1988), caged cAMP and cGMP (Karpen et al., 1988), caged GTP and GTPγS (Dolphin et al., 1988), and caged InsP_3 (Walker et al., 1987). Other caged compounds include caged neurotransmitters (Walker et al., 1986), caged protons (Janko and Reichert, 1987), and caged Ca^{2+} chelators (Kaplan, 1990). With the exception of caged Ca^{2+} compounds, all other caged intracellular messengers mentioned above are formed by esterifying a phosphate group with a photolabile nitrobenzyl group. Photolysis liberates the active messenger molecule, together with a proton and a nitrosoketone leaving group. A technically simple synthesis and purification technique for the preparation of these caged compounds has been described

(Walker et al., 1988,1989), and their structure and photochemistry have been reviewed (Gurney and Lester, 1987; Kaplan and Somlyo, 1989; McCray and Trentham, 1989). The kinetics of photorelease are still not fully understood, and depend on factors that include temperature, pH, and ionic strength (McCray and Trentham, 1989). Under physiological conditions, half-times for photolysis are of the order of several milliseconds (Homsher and Miller, 1990); e.g., 2.5–4 milliseconds for caged InsP_3 (Walker et al., 1989).

Two different families of caged Ca^{2+} compounds now exist. Both are based on the 2-nitrobenzyl chromophores, but otherwise show important differences in their properties (Adams et al., 1988; Kaplan and Ellis-Davies, 1988). Commercially available representatives of each type are nitr-5 and DM-nitrophen. The properties of these, and their relative advantages and disadvantages, are discussed by Kaplan (1990). Perhaps the most important differences between them are that DM-nitrophen shows a much larger change in affinity for Ca^{2+} following photolysis (from $5 \times 10^{-9}M$ to $3 \times 10^{-3}M$, as compared to $1.5 \times 10^{-7}M$ to $6.3 \times 10^{-6}M$ for nitr-5), whereas nitr-5 has a higher selectivity for Ca^{2+} over Mg^{2+}. Both show a very rapid ($3000\ s^{-1}$) rate of Ca^{2+} release on photolysis.

2. Methodology

2.1. Light Sources

Photolysis of caged InsP_3 and other 2-nitrobenzyl compounds requires high light intensities in the near UV region (300–400 nm). Suitable light sources, listed in order of both increasing energy and increasing cost and complexity, include:

1. Continuous arc lamp equipped with shutter;
2. Xenon flash-lamp; and
3. Lasers.

The characteristics and relative advantages of each are discussed below. McCray and Trentham (1989) have reviewed the use of flash-lamp and laser systems, so I cover these only briefly and, instead, concentrate on continuous arc systems.

2.1.1. Continuous Arc

A continuously burning arc lamp provides a simple and very stable source of UV light, with several advantages. The main drawbacks are that the available light energy is limited and, because of mechanical inertia in the shutter, it is not possible to obtain flash durations shorter than 1 or 2 milliseconds.

For use with caged InsP_3, the limited light energy is not a great problem, because of the extremely high sensitivity of cells to intracellular InsP_3. Thus, cells can be loaded with a fairly high (several micromolar) concentration of caged InsP_3, so that photolysis of only a small fraction will liberate sufficient InsP_3 to evoke large responses. However, the situation is very different when using caged Ca^{2+} compounds. Because these act as powerful Ca^{2+} chelators, it is desirable to load cells with the minimum amount required and to photolyze a large fraction of the caged Ca^{2+} with a single flash. In my experience, a continuous arc provides only just enough energy in flashes of 10-milliseconds duration to be able to work usefully with DM-nitrophen and is woefully ineffective with nitr-5.

Commercially available high-speed electric shutters allow reproducible flashes to be obtained with durations as short as 1–2 milliseconds. This time is not much longer than the flash duration obtained from a flash-lamp device (Rapp and Guth, 1988), but is very long compared to the pulses obtained from a laser. In practice, however, there seems to be little advantage in having flash durations much shorter than 1 milliseconds. The half-time for photogeneration of InsP_3 is several millseconds (Walker et al., 1989), and in several cell types, the latency to onset of InsP_3-mediated responses is tens or hundreds of millseconds (Parker and Miledi, 1989; Ogden et al., 1990; Meyer and Stryer, 1990).

An advantage of a continuous arc system not shared by other light sources is that the flash duration may be readily varied by using an electronic timing circuit to control the shutter. This provides a convenient and highly reproducible way of controlling the total energy in each flash, and a range of tenfold or more can be achieved between the briefest shutter opening and a flash duration, which becomes appreciable in comparison to the

onset latency of InsP_3-mediated responses. Another advantage is that paired flashes can be produced at any desired interval (limited only by the operating time of the shutter). The duration of each flash can be independently controlled, and the light intensity is stable for each flash. Furthermore, indefinitely long light exposures may be used to cause a sustained photorelease of InsP_3 and, thus, mimic more closely the situation that is likely to pertain during agonist activation (Parker and Miledi, 1989). For this application, the light output from the arc lamp is usually too intense, but can be attenuated by neutral density filters.

Regarding the choice of arc lamp, a mercury arc is expected to be preferable, since this has an intense spectral line at about 360 nm. However, in practice there seems to be little difference between mercury and Xenon lamps, possibly because the Xenon arc forms a very small (<0.5 mm) intense plasma ball that can be more efficiently focused onto small preparations. There is little point in using an arc lamp of higher power than 50 or 75 W. Although higher power lamps provide a greater total output, the size of the arc is larger. Thus, if the light is to be focused onto a small target (as is the case in most applications of caged compounds), the irradiance is no greater, and may even be less, with a higher power lamp. Quartz-halogen lamps produce very little output in the UV and are of no use for photolysis experiments. This does mean, however, that a conventional fiberoptic illuminator can be used without further filtering to view the preparation without causing appreciable photolysis.

