volume. This duration can be significantly extended by lowering the spatial resolution of release and can be significantly reduced by the addition of calcium-buffering agents, whether intrinsic cellular calcium buffers or extrinsic calcium indicator dyes.

There are many possible uses for TPE of calcium cages. The competition of buffers for locally released calcium could provide a window into the spatial heterogeneities of the intrinsic calcium-buffering dynamics in living cells. This could be accomplished through utilization of the relative uptake of liberated calcium ions by a fluorescent calcium indicator and intrinsic calcium buffers as a measure of the intrinsic buffering rate and capacity of cells. As has been shown in Fig. 6, for at least one cell type these kinetics should happen significantly faster than the diffusional escape of calcium ions from the TPE focal volume, allowing these buffering properties to be measured with submicron, three-dimensional resolution and thereby extending previous whole cell studies.[23]

These measurements and calculations demonstrate that complete two-photon release of calcium with high three-dimensional spatial resolution is possible and can be an extremely useful tool in the study of a variety of cellular processes.

Acknowledgments

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[21] Caged Inositol 1,4,5-Trisphosphate for Studying Release of Ca^{2+} from Intracellular Stores

By NICK CALLAMARAS and IAN PARKER

Introduction

Inositol 1,4,5-trisphosphate ($InsP_3$) is a second messenger that mediates Ca^{2+} release during many physiological processes, including development, gene regulation, secretion, contraction, synaptic transmission, and apoptotic

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cell death.[1,2] The development of caged-$InsP_3$ [c-$InsP_3$: *myo*-inositol 1,4,5-trisphosphate (1,2-nitrophenyl ester)] has provided an elegant and efficient means by which to control levels of this active messenger in the cytoplasm of intact cells, giving an unparalleled degree of temporal and spatial resolution for detailed studies of this ubiquitous intracellular pathway.[3–5] In comparison to other techniques, such as fast-flow perfusion of permeabilized cells[6] or direct introduction from a point source via a pressure or iontophoresis microelectrode,[7] it allows rapid (millisecond), spatially defined elevations in $InsP_3$ concentration to be achieved without compromising the organization and biochemical composition of the cytoplasm or disrupting the electrical properties of the cell.

The essence of the technique is first to introduce the physiologically inert c-$InsP_3$ into a cell (e.g., by microinjection or diffusion from a whole cell patch pipette) and allow it to diffuse and equilibrate throughout the cytosol. Subsequent illumination with near-UV light then provides a means to photorelease active $InsP_3$. Major advantages of this technique are: (1) rapid and reproducible millisecond steps in intracellular concentration of $InsP_3$ are achieved; (2) the relative concentration of $InsP_3$ can be precisely regulated by the intensity and duration of illumination; and (3) manipulation of the illumination field allows for homogeneous release of $InsP_3$ throughout spatially defined regions of the cell.

Several practical points further simplify the use of c-$InsP_3$ and extend its utility. The caged precursor is extremely stable in the cytosol, and most cells are exquisitely sensitive to $InsP_3$ (nanomolar range), so that a large "reservoir" of c-$InsP_3$ may be loaded, allowing numerous photolysis flashes to be delivered, each of which consume only a tiny fraction of the total c-$InsP_3$. Also, c-$InsP_3$ is efficiently photolyzed by light with wavelengths <400 nm, thus freeing up the visible spectrum for simultaneous use of long-wavelength indicator dyes to monitor Ca^{2+} liberation induced by $InsP_3$.

This article describes techniques developed in the authors' laboratory for use of c-$InsP_3$ in *Xenopus laevis* oocytes and illustrates some of their applications in studying the $InsP_3/Ca^{2+}$ second messenger pathway. The

[1] M. J. Berridge, *Nature* **361,** 315 (1993).
[2] T. Michikawa, A. Miyawaki, T. Furuichi, and K. Mikoshiba, *Crit. Rev. Neurobiol.* **10,** 39 (1996).
[3] D. C. Ogden, K. Khodakhah, T. D. Carter, P. T. Gray, and T. Capiod, *J. Exp. Biol.* **184,** 105 (1993).
[4] S. S. Wang and G. J. Augustine, *Neuron* **15,** 755 (1995).
[5] I. Parker, *Neuromethods* **20,** 369 (1992).
[6] T. Meyer, T. Wensel, and L. Stryer, *Biochemistry* **29,** 32 (1990).
[7] M. J. Berridge, *Proc. R. Soc. Lond. B* **238,** 235 (1989).

Xenopus oocyte has long been a common cell type for studies of the InsP_3-mediated Ca^{2+} release system due to its enormous size (>1 mm diameter) and simple geometry, which facilitate many experimental procedures. Additional advantages are the ability of the oocyte to express foreign proteins encoded by microinjected mRNA[8] and a lack of other confounding intracellular Ca^{2+} release channels (i.e., ryanodine receptors). The combined advantages of flash-photolysis of c-InsP_3 and the oocyte system have, for example, permitted high-resolution studies of the kinetics of InsP_3 receptor (InsP_3R) gating in an intact cell system,[9] allowed elucidation of the relationship between InsP_3-evoked Ca^{2+} signals and Ca^{2+}-dependent Cl^- membrane current,[10] and facilitated the resolution of "elementary" Ca^{2+} release events underlying global Ca^{2+} signals.[11–13] Moreover, the ability to evoke reproducible InsP_3 signals has aided pharmacological studies of agents affecting this intracellular messenger pathway.[14,15]

Methodology

Photochemistry of c-InsP_3

In the native oocyte, InsP_3 is generated from receptor-mediated breakdown of phosphatidylinositol bisphosphate. InsP_3 then binds to the intracellular InsP_3R to release Ca^{2+} from a subset of the intracellular Ca^{2+} stores and is subsequently metabolized into a bewildering array of other inositol phosphates, some of which retain physiological activity. The flash photolysis of c-InsP_3 avoids the time delays, nonlinearity, and modulation by other messengers associated with ligand-activated InsP_3 generation. This is achieved due to the efficiency of the photochemistry involved in the cleavage of InsP_3 from its protecting nitrophenyl ester group.[16] In brief, the reaction proceeds in two steps: the rapid (nanosecond) absorption of a high energy photon generating active intermediates followed by a slower (millisecond) dark reaction whereby intermediates decay to release InsP_3

[8] R. Miledi, I. Parker, and K. Sumikawa, *in* "Fidia Neuroscience Award Lectures" (J. Smith, ed.), Vol. 3, p. 57. Raven Press, New York, 1989.
[9] I. Parker, Y. Yao, and V. Ilyin, *Biophys. J.* **70,** 222 (1996).
[10] I. Parker and Y. Yao, *Cell Calcium* **15,** 276 (1994).
[11] I. Parker and Y. Yao, *Proc. R. Soc. Lond. B* **246,** 269 (1991).
[12] Y. Yao, J. Choi, and I. Parker, *J. Physiol. (Lond.)* **482,** 533 (1995).
[13] I. Parker, J. Choi, and Y. Yao, *Cell Calcium* **20,** 105 (1996).
[14] I. Parker and I. Ivorra, *J. Physiol. (Lond.)* **433,** 207 (1991).
[15] V. Ilyin and I. Parker, *J. Physiol. (Lond.)* **448,** 339 (1992).
[16] J. W. Walker, J. Feeney, and D. R. Trentham, *Biochemistry* **28,** 3272 (1989).

