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Novel approach to real-time flash photolysis and confocal $[Ca^{2+}]$ imaging

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Abstract

Flash photolysis of “caged” compounds using ultraviolet light is a powerful experimental technique for producing rapid changes in concentrations of bioactive signaling molecules. Studies that employ this technique have used diverse strategies for controlling the spatial and temporal application of light to the specimen. Here we describe a new system for flash photolysis that delivers light from a pulsed, adjustable intensity laser through an optical fiber coupled into the epifluorescence port of a commercial confocal microscope. Photolysis is achieved with extremely brief (5 ns) pulses of ultraviolet light (355 nm) that can be synchronized with respect to confocal laser scanning. The system described also localizes the UV intensity spatially so that uncaging only occurs in defined sub-cellular regions; moreover, since the microscope optics are used in localization, the photolysis volume can be easily adjusted. Experiments performed on rat ventricular myocytes loaded with the Ca^{2+} indicator fluo-3 and the Ca^{2+} cage NP-EGTA demonstrate the system's capabilities. Localized intracellular increases in $[Ca^{2+}]$ can trigger sarcoplasmic reticular Ca^{2+} release events such as Ca^{2+} sparks and, under certain conditions, regenerative Ca^{2+} waves. This relatively simple and inexpensive system is therefore a useful tool for examining local signaling in heart and other tissues.

Keywords

heart; excitation-contraction coupling; sarcoplasmic reticulum; laser; NP-EGTA

INTRODUCTION

Flash photolysis is a technique in which a sudden change in a chemical's concentration is generated by taking advantage of the light-sensitive properties of specially engineered molecules (Adams and Tsien, 1993; Callaway and Yuste, 2002). For instance, a biologically active molecule such as ATP or glutamate can be synthesized with a covalently attached photosensitive “masking” group that renders the molecule inactive. Such a masking group is often referred to as a “cage.” Absorption of a photon, generally in the ultraviolet range of the spectrum, photochemically transforms or cleaves the cage to restore the full activity of the biological molecule. By applying this principle, Ca^{2+} buffers that decrease their affinity for Ca^{2+} when exposed to light have been engineered (Kaplan, 1990). Applying a brief flash of UV light to a cell loaded with this “caged Ca^{2+} ” produces a rapid jump in intracellular Ca^{2+}

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concentration ($[Ca^{2+}]_i$). Flash photolysis is therefore a useful tool for examining physiological phenomena that depend on fast concentration changes.

A process in which rapid concentration changes are important is Ca^{2+} -induced Ca^{2+} release (CICR), the key step linking electrical excitation to contraction in heart muscle cells (Bers, 2002; Guatimosim et al., 2002). In CICR, depolarization of the cell membrane opens Ca^{2+} channels, and Ca^{2+} entry through these channels triggers the release of a larger amount of Ca^{2+} from the sarcoplasmic reticulum (SR). Together these events transiently increase global $[Ca^{2+}]_i$ from ~ 100 nM to ~ 1 μ M, causing Ca^{2+} ions to bind to myofilaments and initiate contraction. In diseases such as heart failure, Ca^{2+} signaling can become deranged such that the systolic and diastolic levels, as well as the kinetics of changes in $[Ca^{2+}]_i$, are altered (Houser et al., 2000). Thus, studies of CICR can provide insight into both normal cardiac physiology and the mechanisms that underlie pathology.