The stability of light output from an arc lamp can be very good, provided that it is operated from a well-regulated, constant-current power supply and that adequate time is allowed for warm-up. A flash-to-flash repeatability of better than 1% can be achieved, taking into account both variations in lamp output and shutter timing *(see* Section 3.2.*)*. An important point for stability is that the lamp be mounted vertically. It is possible to operate Xenon arc lamps horizontally, but convection currents then cause the arc to "wander," producing large and erratic changes in intensity of the focused beam.

2.1.2. Flash Lamps

Xenon arc lamp systems give a high energy output in a flash duration of 1 ms or shorter. A system designed for use with caged compounds is commercially available (Rapp and Guth, 1988), and produces 100–200 mJ at wavelengths between 300 and 400 nm when focused onto an area of 10 mm^2. The output of this flash-lamp during a 1-ms flash appears to be comparable to the output of a continuous mercury arc for 1 s (McCray and Trentham, 1989). Thus, the flash-lamp is much to be preferred for rapid kinetics studies in which a high proportion of a caged messenger must be rapidly photolyzed. However, the flash-to-flash stability is unlikely to be as good as with a continuous arc system, and independent control of each flash in paired-pulse experiments is difficult.

2.1.3. Lasers

Several types of laser are available that produce high-energy pulses at near UV wavelengths. The main advantages of pulsed lasers are that the parallel output beam can be readily directed and focused onto the specimen, the output is monochromatic, and the pulse duration is very short (generally tens of nanoseconds). Disadvantages include high cost, large size, and in some cases, the need for frequent maintenance and alignment.

A frequency-doubled ruby laser has been used in several studies on muscle (Goldman, 1986). This gives an output pulse at 347 nm with energies up to 300 mJ. However, a major drawback for some applications is that the maximum repetition rate is 1–0.1 Hz, thus effectively precluding paired-pulse experiments. Another alternative is the frequency-tripled neodymium-YAG laser, which gives pulses with energies of over 100 mJ at 355 nm and repetition rates up to 50 Hz. Finally, nitrogen lasers giving an output at 337 nm are much cheaper than the others, but the available pulse energies are only a few milliJoules.

For most purposes, the higher cost of lasers over flash-lamp systems is probably not justified. The total energy outputs are comparable and, as discussed above, the shorter pulse duration of lasers is of little practical benefit. Monochromicity of the laser

output also offers little advantage, except that it may simplify rejection of flash artifacts when simultaneous measurements are made in another region of the spectrum (e.g., use of fluorescent probes to monitor free [Ca^{2+}]).

2.1.4. Two-Photon Excitation

All of the above-described light sources (lamps and lasers) allow photorelease to be spatially restricted by means of appropriate apertures in the light path. However, although the illumination can be controlled in the horizontal plane, it is not possible to obtain a controlled release of messenger throughout a "slice" at some particular depth into the cell. A recent development (Denk et al., 1990) using a colliding-pulse, mode-locked dye laser now offers the possibility of obtaining focal release of messengers at any desired x, y, and z coordinates.

The method involves focusing extremely brief (femtosecond) pulses of light through an objective lens of high numerical aperture. The wavelength of the laser is about double that normally required to cause photolysis, so that photorelease requires the simultaneous absorption of two photons by a caged molecule to combine their energy in order to reach the excited state. Thus, the extent of photorelease varies as the square of the light intensity, rather than being linear, as is the usual case. Because of the highly convergent cone of light formed by a lens with large numerical aperture, appreciable photorelease will occur only in a very restricted region around the beam waist. This will give rise to an effective point source release of messenger but, by rapidly scanning the photolysis spot, it may be possible to obtain near-simultaneous release throughout a thin section of the cell.

2.2. Optics

Several different approaches may be taken to collect the UV radiation from the light source and direct this onto the target cell. Factors to consider when choosing between these include the desired energy density at the specimen, and whether the light needs to be tightly focused (for example, to illuminate only a single cell or part of a cell) or whether it should diffusely cover a broad (several millimeters) area.

With a flash-lamp source, the simplest approach is to collect the light using a condenser lens or ellipsoidal mirror, pass this through a UV-transmitting filter, and place the target at the focus (Ogden et al., 1990). Inclining the light beam to an angle of 38° to the horizontal minimizes energy loss resulting from reflections at the fluid surface above the specimen. Advantages of this method are simplicity, a long working distance, and a high efficiency in collection of available light. Probably the main disadvantage is that the focused spot is fairly large (a few millimeters in diameter), so that the energy density is relatively low and there is little possibility to restrict the illuminated area spatially. Use of an ellipsoidal reflector for focusing gives very high (up to 80%) efficiency of light collection from the flash-lamp, and since additional focusing optics are not needed, there is no problem with glass optics absorbing at short wavelengths. In contrast, a condenser system provides a smaller spot size, at the expense of less efficient light collection (20% at best) and, unless expensive quartz lenses are used, a cutoff at wavelengths shorter than about 340 nm. Rapp and Guth (1988) compared the use of mirror and condenser optics, and found that a mirror gave a slightly higher energy density over a larger focus size.

A similar condenser or mirror system could, in principle, be used also with a shuttered continuous arc system. A difficulty, however, is that fast-acting shutters necessarily have small (1 cm or less) open apertures. Thus, the shutter needs to be mounted inconveniently close to the preparation in order not to obscure a large part of the light beam. Better approaches are to place the shutter at the focus of the condenser system, and then couple the light to the preparation through a fiberoptic or epifluorescence system.

One problem in focusing a flash-lamp or continuous arc directly onto the target is that the light source must be mounted fairly close to the preparation. This may be inconvenient because of the physical size and heat output of the system, and because the high-voltage ignition pulse introduces electrical artifacts into an associated electrophysiological recording system. One way to obviate these problems is to mount the lamp remotely (outside the screened recording "cage"), and to couple it to the setup through a fiberoptic light guide. Both high-grade fused silica and

liquid light guides have good transmission in the UV, but the liquid light guide is probably to be preferred as having the better transmission in the near UV and a larger acceptance angle. At a wavelength of 350 nm, the total transmittance of a 1-m length of liquid light guide is about 50%.