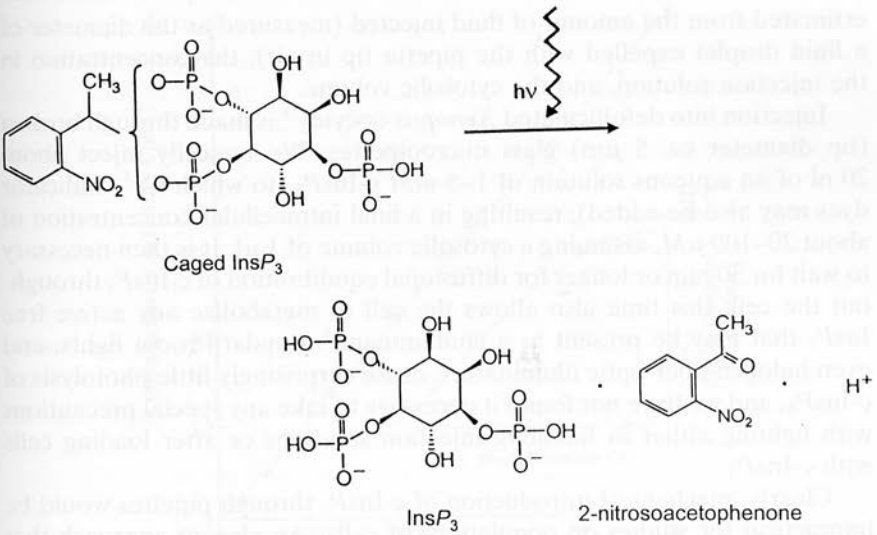

FIG. 1. Schematic photolysis reaction for caged InsP_3. In addition to free InsP_3, photolysis releases a proton and a by-product, 2-nitrosoacetophenone.

(Fig. 1). The by-products of this reaction, a proton and nitrosoacetophenone, can be toxic at high levels (1 $mmol^{-1}$), altering the local pH or reacting with -SH groups of proteins, respectively.[17] Fortunately, the concentrations of InsP_3 necessary to elicit even maximal responses in oocytes and many other cells are sufficiently small that by-product toxicity appears not to be an issue.

Caged InsP_3 can presently be obtained from either Calbiochem (La Jolla, CA) or Molecular Probes (Eugene, OR). In our experience the c-InsP_3 available from Molecular Probes is preferable because of lower contamination by active InsP_3.

Introduction of c-InsP_3 into Cells

Caged InsP_3 is a highly charged molecule and thus does not permeate through the cell membrane. For work with single cells, c-InsP_3 may be introduced into the cytosol by injection through a micropipette (pneumatic presure injection or ionophoresis) or by diffusion from a whole cell patch pipette. In the latter case, the resulting intracellular concentration should approximate that in the pipette. Pressure injection is convenient for use with large cells, and the final intracellular concentration of c-InsP_3 can be

[17] M. G. Hibberd, Y. E. Goldman, and D. R. Trentham, *Curr. Topics Cell. Regul.* **24,** 357 (1984).

estimated from the amount of fluid injected (measured as the diameter of a fluid droplet expelled with the pipette tip in air), the concentration in the injection solution, and the cytosolic volume.

Injection into defolliculated *Xenopus* oocytes[18] is made through broken (tip diameter ca. 5 μm) glass micropipettes. We typically inject about 20 nl of an aqueous solution of 1–5 mM c-InsP_3 (to which Ca^{2+} indicator dyes may also be added), resulting in a final intracellular concentration of about 20–100 μM, assuming a cytosolic volume of 1 μl. It is then necessary to wait for 30 min or longer for diffusional equilibration of c-InsP_3 throughout the cell; this time also allows the cell to metabolize any active free InsP_3 that may be present as a contaminant.[5] Standard room lights, and even halogen fiber-optic illuminators, cause surprisingly little photolysis of c-InsP_3, and we have not found it necessary to take any special precautions with lighting either in handling injection solutions or after loading cells with c-InsP_3.

Clearly, mechanical introduction of c-InsP_3 through pipettes would be impractical for studies on populations of cells. An elegant approach that has recently been developed involves loading c-InsP_3 by extracellular application at a membrane-permeable ester,[19] analogous to the well-known technique for loading indicators such as Fura-2. Alternatively, the plasma membrane may be permeabilized by a variety of techniques[20] to allow access of charged c-InsP_3 to the cytosol.

Linearity and Calibration of Photorelease

The amount of free InsP_3 resulting from a given photolysis flash is expected to be linearly proportional to the starting concentration of c-InsP_3 and the flash intensity and duration. In practice, this linear dependence on flash parameters is followed almost exactly; as illustrated in Fig. 2A, where caged ATP was used to monitor photolysis in place of c-InsP_3 because of the ease of detecting release of ATP by luminescence of a luciferin/luciferase assay system. Thus, an extremely precise control of *relative* amounts of InsP_3 formation within a given cell can be obtained by appropriate adjustment of flash parameters. Our usual method is to adjust the flash intensity using neutral density filters to set an initial working range and then vary the flash duration to control the extent of photorelease. This has advantages in that the duration can be readily altered in very small increments, as opposed to the relatively coarse steps available with filter

[18] K. Sumikawa, I. Parker, and R. Miledi, *Methods Neurosci.* **1,** 30 (1989).
[19] W. H. Li, C. Schultz, J. Lopi, and R. Y. Tsien, *Tetrahedron* **53,** 12017 (1997).
[20] K. A. Oldershaw, D. L. Nunn, and C. W. Taylor, *Biochem. J.* **278,** 705 (1991).

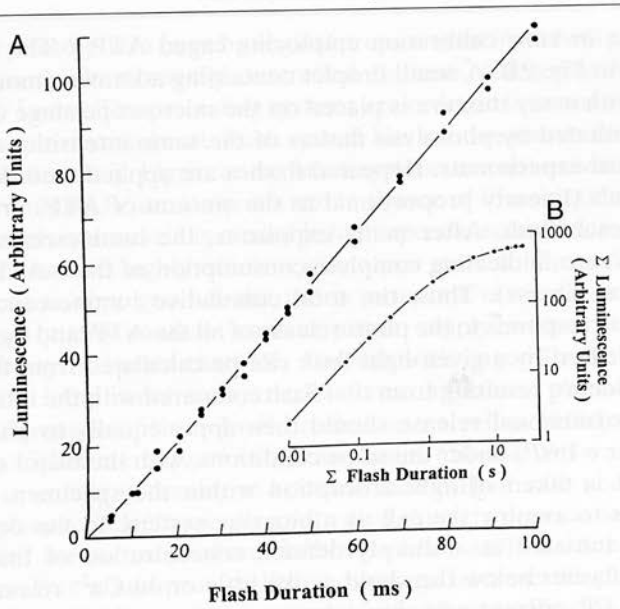

FIG. 2. Linearity of photorelease with flash strength and calibration of extent of photolysis using a luminescence system to assay release of ATP from a caged precursor. (A) Formation of ATP varies linearly with flash duration. Recordings were made from a 20-μl droplet containing 100 μM caged ATP together with undiluted ATP assay reagent (Sigma FL-AAM). The photolysis light was focused by the microscope objective to a small spot within the droplet, and the graph plots the peak amplitude of the ATP-dependent luminescence (monitored by a photomultiplier) evoked following photolysis flashes of identical intensity and varying duration. (B) Estimation of the fractional photolysis of caged ATP resulting from a given photolysis flash. The experiment was performed as in (A), except for use of a smaller droplet that was entirely irradiated by the photolysis spot. A series of repeated flashes of increasing duration were applied, and the peak luminescence recorded following each flash. Double logarithmic plot shows cumulative luminescence (i.e., sum of peak signals evoked by successive flashes) as a function of cumulative duration of exposure to photolysis light. [Reproduced from I. Parker and I. Ivorra, *Am. J. Physiol.* **263,** C154 (1992), with permission.

wheels, and individual flashes within a paired-pulse protocol can be independently controlled. Care should be taken, however, that the longest flashes employed are brief compared to the kinetics of the response under investigation.