Most experimental investigations of cardiac CICR use membrane depolarization, achieved by electrical stimulation or voltage clamp, to initiate Ca^{2+} release. An advantage of this is that the relationship between Ca^{2+} release events and their physiological triggers can be examined. A disadvantage, though, is that the events occurring at the membrane may be incompletely understood, or might not always provide reliable, repeatable triggers. For instance, if two voltage-clamp depolarizations are delivered in rapid succession, Ca^{2+} channels that were inactivated during the first pulse will have not yet fully recovered (Tseng, 1988), and less Ca^{2+} current will be induced. In this light, flash photolysis of caged Ca^{2+} could be a particularly useful technique for CICR studies because nearly instantaneous (< 100 μ s; (Ellis-Davies et al., 1996; Escobar et al., 1997)) changes in $[Ca^{2+}]$ can be produced independent of events occurring at the membrane. Indeed, several groups have used flash photolysis in planar lipid bilayer studies to examine how SR Ca^{2+} release channels, known as ryanodine receptors (RyRs), respond to sudden changes in $[Ca^{2+}]$ (Gyorke and Fill, 1993; Valdivia et al., 1995; Zahradnikova et al., 1999). Other studies on isolated cells have generated uniform increases in $[Ca^{2+}]_i$ throughout the entire cytoplasm (Valdeolmillos et al., 1989; Niggli and Lederer, 1990; Näbauer and Morad, 1990; Patel et al., 1995; Lipp and Niggli, 1996; Diaz et al., 2001) to demonstrate fundamental properties of CICR.

For many cellular processes, uniform illumination of a cell is satisfactory for flash photolysis experiments. In contrast, CICR in heart cells occurs as a collection of discrete, spatially-restricted events rather than as a spatially uniform increase in $[Ca^{2+}]_i$ (Cannell et al., 1995). These local release events, known as Ca^{2+} sparks (Cheng et al., 1993), can be visualized with confocal fluorescence microscopy. Thus, to examine CICR at the level of the spark, a flash photolysis system must increase $[Ca^{2+}]_i$ only in a defined, localized region of the cell. In addition, it would be advantageous to uncage Ca^{2+} with extremely brief flashes of light and to synchronize these flashes to other events. Here we describe a new system that enables such spatially localized and temporally synchronized flash photolysis. This relatively simple system uses an optical fiber to interface a small Nd:YAG laser with a laser scanning confocal microscope so that fluorescence recordings can be synchronized with UV flashes. The system is flexible so that the volume in which photolysis occurs can be varied, and changes in the timing or intensity of the flash can be made during the experiment. We demonstrate some of the capabilities of the new system with experiments in ventricular myocytes and discuss the strengths of this system in the context of local Ca^{2+} signaling in heart cells.

METHODS

Cell isolation

Adult rat heart cells were prepared by standard methods (Cannell et al., 1994). Briefly, rats of either sex weighing between 200 and 300 g were killed by lethal intraperitoneal injection of

pentobarbital (100 mg/kg). The hearts were rapidly removed and perfused using the Langendorff method with Ca^{2+} -free modified Tyrode's solution (see below for solution components) until the blood was washed out. Hearts were then perfused with Tyrode's solution containing 50 μM CaCl_2 along with 1.4 mg/mL collagenase (type 2, Worthington, Lakewood, NJ) and 0.04 mg/mL protease (type XIV, Sigma, St. Louis) until they were soft (approximately 5 minutes). The hearts were removed from the perfusion apparatus, minced into ~ 1 mm chunks, and stirred for 4 minutes in Tyrode's solution containing 50 μM CaCl_2 , 0.7 mg/mL collagenase, and 0.02 mg/mL protease. Cells were filtered through a 200 μm mesh to remove tissue chunks, and extracellular Ca^{2+} was raised to 0.50 mM over 10 minutes through three centrifuge cycles. Cells were stored in DMEM until they were used (within 8 hours). All rats were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland, and all experimental protocols were approved by this committee. Experiments were performed at room temperature (22 - 24°C).

Solutions and Chemicals

During experiments, cells were superfused with modified Tyrode's solution containing (in mM): NaCl 140, KCl 5, HEPES 5, NaH_2PO_4 1, MgCl_2 1, CaCl_2 3, glucose 10 (pH 7.4). To allow for simultaneous flash photolysis and confocal imaging of $[\text{Ca}^{2+}]_i$, cells were loaded for 30 minutes with 5 μM fluo-3 AM and for 10 minutes with 5 μM NP-EGTA AM (both from Molecular Probes, Eugene, OR) at room temperature. Before use, cells were washed and stored for at least 20 minutes in Tyrode's solution to allow for de-esterification of the AM species. Thapsigargin and ryanodine, used to disable SR Ca^{2+} release in some experiments, were from Penick (Newark, NJ).