Unlike other light sources, lasers produce a narrow, highly parallel output beam. This may simply be directed onto the preparation without any intervening optics. On the other hand, the highly collimated beam is ideal for focusing down to small spot sizes, to give a higher energy density, or to give spatially restricted stimulation.

The best approach to obtaining very small illuminated spot sizes is probably to modify an upright or inverted fluorescence microscope. The regular fluorescence illuminator is replaced by a flash-lamp or laser, or in the case of a shuttered continuous arc, it is simply necessary to mount a shutter in front of the existing arc lamp. For safety, it should not be possible to view the photolysis light flash through the eyepieces! By using objectives of different powers, the photolysis light can be focused to cover an area as great as 1 mm^2 or as small as a few micrometers.

2.3. Introduction of Caged Compounds into Cells

The majority of caged compounds (including caged InsP_3) are charged molecules and are thus impermeant through the cell membrane. For work with single cells, they may be introduced into the cytoplasm by injection through a micropipet or by diffusion from a whole-cell patch pipet. In the latter case, the resulting intracellular concentration should approximate that in the pipet, whereas the concentration following injection can be estimated from the amount of fluid expelled and the cell volume.

Clearly, mechanical injection of caged compounds will be impractical for studies on populations of cells and may also be problematic with especially large cells (e.g., skeletal muscle fibers), in which long periods of time would be required for the compound to equilibrate throughout the cell. A recent improvement in the application of caged compounds in such situations has been their introduction into cells permeabilized

with staphylococcal α-toxin or saponin ester β–escin (Ahnert-Hilger et al., 1989; *see* chapter by Taylor et al.). Cells exposed to these agents become permeable to solutes with mol wt of about <1000 dalton and 17,000 dalton, respectively. Thus, caged compounds (e.g., caged $InsP_3$; mol wt 635 daltons) can be introduced while minimizing the loss of higher molecular-weight cytoplasmic constituents.

2.4. Artifacts

Possible artifacts in the use of caged compounds might arise from several mechanisms, including the device used to generate the photolysis light, the effect of light on the preparation, and actions of the caged compound or byproducts of the photolysis reaction on cell metabolism. So far, there appear to be no indications that any of these present major problems.

Potential difficulties with flash-lamp and laser systems include the high voltage pulse associated with their triggering. They may need careful electrical screening to avoid interference with sensitive electrophysiological recordings. Also, the chlorided silver wires commonly used to make contact with recording electrodes are photosensitive and may need to be shielded from the light. Regarding effects of light on the preparation, artifacts may arise through heating (especially if infrared radiation is not well blocked from arc and flash-lamp systems; Parker, 1989a), or from actions of the UV light on proteins and other cell constituents. A simple control for all of these is to illuminate the preparation before loading the caged compound. Effects of the caged compounds themselves may be checked by looking for responses evoked by their introduction into the cell and by testing that agonist-evoked responses (mediated by the second messenger system under study) are not altered following intracellular loading.

The byproducts of the photolysis reaction are 2-nitrosoacetophenone and a proton. Nitrosoketones react with thiols, including cysteines in proteins, and might thus perturb the cell. During experiments using caged ATP in muscle fibers, Goldman et al. (1984) observed a desensitization in contraction that appeared to arise through the formation of 2-nitrosoacetophenone.

Inclusion of reduced glutathione (GSH) in the intracellular solution gave protection from this effect. A good control for actions of photolytic byproducts is to load cells with a caged inactive analog of the messenger being studied. For example, caged $InsP_2$ has been used as a control in experiments with caged $InsP_3$ (Ogden et al., 1990); its photolysis produced no response and did not prevent subsequent agonist activation.

2.5. Flash Photolysis and Calcium Monitoring

One approach to monitoring the rise in intracellular free Ca^{2+} evoked by photorelease of $InsP_3$ is to record some Ca^{2+}-mediated process in the cell, such as the opening of Ca^{2+}-dependent membrane channels. However, interpretation is complicated by factors including possible cooperativity of Ca^{2+} in opening the channels, desensitization, and spatial variations in channel density. Thus, it is desirable to have a more direct measure of intracellular free Ca^{2+}. Until recently this was problematic, because the commonly used fluorescent indicators quin-2, fura-2, and indo-1 all require excitation at wavelengths in the near UV (Grynkiewicz et al., 1985). Measurement will, therefore, necessarily cause photolysis of caged $InsP_3$, although this can be minimized by using low-intensity fluorescence excitation (Gray et al., 1988). The introduction of new fluorescent Ca^{2+} indicators with excitation wavelengths in the visible spectrum (Minta et al., 1989) completely obviates this difficulty, and they have sucessfully been used in conjunction with photochemical generation of intracellular messengers (Kao et al., 1989; Parker and Ivorra, 1990a,b; Ivorra and Parker, 1990a).

Two long-wavelength indicators are currently available, fluo-3 and rhod-2. Their respective excitation and emission maxima are 506 nm and 526 nm for fluo-3, and 553 nm and 576 nm for rhod-2. Unlike the short-wavelength indicators fura-2 and indo-1, both fluo-3 and rhod-2 show only a fluorescence increase on binding Ca^{2+}, with no shift in either excitation or emission spectra. Thus, it is not possible to ratio signals at two wavelengths in order to obtain a calibration of free Ca^{2+} levels that is independent of variations in dye loading and path length. However, for many purposes, an uncalibrated index of intra-

cellular Ca^{2+} transients is likely to be sufficient, and with some difficulty and uncertainty, it may be possible to calibrate the fluorescence by lysing the cell and titrating the Ca^{2+} level in the bathing solution. Dissociation constants of fluo-3 and rhod-2 for Ca^{2+} are 0.4 μM and 1 μM, respectively (Minta et al., 1989), so that both are able to resolve relatively high [Ca^{2+}] that would saturate fura-2.