For many purposes, control of the *relative* amounts of $InsP_3$ photoreleased in a given experiment is sufficient, but it may also be necessary to estimate the *absolute* concentrations of free $InsP_3$. This is a more difficult and less precise task, for which we have taken two approaches. The first

involves an *in vitro* calibration employing caged ATP.[21] The protocol is illustrated in Fig. 2B. A small droplet containing a known amount of ATP together with assay mixture is placed on the microscope stage and is completely irradiated by photolysis flashes of the same intensities as used for physiological experiments. Repeated flashes are applied, and the luminescence signals (linearly proportional to the amount of ATP) are recorded following each flash. After many exposures, the luminescence response declines to zero, indicating complete consumption of the c-ATP (luciferin is present in excess). Thus, the total cumulative luminescence over all responses corresponds to the photorelease of all the ATP, and the fractional amount released by a given light flash can be calculated from the ratio of the luminescence resulting from that flash compared with the total luminescence. This fractional release should then apply equally to photolysis of intracellular c-InsP_3 under the same conditions, with the major caveat that no account is taken of light absorption within the specimen. A second approach is to employ the cell as a bioassay system. In the oocyte, Ca^{2+} waves are initiated at a sharply defined concentration of InsP_3 so that photolysis flashes below threshold evoke little or no Ca^{2+} release or Ca^{2+}-dependent Cl^- current response, whereas just-suprathreshold stimuli give clear responses (cf. Fig. 4). Estimates of the absolute concentration of InsP_3 corresponding to this threshold can then be obtained by injecting increasing amounts of a poorly metabolized but equipotent InsP_3 analog (3-F-InsP_3) to determine the resulting intracellular concentration at which waves are triggered.[12]

Measurements of the InsP_3 concentrations by both of these methods indicate that Ca^{2+} waves are evoked in the oocyte by roughly 50 nM InsP_3, and maximal responses are evoked by concentrations about 20-fold greater.[9] Because the cells can readily be loaded to concentrations of c-InsP_3 as high as 100 μM, flashes giving physiological responses will photolyze only negligible proportions (0.05–1%) of the c-InsP_3. Further, the turbidity of the oocyte cytoplasm limits light penetration to a few tens of μm, so that the greater volume of the cell is effectively shielded from the photolysis light and acts as a virtually inexhaustible reservoir of fresh c-InsP_3. The net result is that it is possible to evoke numerous (hundreds) reproducible responses with repeated flashes, without significant depletion of available c-InsP_3 in the oocyte.

Photolysis Light Sources

The photolysis of caged InsP_3 requires a sufficiently intense source of illumination at wavelengths around 350 nm. Possible light sources capable

[21] I. Parker and I. Ivorra, *Am. J. Physiol.* **263,** C154 (1992).

of efficient photolysis in most biological preparations include (1) continuous arc lamps with electronically controlled shutters, (2) xenon flash lamps, and (3) UV lasers. The preceding systems are ordered in terms of increasing energy, complexity, and cost. Each system has relative merits and disadvantages but, fortunately, bigger and costlier systems are not necessarily better.

A continuous UV arc lamp is a stable source of illumination that, when combined with commercially available high-speed electric shutters, provides an inexpensive and highly efficient photolysis system. The stability of the lamp output is very good if the arc is mounted vertically and powered from a high-quality constant current power supply, with a between-flash reproducibility of better than 1%. For use with caged-InsP_3, the limited power of the lamp is not a major consideration because photolysis of only a small percentage of the c-InsP_3 loaded into a cell is sufficient to evoke responses. Either mercury or xenon arc lamps (50–100 W) work well and, indeed, it is usually necessary to attenute their output with neutral density filters. High-speed electronically controlled shutters provide an affordable and efficient means to produce flashes as brief as 1–2 msec. Durations shorter than this would provide little advantage because the photogeneration of InsP_3 from its caged precursor takes 5–10 msec. Finally, unlike flash lamps or pulsed lasers, it is possible to continuously irradiate the specimen, mimicking physiological InsP_3 generation over several seconds during agonist stimulation. Therefore, a continuous arc lamp is the simplest and most cost-effective choice for most experimental situations. Such systems can be readily constructed from an existing epifluorescence microscope, merely by substituting a UV filter cube to restrict irradiation to wavelengths <400 nm and inserting an electronic shutter into the light path.

Xenon flash lamps[22] provide a much higher (1000 times or greater) energy output in a brief flash than a shuttered arc lamp system but, as noted earlier, this higher energy is not likely to be required when working with c-InsP_3 provided that an efficient optical system is used to channel light to the specimen. Further, flash lamp systems have disadvantages in regard to their greater cost, generation of electrical artifacts, lack of independent control of the duration of each flash in paired-pulse experiments, and an inability to provide continuous illumination of sustained photorelease.

Several types of laser are available that produce high-energy pulses at near-UV wavelengths, including the frequency-doubled ruby laser and the frequency-tripled Nd–YAG (neodymium–ytrrium-aluminum-garnet) laser. In our experience, the only application for which the high cost of these lasers is justified involves focusing the photolysis light to a near-diffraction limited spot in the specimen so as to achieve a virtual point-photorelease

[22] G. Rapp and K. Guth, *Pflugers Arch.* **411,** 200 (1988).

of InsP_3. Because of the perfectly parallel beam from a laser this can be achieved with little loss of light, whereas to achieve the same result with a conventional (arc lamp) source involves insertion of a pinhole aperture with an accompanying drastic loss in light throughput.

Two-photon excitation of caged compounds by femtosecond pulses from mode-locked lasers is a recent development[23] that offers the possibility of even more spatially restricted photorelease because the quadratic dependence of two-photon photolysis on light intensity ensures that this will be restricted only to the plane of focus of the light spot formed by an objective lens. Although this approach has been elegantly employed to map out the sensitivity of neurons to extracellular photorelease of caged neurotransmitter,[24] the possible advantages of this expensive technology for use with c-InsP_3 are unclear and are likely to be limited by the physiology of the InsP_3 pathway. In particular, the rapid intracellular diffusion of InsP_3, together with the long latencies (many milliseconds) before Ca^{2+} release begins, suggests that the spatial localization of InsP_3 will be limited by those factors and not by the minimum volume throughout which photorelease occurs.

Simultaneous Use of c-InsP_3 and Fluorescent Calcium Indicators

The primary action of InsP_3 in the cell is to liberate calcium from intracellular stores. One approach to monitor the resulting rise in cytosolic-free calcium concentration is to measure currents through endogenous calcium-activated membrane channels (e.g., the Ca^{2+}-dependent Cl^- channels in the oocyte membrane.[25] This is advantageous in terms of simplicity and ensures that the measurement technique does not perturb the signals of interest. Interpretation may, however, be complicated by uncertainties regarding the steady-state and kinetic relationships between free [Ca^{2+}] and current amplitude,[10] and voltage-clamp recordings of current provide no information regarding the spatial distribution of calcium within the cell. Thus, for many purposes it is desirable to have a more direct measure of cytosolic calcium. The availability of a wide range of fluorescent calcium indicator dyes operating in the visible spectrum makes it possible to combine fluorescence calcium monitoring or imaging together with simultaneous photolysis of c-InsP_3.