To determine the three-dimensional profile of the flash intensity, DMNB-caged fluorescein dextran (10 kD MW, Molecular Probes, Eugene, OR) was dissolved in a synthetic lacquer (No. 401 Protective Lacquer, AM Cosmetics, North Arlington, NJ). Drops of this mixture were placed on glass coverslips and allowed to harden. This procedure produced a non-diffusing substrate that only became fluorescent after exposure to UV light.

Simultaneous flash photolysis and confocal recordings

The newly developed flash photolysis system is schematically illustrated in Figure 1. The photolysis module is interfaced with a laser scanning confocal microscope (LSM 510, Zeiss, Jena, Germany) so that photolysis can occur during fluorescence measurements. For recordings of $[\text{Ca}^{2+}]_i$, light at 488 nm from a Kr-Ar laser is scanned across the cell, and fluorescence above 505 nm is detected. Confocal measurements of fluorescence are expressed in units of F/F_0 and converted to estimates of $[\text{Ca}^{2+}]_i$ using the equation $[\text{Ca}^{2+}] = F/F_0 * K_d / (K_d / [\text{Ca}^{2+}]_{\text{rest}} + 1 - F/F_0)$ (Cheng et al., 1993). For these calculations we assume $[\text{Ca}^{2+}]_{\text{rest}} = 100$ nM and the K_d for Ca^{2+} binding to fluo-3 is 900 nM in cells loaded with the AM-form of the dye (Loughrey et al., 2003).

For photolysis, light at 355 nm from a frequency-tripled Nd-YAG laser (Minilite, Continuum, Santa Clara, CA) is coupled into a multi-mode optical fiber. The circular output aperture is positioned at one of the microscope's image planes, producing a disk of UV energy at the object plane of the microscope. The UV spot's size depends on the fiber optic diameter and the magnification of the microscope objective. A spot illumination adaptor from Rapp OptoElectronics (Hamburg, Germany) was used to couple the fiber to the epifluorescence port of the microscope. The size of the UV spot projected onto the cell depends on the fiber optic diameter and the magnification of the microscope objective. The experiments displayed here use either a 100 $\times$, 1.3 N.A. oil-immersion objective (Plan-Neofluar, Zeiss) or a 63 $\times$, 1.2 N.A. water-immersion objective (C-Apochromat, Zeiss). These two lenses produce UV spots with diameters of roughly 3 and 5 μm , respectively (see Figure 2). Two precision adjustment screws

allow the axial (Z) location of the UV spot to be changed relative to the microscope plane of focus. Thus, flash photolysis can be performed in the same plane as is being scanned, as illustrated in Figure 2.

The Nd-YAG laser used for photolysis delivers UV energy in 5 ns pulses, with a maximal pulse repetition rate of 15 Hz. In most experiments shown here, the laser was operated in “single shot” mode, with 5 volt TTL pulses used to trigger the 5 ns UV flashes. A pulse generator (Pulsemaster A300, World Precision Instruments, Sarasota, FL) controlled both UV flashes and confocal scanning, thereby allowing photolysis to be tightly synchronized with recordings of intracellular $[Ca^{2+}]$. The total energy in each 5 ns laser pulse is adjustable from 0 to 10 mJ, although the majority of the experiments presented here use energies less than 100 μ J. We estimate that optical components in the system attenuate the light intensity by $\sim 75\%$. According to manufacturer specifications, the optical fiber (Oz Optics, Ottawa, Canada) and the dichroic mirror in the adaptor (Rapp OptoElectronics) transmit, respectively, 60% and 85% of the incident light. Microscope optics are expected to attenuate light by an additional 50%, as measured by Lipp and Niggli (Lipp and Niggli, 1998). Thus, the energy delivered to the specimen in these experiments is roughly 2 to 20 μ J.

RESULTS

This new system allows for the delivery of UV laser pulses with precise spatial and temporal control and for synchronization with confocal imaging to monitor the cellular responses. Here we present results from experiments performed on cardiac myocytes that demonstrate the new system's capabilities.