The visible excitation maxima of fluo-3 and rhod-2 allows the use of a quartz-halogen lamp as an efficient excitation source. Also, the spectra of the dyes match well to the usual fluorescein and rhodamine filter sets available for fluorescence microscopes. A quartz-halogen lamp has the advantages of low cost and, provided that it is operated from a well-stabilized DC power supply, a highly stable light output. For measurements on small cells where the excitation light must be tightly focused, the smaller source size of an arc lamp allows a higher energy density at the specimen, and the mercury arc has an intense peak at about 540 nm that matches well to the excitation maximum of rhod-2. Other possible light sources for fluo-3 include the 488-nm line of an argon ion laser and, for rhod-2, the 543-nm emission of the inexpensive green He-Ne laser (Parker and Ivorra, 1990b).

The green emission from fluo-3 is close to optimal for detection by photomultiplier tubes. However, most photomultipliers have a lower quantum efficiency in the red and will thus show less sensitivity for rhod-2. Photodiodes are much less sensitive than photomultipliers, but their performance improves at longer wavelengths, so that they might be used as an inexpensive detector with cells brightly stained with rhod-2.

Because of the wide separation in wavelengths optimal for photolysis of caged compounds and excitation of fluo-3 or rhod-2, it is possible to obtain good rejection of the photolysis flash in the fluorescence record. If a nonmonochromatic light source (i.e., flash-lamp or continuous arc) is used for photolysis, its output must be filtered to attenuate visible wavelengths. A Schott UG11 filter gives high transmission in the near UV (70% at 350 nm), together with strong (>10^5) attenuation at wavelengths longer than about 400 nm. Alternatively, a UG5 filter offers higher

transmission in the UV over a broader range of wavelengths, but is unsuitable for use with fluo-3 since a "shoulder" in the transmission spectrum extends beyond 500 nm. A point to remember with both filters is that they show a transmission at wavelengths longer than about 700 nm. This needs to be blocked by a separate filter or dichroic mirror if the barrier filter shows appreciable transmission at these wavelengths.

3. Application of Caged Compounds to Study InsP_3 Signaling in Oocytes

Oocytes of *Xenopus laevis* possess a phosphoinositide signaling system by which the activation of cell surface receptors leads to the generation of an oscillatory Cl^- membrane current. InsP_3, which is formed in response to receptor activation, acts as an intracellular messenger to release Ca^{2+} from intracellular stores (Oron et al., 1985; Parker and Miledi, 1986) and to activate an influx of extracellular Ca^{2+} (Parker and Miledi, 1987). The resulting rise in cytoplasmic free Ca^{2+}, in turn, activates Ca^{2+}-dependent Cl^- channels to give the final membrane current response. For the purposes of studying this messenger pathway, the oocyte is a convenient model cell system, since its large size (>1 mm diameter) greatly facilitates such procedures as intracellular recording and microinjection.

The following sections describe equipment and procedures developed in the author's laboratory, together with results that illustrate some of the applications of caged compounds. The methods should be generally applicable also to cells much smaller than the oocyte, with the exception that different techniques may be required for intracellular recording, and for loading of caged compounds and indicators.

3.1. Optical System

Figure 1 shows the optical system employed to allow photolysis of caged InsP_3 or caged Ca^{2+}, together with recording of Ca^{2+}-dependent changes in fluorescence of long-wavelength indicators. The system is based on a Zeiss upright microscope, using two epifluorescence illuminators stacked one above the

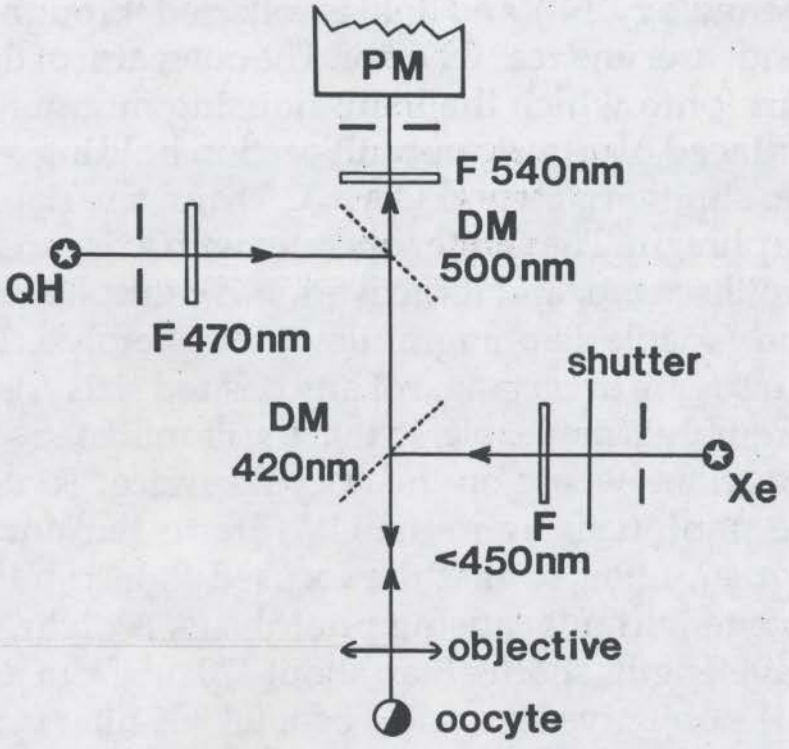

Fig. 1. Diagram of optical system for photolysis of caged compounds and fluorescence monitoring of intracellular Ca^{2+}. PM = photomultiplier, QH = quartz-halogen lamp (100 W), Xe = xenon arc lamp (75 W), DM = dichroic mirror, F = filter. *See text* for further details.

other. The lower illuminator provides precisely controlled flashes of UV light for photolysis, whereas the other provides excitation light for the Ca^{2+} indicator. To allow the two epifluorescence units to be physically mounted together and to improve the optical performance, the diverging and converging Telan lenses are removed from the upper and lower units, respectively. Further, the diverging Telan lens on the lower unit is replaced by a fused silica lens, since this element otherwise limits the short-wavelength cutoff for the entire optical path. A 6.3× Neofluar objective is usually fitted to the microscope. This has quite a good UV transmission and a long working distance to facilitate the insertion of micropipets. It also gives a field of view sufficient to visualize the entire oocyte.