Currently, our favorite dyes are the Oregon green family (Molecular Probes), which are optimized for excitation by the 488-nm line of the argon ion laser and fluoresce in the green. The excitation spectra of these dyes are sufficiently separated from that of c-InsP_3 that we find little practical

[23] W. Denk and K. Svoboda, *Neuron* **18,** 351 (1997).
[24] W. Denk, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 6629 (1994).
[25] R. Miledi and I. Parker, *J. Physiol. (Lond.)* **357,** 173 (1984).

difficulty with photolysis of c-$InsP_3$ resulting from the blue excitation light and, conversely, photolysis flashes at wavelengths <400 nm cause little or no artifacts in fluorescence recordings. Furthermore, the dyes are available as variants with different calcium affinities (e.g., Oregon green 488 BAPTA-1 has an affinity of a few hundred nanomolar, and Oregon green BAPTA-5N has a low affinity of a few tens of micromolar), so that they may be used selectively to examine small calcium signals close to the basal calcium level or large calcium transients evoked by maximal $InsP_3$ levels. A disadvantage of all currently available long-wavelength indicators is that, unlike the UV-excited dyes Fura-2 and Indo-1, none show shifts in either excitation or emission spectra on binding calcium. Thus, using a single dye, it is not possible to ratio signals at two wavelengths to obtain a calibration of free calcium levels independent of variations in dye loading and path length. A reasonable compromise, however, is to form a "pseudo ratio" signal by expressing calcium-dependent fluorescence signals relative to the resting fluorescence before stimulation.[2]

The basic principle in designing an optical system for simultaneous fluorescence monitoring and photolysis is to "stack" dichroic mirrors so as to split off appropriate parts of the visible and UV spectra. Thus, a UV dichroic mirror placed close to the microscope objective will reflect short wavelengths (<400 nm) from a photolysis light source onto the specimen but transmit longer wavelengths, which may then be further split by an additional dichroic mirror to separate fluorescence excitation and emission wavelengths appropriate for specific dyes. In our earlier optical system[5] employing a Zeiss Universal microscope, this "stacking" was possible by physically mounting one epifluorescence attachment on top of another. More modern microscope designs fail to anticipate the need for such flexibility, and in our present system (described in the following section) a UV dichroic mirror is placed in the regular epifluorescence unit, whereas the fluorescence excitation dichroic mirror is located externally to the microscope with light directed through the video port. Excellent dichroic mirror and filter sets tailored for use with particular dyes are available from Omega Optical Inc. and Chroma Technology Corp. (both at Brattleboro, VT).

Fluorescence calcium monitoring systems may vary in their degree of spatial and temporal resolution (with corresponding increases in cost and complexity) from photomultiplier-based detectors to monitor calcium throughout an entire cell or defined region of a cell,[5] through CCD camera-based wide-field imaging systems[11] to laser-scan confocal microscopes providing millisecond and submicron resolution.[26]

[26] I. Parker, N. Callamaras, and W. Wier, *Cell Calcium* **21,** 441 (1997).

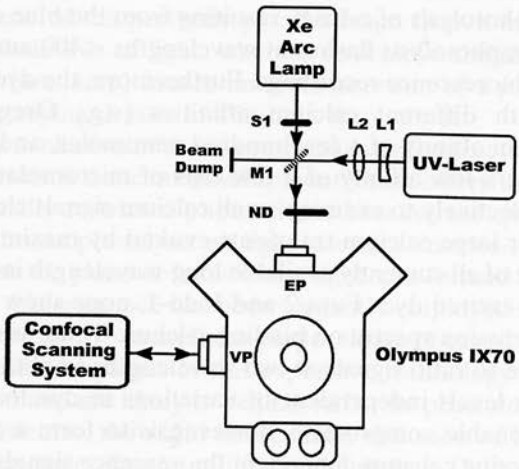

FIG. 3. Schematic diagram of a versatile photolysis and imaging system constructed around an Olympus IX70 inverted microscope. The photolysis system is shown at the top, and interfaces through the epifluorescence port (EP) of the microscope. A confocal scanning system on the left interfaces through the video side port (VP). L1, plano-concave lens; f, −1 cm; L2, biconvex lens; f, 5 cm; S1, electronic shutter; M1, cover glass acting as beam splitter; ND, neutral density filter wheels.

Versatile Photolysis System for Imaging and Electrophysiological Studies

This section describes details of our current photolysis system, which operates through the epifluorescence port of an Olympus IX70 inverted microscope (Lake Success, NY), and can be used either independently or together with a confocal Ca^{2+} imaging system interfaced through the microscope video port (Fig. 3). For further details of the confocal scanner, see Parker *et al.*,[26] and for more general information pertaining to the principles and applications of biological confocal microscopy, see Pawley.[27]

The photolysis system employs both a continuous arc lamp source (for wide field photorelease) and a pulsed UV laser (for "point" photorelease), with light from both systems being combined by a beam-splitting mirror and directed into the epifluorescence port of the Olympus IX70, which is equipped with a standard UV filter cube and fluor objective lenses. Components are mounted on an optical breadboard, using standard post-mounts (New Focus Inc., Santa Clara, CA). Note that with the exception of the filter cube and holder, no Olympus epifluorescence components are required.

The upper section of Fig. 3 shows the layout of the photolysis light

[27] J. B. Pawley, "Handbook of Biological and Confocal Microscopy." Plenum Press, New York, 1990.

paths. All lenses are fused silica for optimal UV transmission, and optical components are postmounted at a height corresponding to the center line of the Olympus epifluorescence port. For experiments where photorelease of caged compound is required over a wide area (10- to 100-μm-diameter spot using a 40× objective), UV light is derived from an arc lamp (75-W xenon lamp, mounted in a Zeiss housing and operated from a stabilized constant-current power supply). An electronic shutter (Uniblitz, Vincent Associates, NY), triggered manually or via TTL input from a Digitimer or computer, controls exposure duration while a set of neutral density wheels (3.0 OD in steps of 0.1 OD, New Focus Inc.) allow control of light intensity. Adjustment of a collector lens in the arc lamp housing provides uniform (Koehler) illumination throughout the photolysis spot in the microscope image plane.

The laser system employs a Mini-Lite frequency-tripled (355 nm) Nd : YAG laser (Continuum, Santa Clara, CA), with the laser head mounted on the optical table by nylon screws and insulating stand-offs to avoid hum loops for electrophysiological recording. Lenses L1 and L2 form a beam expander so that the laser beam fills the back aperture of the objective lens, thus making use of its full numerical aperture. Because the laser beam at full power may crack an objective lens with poor UV transmission, a microscope cover glass is used as a mirror (M4) to reflect only a small percentage of the laser beam into the microscope through the neutral density filter wheels, which allow further attenuation. The invisible beam from the Mini-Lite presents a considerable safety hazard, and appropriate precautions, including use of laser safety goggles, should be taken when the beam is exposed for alignment. A beam dump is placed after M4 to avoid the possibility of the beam passing into the room, and the entire beam path is covered while the laser is operating. The laser spot formed by an objective lens can be viewed using a coverslip marked with a yellow "highlighter" pen, and its position centered by small adjustments of laser position and deflection of M1. Finally, the spot can be brought to a sharp focus by axial adjustment of L2. A UV-blocking filter in the Olympus filter cube, together with an additional long-pass filter (λ >510 nm) inserted in the microscope binocular head, permits safe viewing while the laser is in use. The laser can be operated in single-shot mode (triggered by a push switch or TTL input) or pulsed repeatedly at up to 10 Hz.