The three-dimensional spatial extent of the UV flash energy was determined as shown in Figure 2. Caged fluorescein was dispersed in a lacquer and allowed to dry so that diffusion was severely hindered (see Methods). This compound is non-fluorescent until UV light converts it to a bright fluorophore that can be visualized in the microscope. In the experiment shown in Figure 2a, a series of low-energy UV flashes (50 μ J; 30 seconds at 10 Hz) were delivered to the sample through the 100 μ m diameter fiber optic and the 100 $\times$ microscope objective. A three dimensional Z-stack of optical sections was then acquired to determine the spatial profile of the photolysis. The two-dimensional image of fluorescence in the microscope plane of focus (top left) shows that flash energy was confined to a circular region with a diameter of roughly 3 μ m. Linear intensity profiles in the x (blue) and y (red) directions, taken through the center of the spot, are shown above the 2-D image. Identically scaled images of fluorescence in the x - z and y - z planes are displayed below and to the right to confirm that flash energy is maximal in the plane of focus and declines with distance along the microscope's optical axis. The spatial localization of flash energy in the z direction is superior to that seen previously in similar photolysis systems (Wang and Augustine, 1995), presumably because the greater numerical aperture of the microscope objective (1.3 versus 0.7) leads to a thinner plane of focus.

Additional experiments with the calibration substrate were performed to demonstrate how changes in the microscope optics or the optical fiber position affect the size and location of the UV flash. The size of the UV spot was inversely related to the magnification of the microscope objective, as illustrated in Fig. 2b. The diameter of the uncaging spot in the plane of focus (defined as the region in which fluorescence intensity was $> 50\%$ of the peak) was 3.2 μ m with the 100 $\times$ objective but 4.8 μ m when the 63 $\times$ objective was used. Fig. 2c shows that the plane of maximal flash energy can be changed by using the micromanipulator to position the fiber optic output aperture relative to the microscope back focal plane. Thus, maximal uncaging can be produced above or below the microscope plane of focus if desired.

High speed frame scanning (31 ms/frame) of a rat ventricular myocyte loaded with fluo-3 and NP-EGTA is shown in Figure 3. This displays the response of a quiescent cell to a single 5 ns, 42 μ J UV flash delivered through the 100 μ m fiber optic and the 63 $\times$ objective. The three frames displayed, which show fluorescence images of $[Ca^{2+}]_i$ before the flash, ~ 15 ms after the flash, and ~ 75 ms after the flash, demonstrate that a single 5 ns pulse from the laser produces a localized, transient elevation of $[Ca^{2+}]_i$. In this example, the spatial extent of the $[Ca^{2+}]_i$ increase is roughly 8 μ m, slightly larger than the ~ 5 μ m spot observed after photolysis of immobilized caged fluorescein. This occurs because Ca^{2+} -bound fluo-3 begins to diffuse out of the flashed region immediately after uncaging.

The repeatability of the photolysis response and its dependence on flash energy were examined in cells pre-treated for one hour with ryanodine and thapsigargin (1 μ M each). These agents deplete the SR and prevent Ca^{2+} release. Under these conditions, UV flashes of the same energy delivered two seconds apart produce nearly identical spatiotemporal patterns of fluorescence (Figure 4a). Thus, repeated flashes liberate equivalent quantities of Ca^{2+} . The peak F/F_0 value of 2.6 in these examples corresponds to $[Ca^{2+}]_i$ of roughly 316 nM in the flash region (see Methods). Fig. 4b shows example images from a different experiment to demonstrate that when flash energy is increased, Ca^{2+} uncaging increases monotonically. A semi-quantitative estimate of the total quantity of Ca^{2+} uncaged by these flashes can be obtained using the equations with which Chandler et al. analyzed Ca^{2+} sparks in frog skeletal muscle (Chandler et al., 2003). For example, the responses shown in Fig. 4a have spatial width (measured as full-width at half maximum, FWHM) of ~ 8 μ m at the time they reach peak F/F_0 . This corresponds to a "signal mass" of approximately 1000 μ m³ (mass = $1.206 \cdot \Delta F \cdot FWHM^3$). If AM-loaded myocytes contain 50 μ M fluo-3, then 5 μ M fluo-3 is bound to Ca^{2+} in resting cells, and the flashes in Fig. 4a increase $[Ca^{2+}\text{-fluo3}]$ by $5 \mu\text{M} \cdot 1000 \mu\text{m}^3 = 5 \times 10^{-18}$ mol, or 3×10^6 molecules. The total number of Ca^{2+} ions uncaged depends on the percentage of the released ions that become bound to fluo-3, which previous analyses have estimated to be 25% (Chandler et al., 2003). Thus, roughly 12 million Ca^{2+} ions are released during these (relatively strong) flashes. Computer simulations are currently being performed to develop more precise estimates of the spatial profile of Ca^{2+} concentrations induced by such UV flashes.