The light source for photolysis is a continuous 75-W Xenon arc lamp mounted in a 100-W Zeiss lamp housing. The arc is operated from a constant-current DC power supply (Photon

Technology, NJ), and light is collected through a three-lens glass condenser and rear reflector. The outer arm of the epifluorescence unit (onto which the lamp housing mounts) is removed and replaced by a custom-built section holding a high-speed electric shutter (Newport Corp, CA) and a variable rectangular slit diaphragm. The shutter operates with a rise and fall time of about 1 millisecond, and its activation is controlled by a digital timer. The variable diaphragm allows the photolysis light to be focused as a square or rectangle of any desired size. Also, the diaphragm assembly is moveable, so that the illuminated square can be positioned anywhere on the oocyte surface. To assist in adjusting the photolysis light, the UV filter is temporarily replaced by a green filter, so that the focused light can be viewed on the oocyte without causing photolysis. A dichroic mirror reflects wavelengths shorter than about 420 nm from the photolysis light to the objective lens, and a Schott UG5 filter further restricts illumination to UV wavelengths. Because the dichroic mirror is transparent to visible wavelengths, it does not attenuate the fluorescence excitation light directed down from the upper epifluorescence unit or the emitted light collected by the objective. Neutral density filters are used to vary the intensity of the photolysis light, though, in most experiments, it is more convenient to keep the intensity constant and vary the extent of photolysis by altering the flash duration. Some control of light intensity is also possible by varying the arc-lamp current, but the available range is restricted, because at currents less than about one-half of the rated maximum, the arc becomes unstable. It is also possible to double the lamp output briefly (a few seconds) by overrunning it at 10 A. This is entirely in contradiction to the manufacturer's warnings, but so far, has not provoked any lamps to explode.

When operated at normal lamp current, the energy density available at the cell is about 10 $\mu J\ mm^{-2}$ from a 2-milliseconds flash (at wavelengths of about 340–400 nm). The illuminated area (using a 6× microscope objective) can be varied from 0 to a maximum of about 0.5 mm^2.

Fluorescence excitation for Ca^{2+} monitoring is provided by a quartz-halogen lamp mounted on the upper epifluorescence

unit. Excitation and emission wavelengths are determined by standard Zeiss filter sets, which can therefore be readily interchanged to permit measurements with either fluo-3 or rhod-2. For convenience, the second position in the sliding filter holder is left empty, so that the filters can be slid out of the way for viewing the oocyte while positioning electrodes. The photolysis light is usually focused on the oocyte as a spot concentric with, and slightly smaller than, the photolysis light. However, because the diaphragms in the photolysis and fluorescence emission systems are independent, it is also possible to record from regions of the cell distant from that stimulated. Fluorescence emission is monitored by a photomultiplier (EMI 9524B) mounted on the microscope phototube.

3.2. Intracellular Loading of Caged InsP_3

Caged InsP_3 obtained from Calbiochem is dissolved at a concentration of 1 m*M* in an aqueous solution including 5 m*M* HEPES (at pH 7.0) and 50 μ*M* EDTA (to chelate contaminating Ca^{2+} and prevent activation of Cl^- currents on injection). This solution is passed through a 0.22-μm Millipore filter to remove any particles that might block the injection pipet, and a few microliters are loaded into the back of a micropipet. Pipets for injection are pulled using a conventional microelectrode puller, from fiber-filled glass tubing, and the tip is broken to a diameter of a few micrometers. Judicious tapping of the pipet will usually (but not always) ensure that the shank of the pipet becomes filled without bubbles. After mounting in the micromanipulator, the pipet is connected by flexible tubing to a "Picopump" (World Precision Instruments, New Haven, CT), which supplies pneumatic pressure pulses (usually set to 20 psi for 50 milliseconds) to eject fluid. The volume of fluid expelled by each pulse is estimated by measuring the diameter of the fluid droplet expelled with the pipet tip raised in the air.

Insertion of the pipet into the oocyte is monitored by a transient inward current in the voltage-clamp record. After allowing a few minutes for the cell membrane to seal around the pipet, sufficient pressure pulses are then applied to load the oocyte with a total of 0.5–10 pmol of caged InsP_3. This corresponds to a

fluid vol of 0.5–10 nL of solution, giving a final intracellular concentration of roughly 0.5–10 μ*M*.

Caged InsP_3 appears to be stable under normal room lighting, and even illumination of the oocyte by a powerful fiberoptic lamp causes no detectable response, but to be safe, stock solutions should be kept frozen in the dark when not in use. Greater care is needed, however, with stray light from the arc lamp, which, even though it may appear dim, contains a high proportion of UV. Thus, remove the injection pipet from the oocyte after loading, and displace it several millimeters away from the objective lens. Stray light from the base of the lamp housing can also cause appreciable photolysis of caged InsP_3 in the pipet over a few hours and needs to be shielded.