Applications of c-$InsP_3$ for Study of Ca^{2+} Signaling in Oocytes

Dose–Response Relation of Ca^{2+} Release

Bath application of calcium-mobilizing agonists to oocytes results in a complex oscillatory Cl^- current, which varies in a highly nonlinear manner

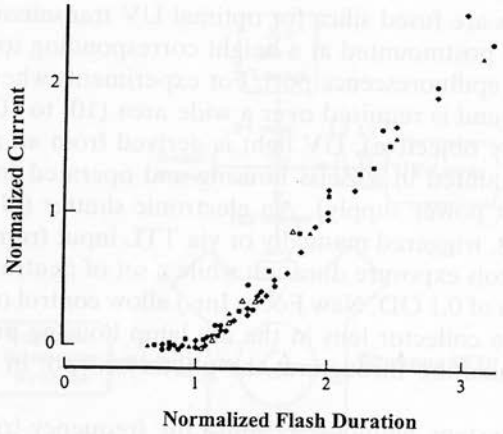

FIG. 4. Dose–response relationship of membrane currents evoked by photorelease of InsP_3 by different durations of irradiation. Pooled data from five oocytes (indicated by different symbols) showing the relationship between flash duration and peak size of evoked currents. Flash durations are normalized with respect to that which evoked just-suprathreshold responses in each oocyte, and currents are normalized with respect to currents evoked in each oocyte evoked by a flash of twice threshold duration. In this experiment the entire vegetal hemispheres were exposed to UV light. [Reproduced from I. Parker and I. Ivorra, *Am. J. Physiol.* **263,** C154 (1992), with permission.]

with agonist concentration.[28] The intracellular location of the numerous stages in the signaling pathway between cell surface receptors and activation of the current, however, makes it difficult to determine at which stage the nonlinearity arises. Photolysis of caged InsP_3 offers an elegant means to circumvent this problem bypassing earlier stages in the pathway. Indeed, the reproducibility and linearity of photolysis allow intracellular dose–response relationships to be determined with the same ease as bath application of compounds to an extracellular receptor. For example, Fig. 4 illustrates the dependence of Ca^{2+}-activated membrane currents on the strength of photolysis flashes and demonstrates a nonlinear relationship in that a threshold amount of $InsP_3$ is needed before any current is generated. These data also illustrate the reproducibility of the photolysis technique, as evident in the close overlap of measurements from five oocytes when flash strengths are normalized relative to the threshold required to evoke a detectable current in each cell so as to compensate for differing amounts of microinjected c-$InsP_3$.

[28] I. Parker, K. Sumikawa, and R. Miledi, *Proc. R. Soc. Lond. B.* **231,** 37 (1987).

Pharmacological Studies of InsP_3R in Intact Oocytes

The InsP_3R through which calcium liberation occurs is a potential site for modulation by many endogenous messenger compounds and exogenous pharmacological agents. Studies of such effects on calcium mobilization are complicated, however, if extracellular agonists are used to activate the InsP_3 pathway because different agents may act on stages between the cell surface receptor and InsP_3 formation, as well as on the InsP_3R itself. Again, caged InsP_3 provides a means to circumvent these difficulties by allowing the effects of microinjected or (for membrane-permeant substances) bath-applied agents to be monitored on signals evoked by repeated and identical pulses of intracellular InsP_3. Two examples are shown in Fig. 5.

The first concerns the possible physiological roles of Ins(1,3,4,5)P_4 (InsP_4), a higher-order inositol polyphosphate that is formed transiently by phosphorylation of InsP_3 during activation of the signaling pathway. As shown in Fig. 5A, evidence that InsP_4 acts as a weak agonist at the InsP_3R to potentiate calcium mobilization is obtained by microinjecting InsP_3 into oocytes while monitoring calcium-dependent Cl^- currents evoked by InsP_3 photoreleased by successive, just-suprathreshold flashes.[14] A strong potentiation of the currents is observed, even with small doses of InsP_4 which, themselves, evoke almost detectable responses. This experiment further illustrates a technical point, in that it is necessary to microinject the highly charged InsP_4 into the oocyte. The spatial spread of InsP_4 in the cell is restricted to a region around the pipette tip because of diffusion and metabolism, and the photolysis light is therefore focused as a small spot centered around the injection pipette. Even though voltage-clamp recordings reflect current from the whole membrane area of the oocyte, the evoked Cl^- currents thereby arise only from the local region exposed to InsP_4.

A second example of the use of c-InsP_3 for pharmacological studies concerns the actions of caffeine, which is widely used as a tool to discriminate between calcium mobilization mediated through InsP_3R and ryanodine receptors, by virtue of its ability to potentiate ryanodine receptor-mediated responses. Because caffeine readily permeates the cell membrane, we were able to study its actions on InsP_3-evoked Cl^- current responses by bath application during trains of repetitive photolysis flashes.[29] This causes a dramatic and reversible reduction in responses (Fig. 5B) and a rightward shift in the dose–response curve for InsP_3 (Fig. 5C), suggesting that caffeine may act as a reversible antagonist at the InsP_3 receptor. In this and other experiments where calcium liberation is monitored by endogenous calcium-

[29] I. Parker and I. Ivorra, *J. Physiol. (Lond.)* **433,** 229 (1991).

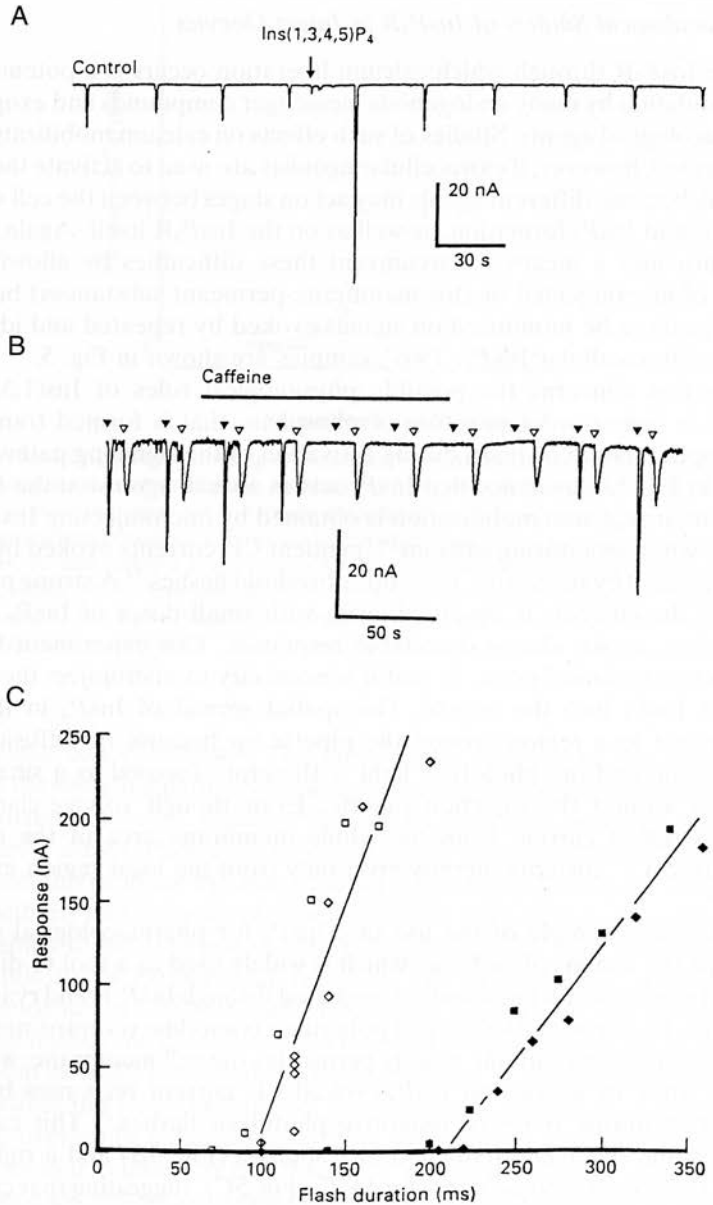

FIG. 5. Utility of c-InsP_3 for pharmacological studies. (A) Responses evoked by photorelease of InsP_3 are facilitated by injection of low doses of InsP_4. Trace shows calcium-dependent membrane current responses (inward currents at a clamp potential of −60 mV) evoked by repetitive, identical photolysis flashes, with a duration just above the threshold to evoke

activated currents it is, however, important to countrol for possible effects on the calcium-activated Cl^{-1} channels themselves. Monitoring of intracellular calcium by means of indicator dyes is one obvious approach, but in the experiment shown in Fig. 5B, intracellular injections of calcium through a micropipette are interspersed with photolysis flashes to directly activate Cl^- currents and serve as a control.