In contrast to cells in which SR Ca^{2+} release is blocked, repeated flashes delivered to control cells with SR release intact can produce dramatically different changes in $[Ca^{2+}]_i$. Figure 5 displays responses to four consecutive 61 μ J flashes delivered 10 seconds apart in a resting cardiac myocyte. The long interval between flashes and the small volume illuminated by each flash ensure that NP-EGTA depletion is negligible and that the cell returns to the same state before each flash is delivered. In addition, no change in fluorescence was observed in control experiments performed in cells loaded with fluo-3 but not NP-EGTA (not shown). These experiments verified that the observed changes in fluorescence were due to uncaging and/or SR Ca^{2+} release rather than laser photodamage. Because there was essentially no variability in the photolysis-induced responses when SR Ca^{2+} release was blocked (Figure 4a), the differences amongst trials in the present experiment are due to SR Ca^{2+} release events triggered by the photoreleased Ca^{2+} . A rough estimate of the SR Ca^{2+} release triggered by a flash can be obtained by uncaging Ca^{2+} several times at a given location and pulse energy, then assuming that the dimmest response represents a trial in which no Ca^{2+} release was triggered. With this assumption, the SR Ca^{2+} release component can be derived by subtracting the dim response, which reflects only uncaging, from the remaining trials. Through this simple analysis, fluorescence signals with spatiotemporal characteristics similar to Ca^{2+} sparks are obtained (Fig. 5; image and trace at bottom right). Thus, the simplest explanation of the data is that Flash 1 uncaged Ca^{2+} from NP-EGTA but did not trigger any release whereas Flash 2 also triggered a Ca^{2+} spark at the location indicated in Fig. 5.

The new system, by producing a spatially confined and controllable increase in $[Ca^{2+}]$ in the cytoplasm, also allows repeatable and reliable triggering of regenerative Ca^{2+} waves under appropriate conditions (Figure 6). When a rat ventricular myocyte bathed in 3 mM external Ca^{2+} had been quiescent for several minutes, a single 37 μ J flash produced a small increase in fluorescence at the flash location (Fig. 6a, black) and no response at a distal location (red). The cell was then field stimulated at 1 Hz for 1 minute to increase the SR Ca^{2+} load. Thereafter, a 37 μ J flash caused a larger, but still localized, increase in F/F_0 , indicating more photolytic uncaging of Ca^{2+} and/or more SR Ca^{2+} release under these conditions (Fig. 6b). Occasionally, however, identical flashes delivered under these conditions caused regenerative Ca^{2+} waves rather than localized increases in $[Ca^{2+}]_i$ (Fig. 6c). In this experiment, 2 flashes out of 6 produced waves whereas the remainder did not. With this photolysis system, then, we can initiate Ca^{2+} waves at defined locations and investigate how factors such as SR load and diastolic $[Ca^{2+}]$ influence the initiation and propagation of these events.