Even after taking all the above precautions, injections of freshly prepared solutions of caged InsP_3 obtained from Calbiochem still evoked oscillatory chloride currents, like those generated by free InsP_3 (Parker and Miledi, 1989). Most probably, these currents arose because the samples of caged InsP_3 were slightly contaminated by free InsP_3, or by InsP_3 caged at the 1-phosphate position. Whatever the explanation, these responses do not pose any great problem for work in the oocyte, because the oscillations die away after several minutes, presumably as the physiologically active contaminant is metabolized. We normally wait for at least 30 min after loading caged InsP_3 to allow the compound to distribute throughout the cell and to allow the size of the response evoked by light flashes to stabilize. However, in other preparations, this trick of using the cell itself to "clean up" the caged InsP_3 may not work so effectively, and it would certainly be better to have more pure preparations available.

Figure 2 illustrates the changes in peak size of Ca^{2+}-dependent current responses evoked by identical light flashes at intervals after loading an oocyte with about 5 pmol of caged InsP_3. The injection of caged InsP_3 itself elicited an oscillatory current, which ceased after about 8 min. No responses were evident to the light flashes until 15 min following injection, and the currents then grew progressively before stabilizing at a maximal value after about 30 min. In this experiment, the part of the

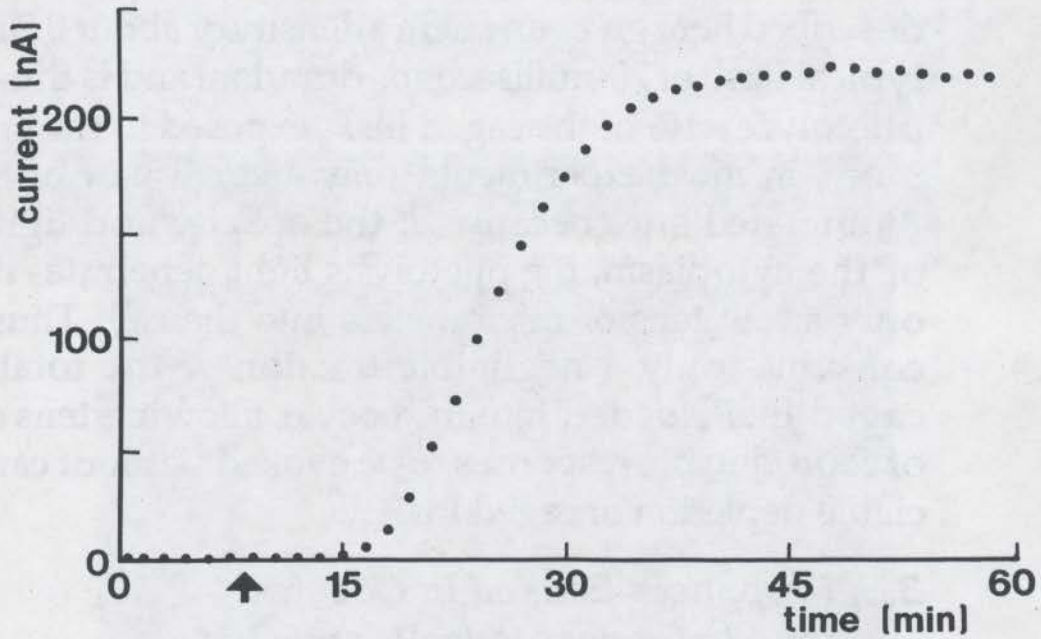

Fig. 2. Sizes of peak membrane currents evoked by constant light flashes applied at 90-s intervals following intracellular loading of an oocyte with caged InsP_3. The arrow indicates the time at which oscillatory currents ceased.

oocyte illuminated by the photolysis light was close (about 100 μm) to the injection site, so that the lack of responses during the first 15 min was probably not owing to a restricted spread of the caged InsP_3 to the illuminated area. Further, a similar delay to the onset of responses was seen even when the photolysis light was focused at the injection site. Instead, it may be that responses to photorelease of InsP_3 are inhibited because the intracellular free Ca^{2+} level is elevated for some time after loading (Parker and Ivorra, 1990), as a result of Ca^{2+} liberation from intracellular stores and from influx of extracellular Ca^{2+} (Parker and Miledi, 1986, 1987).

After allowing sufficient time for stabilization, the current responses evoked by successive stimuli are very stable. For example, the SD of the last 12 responses in Fig. 2 is only about 0.7% of the mean current amplitude.

A rough estimate of the amount of caged InsP_3 photolyzed can be obtained from the energy density of the photolysis light at the preparation. Ogden et al. (1990) estimate that a flash with an energy density of about 20 mJ/mm^{-2} produces about 50% conversion of caged InsP_3 to free InsP_3. The photolysis system

described here gives an energy density of about 0.2 mJ mm^{-2} in a typical flash of 20-milliseconds duration, and is thus expected to photolyze <1% of the caged InsP_3 exposed to the light. Furthermore, in most experiments, only a small part of the oocyte is illuminated and, because of the opacity and light scattering of the cytoplasm, the photolysis light penetrates to a depth of only a few tens of micrometers into the cell. Thus, each flash consumes only a negligible fraction of the total amount of caged InsP_3 loaded into the oocyte, allowing tens or hundreds of reproducible responses to be evoked without causing appreciable depletion of caged InsP_3.

3.3. Responses Evoked in Oocytes by Photoreleased InsP_3 and Ca^{2+}

Figure 3 shows superimposed records obtained from two oocytes, loaded with caged InsP_3 or caged Ca^{2+} (DM-nitrophen), and illustrates the time resolution made possible by the use of caged compounds. Both oocytes were stimulated identically (by a 10-milliseconds flash of near UV light at the arrow). Simultaneous measurements of the resulting elevations in intracellular free Ca^{2+} were obtained by recording the Ca^{2+}-activated Cl^- membrane current under voltage clamp (lower traces), and by monitoring fluorescence of fluo-3 loaded into the oocyte together with the caged InsP_3.

Photolysis of caged InsP_3 within the oocyte evokes transient membrane current and fluorescence signals. Following brief light flashes like that in Fig. 3, the responses arise from liberation of Ca^{2+} from intracellular stores, since they are not altered by removal of extracellular Ca^{2+} (Parker and Miledi, 1989). However, release of larger amounts of InsP_3 by prolonged illumination also induces responses that arise through the activation of an influx of extracellular Ca^{2+} (Parker, I., unpublished observation).