Temporal Control and Kinetics of the Release Process

The photorelease of $InsP_3$ is rapid (a few milliseconds) following a photolysis flash, so that the kinetics of calcium liberation in response to a virtually instantaneous step of $InsP_3$ concentration can be studied. Furthermore, photolysis by a broad light spot results in a homogeneous release of $InsP_3$ throughout a volume of the cell, thus obviating problems of diffusion delays that arise in other techniques such as intracellular microinjection or extracellular applications of $InsP_3$ to permeabilized cells. An example is shown in Fig. 6, where a laser confocal microscope is used to monitor calcium fluorescence signals from a minute volume within the oocyte (ca. 1 fl) in response to $InsP_3$ photoreleased throughout a larger region of the cell surrounding the laser spot. Calcium signals following photolysis flashes of increasing strength begin with progressively shorter latencies and show increasing rates of rise (Fig. 6A). Because the fluorescence signal reflects accumulation of calcium in the cytosol, differentiation of the signal further provides a measure of the rate of calcium efflux (Fig. 6B), allowing a more detailed study of the dependence of the kinetics of opening of the release channels on the concentration of $InsP_3$.[9]

As is evident in Fig. 6B, calcium liberation following a photolysis flash terminates within a few hundred milliseconds, even though the levels of $InsP_3$ remain elevated for several seconds.[21] Thus, the $InsP_3R$ enters a refractory state from which its subsequent recovery can be investigated by applying paired photolysis flashes at varying intervals, in a manner analo-

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detectable signals. $InsP_4$ (about 1 fmol) was injected into the oocyte when marked by the arrow. [Reproduced from I. Parker and I. Ivorra, *J. Physiol.* (*Lond.*) **433,** 207 (1991), with permission of the Physiological Society.] (B) Bath-applied caffeine inhibits $InsP_3$-evoked membrane current responses, but not the activation of the Ca^{2+}-dependent Cl^- current. Trace shows currents evoked by alternate stimulation by photolysis flashes (filled arrowheads) and intracellular injections of calcium (open arrowheads). Caffeine (5 m*M*) was bath-applied when marked by the bar. (C) Caffeine increases the threshold amount of $InsP_3$ required to evoke membrane currents. Graph shows amplitudes of currents evoked by photolysis flashes of varying durations in the absence (open symbols) and presence (filled symbols) of 2.5 m*M* caffeine. [B, C reproduced from I. Parker and I. Ivorra, *J. Physiol.* (*Lond.*) **433,** 229 (1991) with permission of the Physiological Society.]

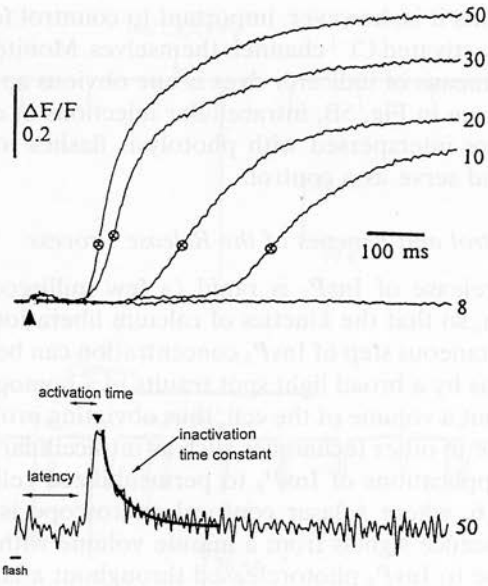

FIG. 6. Calcium transients and kinetics of calcium liberation in response to photorelease of varying amounts of InsP_3. (A) Superimposed fluorescent signals evoked by light flashes of varying durations (indicated in milliseconds at the right of each trace). The arrowhead indicates the time of the flash and circles indicate the point of maximal rate of rise of the signal. The oocyte was loaded with the low-affinity indicator calcium green-5N, together with c-InsP_3. (B) The time differential $[d(\Delta F/F)/dt]$ of the 50-msec trace from (A), indicating the kinetics of Ca^{2+} flux. [Modified from I. Parker, Y. Yao, and V. Ilyin, *Biophys. J.* **70,** 222 (1996), with permission of the Biophysical Society.]

gous to the paired-pulse experiments used to examine the refractory state of voltage-gated channels.[30,31] The onset and recovery from inactivation are determined by delivering paired, identical photolysis flashes at varying intervals and monitoring the calcium-activated currents evoked by the second flash in each pair relative to that evoked by a single flash (Fig. 7). At short intervals the calcium signal is potentiated by a preceding flash, but becomes almost completely suppressed as the interval is lengthened to about 2 sec, and subsequently recovers over several seconds.

Entry into and recovery from the refractory state undoubtedly have an important role in generation of the repetitive calcium spikes observed in various cells during sustained activation by extracellular agonists.[32] A characteristic feature is that the frequency of spiking increases with agonist

[30] I. Parker and I. Ivorra, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 260 (1990).
[31] V. Ilyin and I. Parker, *J. Physiol. (Lond.)* **477,** 503 (1994).
[32] M. J. Berridge and A. Galione, *FASEB J.* **2,** 3074 (1988).

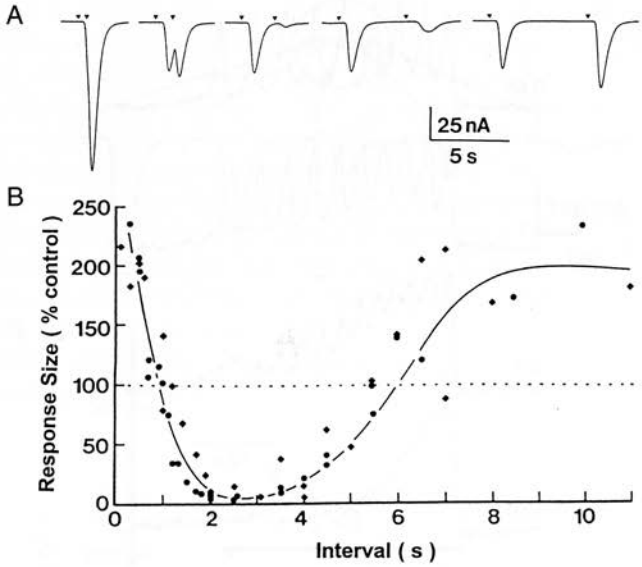

FIG. 7. Paired-flash experiment to investigate the onset and recovery of $InsP_3$-mediated calcium release. (A) Examples of membrane currents evoked by paired, identical flashes delivered at different intervals. Arrowheads mark the times of each flash. (B) Size of the response to the second flash plotted against interval between flashes. Data are shown from two oocytes (different symbols) and are scaled as a percentage of the response evoked by a single flash. [Reproduced from I. Parker and I. Ivorra, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 260 (1990), with permission.]

concentration but, as noted before, interpretation of these findings is complicated by the complex stages in the messenger pathway between the cell surface receptor and the generation of $InsP_3$. An alternative approach is to use sustained illumination with low-intensity UV light to cause a prolonged elevation of intracellular [$InsP_3$] to levels proportional to the light intensity.[33] This results in repetitive spikes in the fluorescence calcium signal during the period of photolysis, which increase in frequency with increasing photorelease but become smaller and superimposed upon a more sustained calcium elevation (Fig. 8).