DISCUSSION

The system described in this report allows intracellular uncaging of Ca^{2+} , and the consequent triggering of SR Ca^{2+} release, with precise temporal and spatial control. Because the quantity of Ca^{2+} uncaged, which represents the trigger for SR release, is highly reproducible at a given pulse energy (Fig. 4b), we can investigate quantitatively the relationship between local triggers and the resulting Ca^{2+} release events. This system therefore represents an advance over previous endeavors to examine CICR with flash photolysis in heart cells. In most prior studies, technical limitations, primarily inadequate spatial resolution, have prevented the examination of local Ca^{2+} release events. The most significant progress in this regard has been made by Ernst Niggli and coworkers, who have demonstrated that two-photon photolysis of DM-nitrophen, by generating $[Ca^{2+}]_i$ increases in very small volumes, can trigger local release events (Lipp and Niggli, 1998; DelPrincipe et al., 1999b; Lindegger and Niggli, 2005). However, in these experiments laser pulse trains with durations on the order of 30-50 ms were applied. It is unclear whether other processes, such as uptake of Ca^{2+} into the SR, were also being activated during these long stimuli. Similarly, in these experiments consecutive two photon pulse trains were only delivered with a minimum gap of 200 ms between them (DelPrincipe et al., 1999b). This long delay between optical stimuli prevents this technique from being used to examine phenomena that occur more quickly, such as the recovery of Ca^{2+} spark amplitude (Brochet et al., 2005; Sobie et al., 2005). Thus, despite the significant findings generated by these studies, a re-examination, which will be feasible with our new photolysis system, may be warranted.

In particular, the new system will allow us to investigate the controversial proposal that localized $[Ca^{2+}]$ stimuli can trigger not only Ca^{2+} sparks, but also smaller release events termed “ Ca^{2+} quarks.” When short UV pulses and wide-field illumination were used to uncage Ca^{2+} from DM-nitrophen throughout the entire cytoplasm, Lipp and Niggli (Lipp and Niggli, 1996) did not observe any spatial heterogeneity in the resulting fluorescence, leading them to suggest that only small release events, but not Ca^{2+} sparks, were triggered. We observe significant spatial variation in the cellular Ca^{2+} response to flash photolysis, even when a relatively large region in the cell is affected by the UV flash. The reasons for the differences with the results of Lipp and Niggli remain to be determined. In their follow up study employing two-photon photolysis (Lipp and Niggli, 1998), these investigators were able to trigger Ca^{2+} release events similar to sparks as well as smaller release events, which they termed quarks. More experiments similar to that shown in Fig. 5 will be necessary to determine the nature of the Ca^{2+} release events triggered by uncaged Ca^{2+} . Localized release under these conditions may consist entirely of Ca^{2+} sparks or of sparks in addition to smaller events. The nature of the events that are triggered may also depend on the sub-cellular location that is examined and the intensity of the flashes used. A thorough examination of the release events that can be

triggered under different conditions will lead to a better understanding of how CICR is controlled in heart.

NP-EGTA served as the Ca^{2+} cage in the experiments presented here, in contrast to previous studies which have used DM-nitrophen. The primary advantage of NP-EGTA is that it is highly selective in binding Ca^{2+} over Mg^{2+} . Thus, flash photolysis experiments using this cage can be performed with physiological levels of Mg^{2+} present, whereas experiments using DM-nitrophen require that intracellular $[\text{Mg}^{2+}]$ be reduced to unphysiological levels. Because Mg^{2+} causes a substantial decrease in both RyR open probability and conductance (Zucchi and Ronca-Testoni, 1997; Fill and Copello, 2002), data obtained under conditions of low Mg^{2+} must be interpreted cautiously. The disadvantage of NP-EGTA is that its K_d for Ca^{2+} is 80 nM (Ellis-Davies and Kaplan, 1994), which means that at resting $[\text{Ca}^{2+}]_i$ of 100 nM, roughly 45% of the cage exists in the Ca^{2+} -free form and acts as a mobile cytoplasmic Ca^{2+} buffer. The excess buffering capacity contributed by NP-EGTA is unlikely to interfere with triggering of Ca^{2+} release because slow buffers such as EGTA and its derivatives are ineffective in altering signaling processes on a local scale (Smith et al., 1996; Naraghi and Neher, 1997). The free NP-EGTA will, however, affect the spatial spread of released Ca^{2+} and the time course of $[\text{Ca}^{2+}]$ decay after release is triggered (Diaz et al., 2001). This buffering effect can be studied quantitatively in future experiments in cells treated with ryanodine and thapsigargin (as in Figure 4). Under these conditions, the responses to several flashes of the same energy can be averaged to determine the spatiotemporal spread of the uncaged Ca^{2+} with precision. Effects of excess buffering can be determined by comparing the flash-induced changes in $[\text{Ca}^{2+}]_i$ in cells loaded with NP-EGTA with those seen in cells loaded with DM-nitrophen. Because SR Ca^{2+} release will be disabled in these experiments, effects of $[\text{Mg}^{2+}]_i$ on RyR gating will not play a role, and differences in spatial and temporal changes in fluorescence will result from the excess buffering provided by the free NP-EGTA. A newer cage known as DMNPE-4 (DelPrincipe et al., 1999a), which combines the advantages of high affinity for Ca^{2+} (19 nM) with poor binding to Mg^{2+} , should be ideal for experiments using flash photolysis to investigate CICR in cardiac myocytes, although relatively few studies have used this compound thus far (Ng et al., 2002; Xu et al., 2002).