3.3.1. Kinetics

Both the fluorescence calcium signal and the membrane current begin following a latency of a few hundred milliseconds after photolysis of caged InsP_3. Since photolysis of caged InsP_3 is

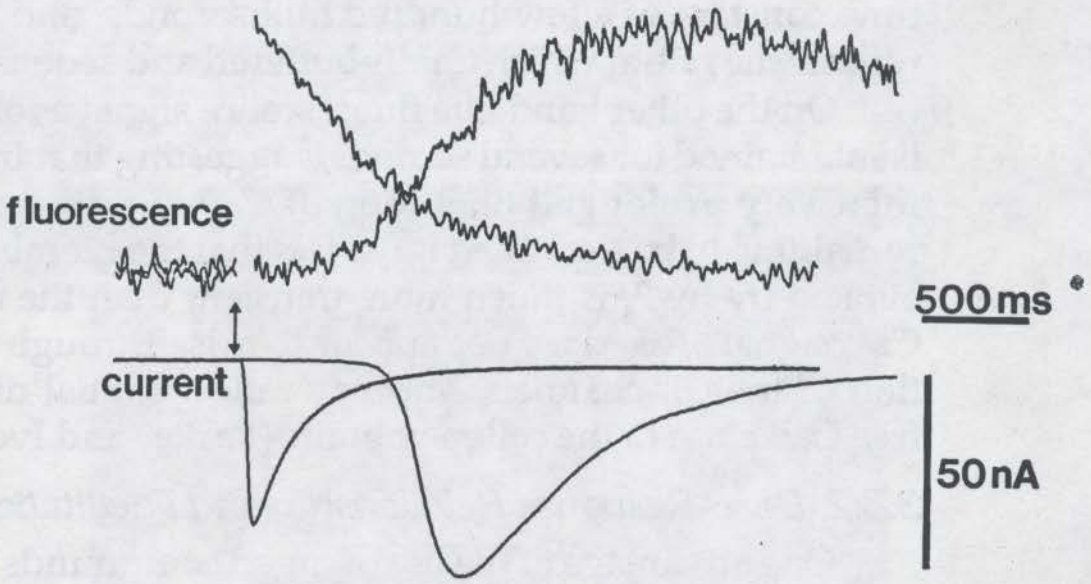

Fig. 3. Records of fluorescence and membrane current (clamp potential = –60 mV) obtained in two oocytes loaded intracellularly with caged Ca^{2+} (DM-nitrophen) or caged $InsP_3$. Traces from each oocyte are shown superimposed to facilitate comparison of responses evoked by photoreleased Ca^{2+} and $InsP_3$. Brief (10-milliseconds) light flashes were given at the arrow. Both oocytes were loaded with fluo-3, and an increase in fluorescence (upward deflection) corresponds to increasing free Ca^{2+}. For both the fluorescence and current traces, the more rapidly rising and falling records were obtained in the oocyte loaded with caged Ca^{2+}. The amplitudes of the fluorescence signals are not calibrated, but were scaled to give similar peak sizes for both oocytes. The fluorescence traces are blanked out during and shortly after the light flashes, because stray light saturated the photomultiplier. Records are from Ivorra and Parker (1990a).

expected to be complete within a few milliseconds, this long latency presumably arises from the processes within the cell responsible for $InsP_3$-induced Ca^{2+} release. In agreement, experiments using $InsP_3$ microinjected into the oocyte by pressure injection or ionophoresis showed a similar latency (Miledi and Parker, 1989). Furthermore, photorelease of Ca^{2+} from DM-nitrophen evoked fluorescence and current signals beginning with short latency, demonstrating that activation of the Ca^{2+}-dependent Cl^- conductance occurs rapidly.

A further difference between the responses to photoreleased Ca^{2+} and $InsP_3$ is the much longer durations of the signals evoked by $InsP_3$. The fluorescence signal evoked by Ca^{2+} declines with a

time constant of a few hundred milliseconds, and presumably reflects the rate at which Ca^{2+} is buffered and sequestered by the cell. On the other hand, the fluorescence signal evoked by InsP_3 is maintained for several seconds, suggesting that InsP_3 causes a relatively prolonged liberation of Ca^{2+}. An interesting point remaining to be explained is the fact that the membrane current evoked by InsP_3 is much more transient than the fluorescence Ca^{2+} signal. This does not appear to arise through desensitization of the Cl^- channels, but may reflect spatial differences in free Ca^{2+} close to the cell membrane (Parker and Ivorra, 1990a).

3.3.2. Dose–Response Relationship and Facilitation

One advantage of the use of caged compounds is the ability to control precisely the amount of messenger released. Thus, by varying the duration or intensity of light flashes applied to oocytes loaded with caged InsP_3, we have been able to construct dose–response relationships for the membrane current and intracellular Ca^{2+} signals (Parker and Miledi, 1989; Parker, 1989b). For several reasons (including uncertainties as to the intracellular concentration of caged InsP_3 loaded and the light energy penetrating into the cell), it is difficult to estimate the absolute concentration of intracellular InsP_3 resulting from a given light flash. Nevertheless, the flash duration or intensity provides a relative indication of the resulting concentration of InsP_3 and, because even the strongest stimuli photolyze only a small percentage of the caged InsP_3, the extent of photolysis is expected to be linearly proportional to the energy of the flash.

An important finding is that the light flash must be greater than a certain threshold intensity or duration before any detectable calcium or membrane current signals are seen (Parker and Miledi, 1989; Ivorra and Parker, 1990b). Previous experiments (Parker and Miledi, 1987) measuring responses evoked by different doses of microinjected InsP_3 had indicated that the phosphoinositide signaling pathway in the oocyte operated in a highly nonlinear manner, but the use of caged InsP_3 provided a more precise approach to the study of this phenomenon, and facilitated the identification of the stage in the pathway responsible.