Spatial Control and Heterogeneity of Calcium Release Sites

The use of light as a stimulus to evoke photolysis of c-$InsP_3$ permits not only control of the magnitude and kinetics of $InsP_3$ formation, but also control of its spatial distribution. Thus, information can be obtained

[33] I. Parker and I. Ivorra, *J. Physiol. (Lond.)* **461,** 133 (1993).

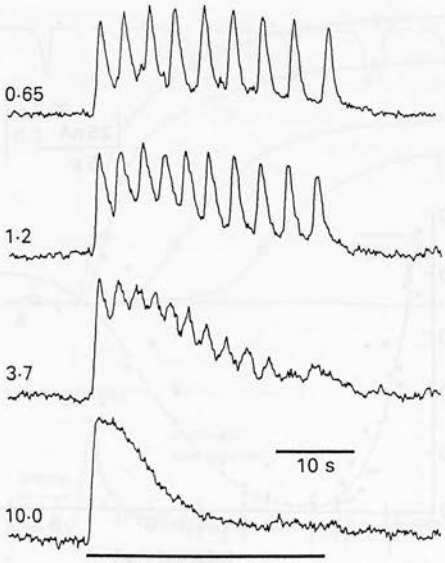

FIG. 8. Repetitive Ca^{2+} spikes during prolonged photorelease of $InsP_3$. Tracaes show point-confocal Ca^{2+} signals (rhod-2 fluorescence) evoked at a single recording spot by various intensities of photolysis light. Horizontal bar indicates the duration of exposure, and numbers next to each trace indicate the intensity of illumination as a percentage of maximum. [Reproduced from I. Parker and I. Ivorra, *J. Physiol. (Lond.)* **461,** 133 (1993), with permission of the Physiological Society.]

regarding the spatial aspects of subcellular calcium liberation, either by restricting photolysis to defined regions of the cell or by fluorescence imaging of the patterns of calcium liberation evoked by spatially homogeneous photolysis. The following examples illustrate use of these approaches to study calcium liberation at increasingly fine levels of resolution, from global signals involving the whole cell to elementary calcium events located to within a few microns.

Figure 9 illustrates an experiment in which the regional sensitivity to $InsP_3$ is mapped across the two hemispheres (animal and vegetal) of the polarized oocyte cell. The oocyte was loaded with caged $InsP_3$ and stimulated by identical light flashes focused as a 50 × 50-μm square. As the photolysis square is moved from the vegetal to the animal pole, the whole-cell Cl^- currents evoked by the flashes grow progressively larger (Fig. 9A), indicating increased sensitivity to $InsP_3$. Thus, even though the voltage-clamp recording summates membrane currents from the whole cell, spatial information is provided by the localized photorelease of $InsP_3$. One complication in such experiments, however, is that the extent of photorelease may

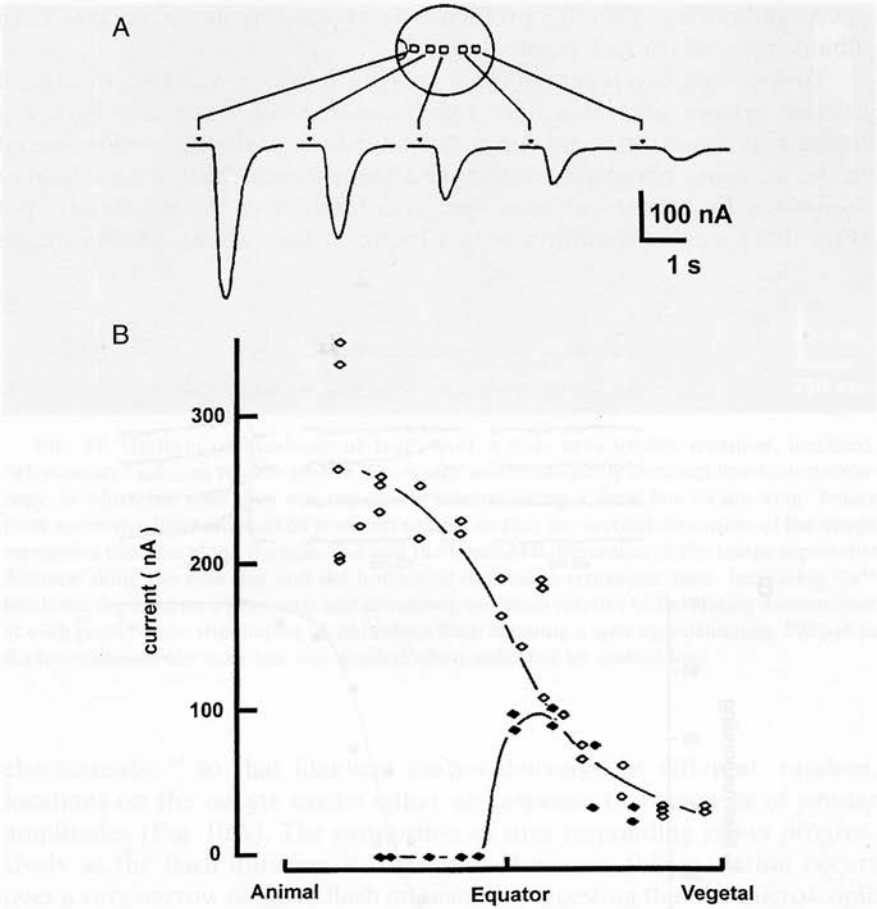

FIG. 9. Spatial variation in currents evoked by $InsP_3$ photoreleased at different locations across the animal/vegetal axis of the oocyte. (A) Traces show currents evoked by identical light flashes, with the photolysis light focused as a small square at different locations on the albino oocyte as denoted in the diagram. The oocyte was oriented with the germinal vesicle (animal pole) to the left. (B) Variation in the peak size of the current at different photolysis positions. Filled symbols are measurements from a pigmented oocyte and open symbols from an albino oocyte. Curves were drawn by eye.

be affected by optical factors in the cell. Wild-type oocytes are strongly pigmented in the animal hemisphere. In these cells, sensitivity to photolysis flashes is greatly reduced in the animal hemisphere (filled symbols, Fig. 9B), presumably because the superficial pigment blocks penetration of photolysis light into the cell. In the experiment illustrated (Fig. 9A and

open symbols Fig. 9B), this problem was avoided by use of oocytes from albino frogs, which lack pigmentation.

To examine heterogeneity in sensitivity between different functional calcium release sites at a finer (micrometer) scale,[13] the UV light was focused to a spot of about 2 μm diameter by a pinhole aperture placed in the arc lamp photolysis system, and fluorescence calcium signals were monitored by a confocal laser spot concentric with the photolysis spot (Fig. 10). Calcium liberation at this localized level shows an all-or-none