Our system localizes the spatial extent of the flash energy by using the microscope optics to image the exit end of an optical fiber at the specimen. Alternative methods for achieving spatial localization include two-photon (2P) photolysis and a “proximity focused” fiber optic system (Margulis and Tang, 1998; Parpura and Haydon, 1999; Eberius and Schild, 2001; Hoesch et al., 2002; Bernardinelli et al., 2005). Our method offers both advantages and disadvantages compared with these approaches. As mentioned, 2P photolysis has been used by Niggli's group in studies with caged Ca^{2+} in heart cells (Lipp and Niggli, 1998; DelPrincipe et al., 1999b; Lindegger and Niggli, 2005) and by several other groups in different types of tissue (Denk, 1994; Wang et al., 2000; Matsuzaki et al., 2001; Soeller et al., 2003). The main advantage of 2P photolysis is the potentially superb spatial resolution it allows—photolysis is confined to an ellipsoidal volume extending ~ 700 nm along the optical axis of the microscope and ~ 300 nm in the plane of focus. A potential disadvantage is that this sub-femtoliter volume may not contain enough caged molecules for a short light pulse to activate the biological process of interest. Moreover, the 2P cross sections of most existing caged molecules are low, making photolysis by 2P excitation inefficient. For instance, Brown et al. measured a 2P action cross section of 0.013 GM (Goppert-Meyer; $10^{-50} \text{ cm}^4 \cdot \text{s}/\text{photon}$) for DM-nitrophen and were unable to measure any 2P photorelease of Ca^{2+} from NP-EGTA (Brown et al., 1999). If, from this result, we assume that the 2P action cross-section of NP-EGTA is ≤ 0.001 GM and compare this with the product of extinction coefficient and quantum yield, $224 \text{ M}^{-1} \text{ cm}^{-1}$, measured for one-photon photolysis (Ellis-Davies and Kaplan, 1994), we can calculate that a single UV pulse at 355 nm (50 μJ , 5 ns) will be as effective at uncaging as a 2P pulse train (at 705 nm) lasting for 16 ms (see Appendix for details of calculation). These factors may explain why the 2P

studies discussed above employed 30-50 ms pulse trains to trigger Ca^{2+} release events (DelPrincipe et al., 1999b; Lindegger and Niggli, 2005) and other 2P photolysis studies have required laser power levels approaching the photodamage threshold (Hopt and Neher, 2001; Kiskin and Ogden, 2002). We have been able to trigger Ca^{2+} release using much shorter (5 ns) light flashes for two likely reasons: 1) the volume in which Ca^{2+} uncaging occurred was much larger and thus contained many more caged molecules, and 2) the single-photon cross section (i.e. the extinction coefficient) for absorbing UV light is sufficiently high.