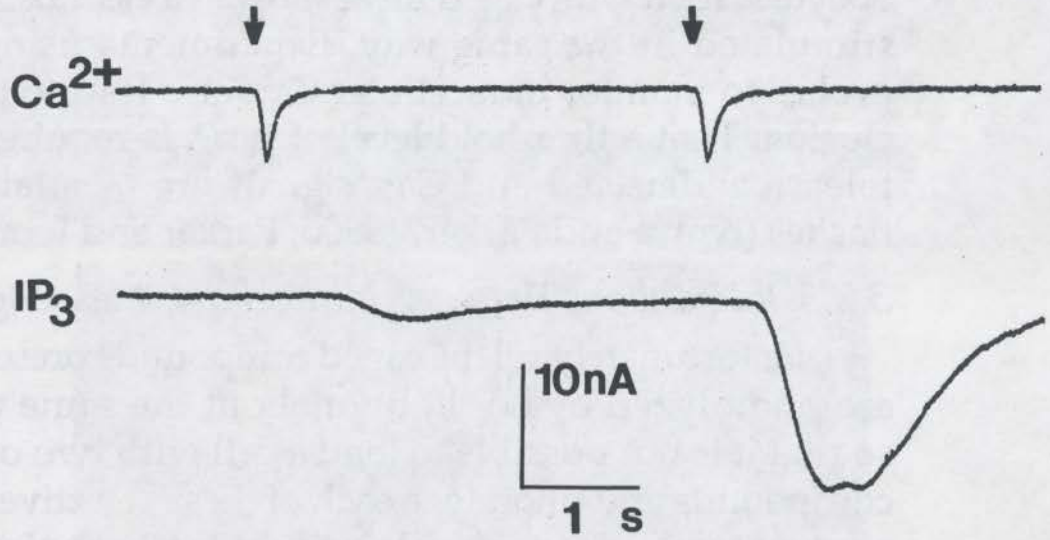

Fig. 4. Comparison of membrane current responses evoked by paired light flashes in two oocytes loaded with caged Ca^{2+} (DM-nitrophen) (upper trace) or caged InsP_3 (lower trace). In both cases, identical light flashes (10-milliseconds duration) were applied at an interval of 3 s, as indicated by the arrows.

Associated with the threshold phenomenon is a pronounced facilitation seen with paired light flashes that are each close to threshold. For example, the lower trace in Fig. 4 shows membrane currents evoked by two identical light flashes applied to an oocyte loaded with caged InsP_3. The first flash evoked only a small current, whereas the second gave a much larger response. This facilitation might arise if a preceding flash somehow "primed" the caged InsP_3 so that a subsequent flash caused greater photolysis. Such an effect (at intervals of several seconds) is, however, not expected from what is known of the photochemistry of caged InsP_3. Instead, it seems that the facilitation arises within the cell, a conclusion that is supported by the finding that intracellular injections of low doses of InsP_3 can also facilitate responses to subsequent light flashes. The question then is whether the threshold and facilitation arise because a certain level of InsP_3 is required before Ca^{2+} is released from intracellular stores, or because a threshold level of Ca^{2+} is needed to activate the Cl^- membrane conductance. One experiment to distinguish between these hypotheses is shown in Fig. 4. Paired light flashes delivered to an oocyte loaded with caged Ca^{2+} evoked membrane current responses of almost identical sizes, whereas an

oocyte loaded with caged InsP_3 showed a clear facilitation when stimulated in the same way. Experiments using fluorescent probes to monitor intracellular Ca^{2+} also lead to the same conclusion: That a threshold level of InsP_3 is required before Ca^{2+} release is detected and Ca^{2+} signals are facilitated by paired flashes (Ivorra and Parker, 1990b; Parker and Ivorra, 1990b).

3.3.3. Interactions Between Intracellular Messengers

Unfortunately, all the caged compounds presently available are photolyzed by UV light of about the same wavelengths, so that it is not possible to load a cell with two or more caged compounds and photolyze each of these selectively. The development of caged compounds activated by light of well-separated wavelengths would allow some fascinating experiments, but for the moment, it is still feasible to study interactions between a photoreleased messenger compound and another compound that is microinjected into the cell. Using this approach we have, for example, shown that responses to photolysis of caged InsP_3 are facilitated by microinjection of InsP_3 (Parker and Miledi, 1989), but are depressed by microinjection of Ca^{2+} (Parker and Ivorra, 1990a).

3.3.4. Spatial Localization

A great advantage of using light as a stimulus is the ease with which it can be focused to any desired shape and location, thus permitting spatially localized release of intracellular messengers. One application is to "map" the spatial sensitivity of the oocyte to intracellular InsP_3. The photolysis light is focused on the oocyte surface as a small (ca. 50 μM) spot, and membrane currents are recorded in response to identical flashes delivered with the spot moved to various positions across the cell. (Pigmentation in the animal hemisphere of normal oocytes complicates this experiment, but this problem can be avoided by the use of oocytes from albino frogs.) Another example is the measurement of the localization of Ca^{2+} liberation induced by a spatially restricted photorelease of InsP_3. The optical system described above allows the light spots used for photolysis and fluorescence Ca^{2+} monitoring to be positioned independently. Thus, InsP_3 can be released at one area on the oocyte and

Ca^{2+} signals recorded from sites at various distances (Parker and Ivorra, 1990b).

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Abbreviations

cAMP	Adenosine 3',5' cyclic monophosphate
cGMP	Guanosine 3',5' cyclic monophosphate
GTP	Guanosine 5'-triphosphate
GTPγS	Guanosine 5'-[γ-thio]triphosphate
$InsP_2$	Inositol bisphosphate (isomer specified in text)
$InsP_3$	Inositol trisphosphate (isomer specified in text)

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