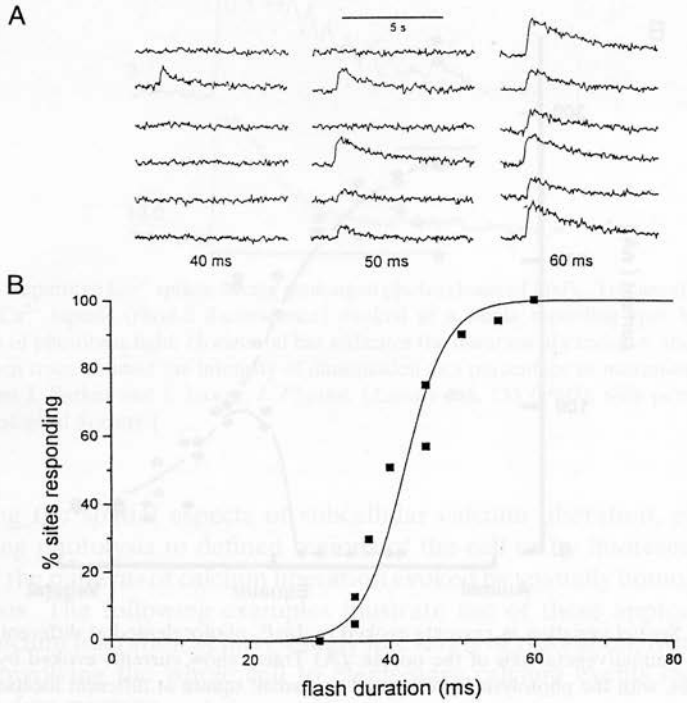

FIG. 10. Local pinhole photorelease of InsP_3 reveals little variation in sensitivity to InsP_3 at different sites. (A) Traces show Ca^{2+} fluorescence monitored from a stationary confocal spot in response to photorelease of InsP_3 evoked by flashes of UV light focused to a 2-μm spot concentric with the confocal spot. Each trace was obtained with the oocyte moved to a new random position, and the columns of traces show responses to three different flash durations as indicated. Note the all-or-none characteristics of the responses. (B) Percentage of sites responding to flashes of varying durations. Data were obtained from traces similar to those in (A). Points indicate the percentage of trials ($n > 20$) with each flash duration in which responses were observed. All responses were from a single albino oocyte, at randomly chosen locations, constrained to fall within a 200 × 200-μm area of the animal hemisphere. [Reproduced by I. Parker, I. Choi, and Y. Yao, *Cell Calcium* **20,** 105 (1996), with permission of *Cell Calcium.*]

FIG. 11. Uniform photorelease of $InsP_3$ over a wide area evokes transient, localized, "elementary" calcium release events. The image was obtained by confocal line-scan microscopy, in which the laser spot was repeatedly scanned along a fixed line 50 μm long. Traces from successive lines are stacked from left to right so that the vertical dimension of the image represents distance along the scan line and the horizontal dimension of the image represents distance along the scan line and the horizontal dimension represents time. Increasing Ca^{2+} levels are depicted on a gray scale and are shown as a ratio relative to the resting fluorescence at each pixel before stimulation. A photolysis flash covering a spot approximately 100 μm in diameter around the scan line was applied when indicated by dashed line.

characteristic,[34] so that identical flashes delivered at different, random, locations on the oocyte evoke either no response or responses of similar amplitudes (Fig. 10A). The proportion of sites responding grows progressively as the flash duration is increased. However, this gradation occurs over a very narrow range of flash intensities, suggesting that the microscopic sensitivity to $InsP_3$ varies only slightly from site to site.

A different approach to obtain spatial information is to image calcium release evoked by homogeneous photolysis of c-$InsP_3$ over a relatively wide (50–100 μm) area, as illustrated in Fig. 11. In this case, fluorescence calcium images are obtained using a rapid (8.0 msec per line), high-resolution (0.2 μm/pixel) confocal line-scan system. Such studies reveal that homogeneous stimulation gives rise to localized "elementary" release events, originating in a stochastic manner at particular sites that probably represent clusters of $InsP_3R$.[13]

Finally, line-scan calcium imaging can be combined with localized photorelease induced by a focused spot from a UV laser to visualize calcium

[34] I. Parker and I. Ivorra, *Science* **250,** 977 (1990).

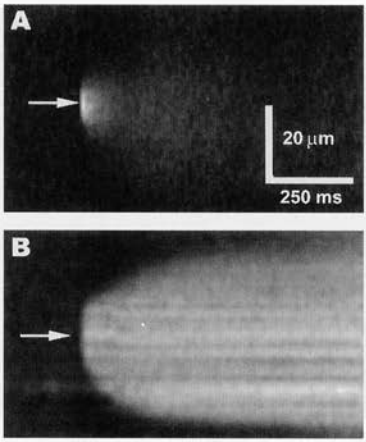

FIG. 12. Examples of UV laser "point" photolysis of caged compounds monitored by confocal line-scan imaging. (A) Control experiment monitoring fluorecsence of fluorecein photoreleased in a droplet of caged precursor. The UV laser flash was delivered at the location and time indicated by the tip of the arrowhead. (B) Ca^{2+} release evoked following photolysis of caged InsP_3 by a focused spot from a UV laser. The oocyte was loaded with Oregon green-1 (50 μM) and caged InsP_3 (5 μM), and recordings were made at a depth of 7 μm from the surface. The laser spot was positioned in the center of the line scan, and the tip of the arrowhead denotes the location and time of the flash. Line-scan imaging was performed as in Fig. 11. [Reproduced from I. Parker, N. Callamaras, and W. Wier, *Cell Calcium* **21,** 441 (1997), with permission of *Cell Calcium.*]

liberation as InsP_3 diffuses from a virtual point source. Figure 12A shows a control experiment in which fluorescein is photoreleased from a droplet of caged precursor (Molecular Probes). The image shows that fluorescein is formed almost immediately following the flash and subsequently spreads rapidly, consistent with diffusion in free solution. However, calcium liberation evoked by "point" photorelease of InsP_3 in the oocyte shows more complex characteristics (Fig. 12B). Calcium signals do not begin until after a latency of several tons of milliseconds and are first seen at the site of photorelease. Liberation then occurs at increasing distances from the photolysis spot, giving an indication of the "range of action" of InsP_3, reflecting both its diffusion and the requirement for [InsP_3] to exceed a specific threshold to evoke calcium release.

Final Comments

In summary, c-InsP_3 is an invaluable tool for the study of intracellular Ca^{2+} signaling due to the degree of control that can be exercised over the intensity, duration, and spatial extent of stimulation. This technique has

been used to extend whole cell studies of the InsP_3-mediated Ca^{2+} signaling system in *Xenopus* oocytes. Combination of flash photolysis together with fluorescence Ca^{2+} imaging or membrane current recording provides a means to study the transient kinetics and modulation of spatially complex, dynamic Ca^{2+} signaling events in an intact cell. Moreover, given the flexibility, simplicity, and relative affordability of the basic apparatus necessary to conduct these experiments, the approach should be well within the reach of many investigators.

Acknowledgments

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[22] Characterization and Application of Photogeneration of Calcium Mobilizers cADP-Ribose and Nicotinic Acid Adenine Dinucleotide Phosphate from Caged Analogs

By KYLE R. GEE and HON CHEUNG LEE

Introduction

Two independent mechanisms for the mobilization of internal calcium stores have been identified in sea urchin eggs. The calcium-mobilizing metabolites in these mechanisms are the novel nucleotides cyclic ADP-ribose (cADPR, **1**)[1] and nicotinic acid adenine dinucleotide phosphate (NAADP, **2**),[2] derived from NAD and NADP, respectively. Cyclic ADP-ribose can function as a modulator of the calcium-induced calcium response mechanism and it also functions as a calcium messenger itself.[3] In addition to the calcium-releasing activity of cADPR in sea urchin eggs, a variety of mammalian, amphibian, and plant cells have been shown to be responsive to the molecule.[4] The calcium release mechanism activated by NAADP is

[1] H. C. Lee, T. F. Walseth, G. T. Bratt, R. N. Hayes, and D. L. Clapper, *J. Biol. Chem.* **264,** 1608 (1989).
[2] H. C. Lee and R. Aarhus, *J. Biol. Chem.* **270,** 2152 (1995).
[3] H. C. Lee, *Recent Prog. Horm. Res.* **52,** 357 (1996).
[4] H. C. Lee, *in* "CRC Series on Pharmacology and Toxicology" (V. Sorrentino, ed.), p. 31, CRC Press, Boca Raton, FL, 1995.

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