In a proximity focused system, UV light is coupled into an optical fiber that is brought extremely close to the specimen, usually from above via a micromanipulator. One potential advantage of this approach is that UV flashes can be delivered in multiple locations if several fibers are positioned at different sites. A disadvantage is that light exiting the fiber will form a divergent cone, the shape of which is determined by the fiber's numerical aperture. Light intensity will therefore be greatest at the top of the specimen, which will not necessarily correspond to the microscope plane of focus or the region of greatest biological interest. In addition, the energy density of the light reaching the specimen will depend on the distance from the exit aperture to the sample, a variable that may be difficult to control precisely. These difficulties can be partially surmounted by using "microlensed" fibers, but the uncertainty caused by the vertical placement of the fiber makes other strategies attractive. An advantage of the system described here is that the plane of peak flash energy can be adjusted by translating the optical fiber (Fig. 2c). An additional advantage of our system is that the physical size of the flash spot can be altered (to a limited extent) by changing the magnification of the microscope objective or (to a much greater extent) by changing the core diameter of the optical fiber into which the laser pulse is launched. Thus, spatially localized and spatially diffuse flashes can be delivered sequentially to the same cell with minimal disruption. Neither a 2-photon nor a proximity-focused approach affords such flexibility.

In summary, we have described a new system that enables UV photolysis of caged compounds with precise spatial and temporal control. This can be used to study not only Ca^{2+} -induced Ca^{2+} release in heart cells, but also other biological processes in which local and rapid changes in the concentrations of effector molecules are important.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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APPENDIX

To compare the relative rates of Ca^{2+} liberation from NP-EGTA using one-photon (1P) or two-photon (2P) photolysis, we made several assumptions about the conditions under which photolysis occurs in the two cases, as described here.

The susceptibility of a cage to 1P photolysis is often expressed as the product of the extinction coefficient and quantum yield, in units of $\text{M}^{-1}\cdot\text{cm}^{-1}$. To convert this to an "action cross-section" in cm^2 , we use the conversion factor 3.82×10^{-21} (Lakowicz, 1999). Thus, the reported extinction coefficient times quantum yield of $224 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 347 nm (Ellis-Davies and Kaplan, 1994) corresponds to a 1P action cross section for uncaging of $\sigma_{1,u} = 8.56 \times 10^{-19} \text{ cm}^2$. To compute a rate constant for 1P photolysis when a UV pulse at approximately this wavelength is applied, we use the following formula (Koester et al., 1999):

$$k_{1P} = \sigma_{1,u} [I] / (hc/\lambda) \quad (1)$$

where $[I]$ is the time-average light intensity, in W/cm^2 , and h , c , and λ are Planck's constant (6.626×10^{-34} J·s), the speed of light (3.00×10^8 m/s), and the wavelength, respectively.

A 50 μJ , 5 ns pulse has an average power $P = 10$ kW during the pulse. Based on our measurements (see Figure 2), we assume that at the microscope plane of focus, this light is concentrated in a circle with radius $r = 1.6$ μm , yielding an intensity of $[I] = P/(\pi r^2) = 124$ GW/cm^2 . To account for imperfect delivery of light to the specimen, we assume that 90% of the UV light (355 nm) is lost coupling the laser into the optical fiber, and 80% of the remaining light is attenuated by the microscope optics. Together, these values yield a 1P photolysis rate constant of:

$$k_{1P} = 8.56 \times 10^{-19} (0.1) (0.2) (1.24 \times 10^{11}) / \left[(6.626 \times 10^{-34}) (3 \times 10^8) / (355 \times 10^{-9}) \right] = 3.80 \times 10^9 \text{ s}^{-1}$$

For 2P photolysis using a pulsed laser, when the duration of each pulse, τ , is much less than the reciprocal of the pulse repetition rate, $1/f$, the rate constant of 2P photolysis is given by the following formula (Koester et al., 1999):

$$k_{2P} = g^{(2)} \sigma_{2,u} [I]^2 / \left[\tau f (hc/\lambda)^2 \right] \quad (2)$$

where $g^{(2)}$ is the time-zero second order temporal coherence, equal to 0.59 for lasers commonly used for 2P photolysis, and $\sigma_{2,u}$ is the 2P action cross section for uncaging, in units of $\text{cm}^4 \cdot \text{s}/\text{photon}$. Brown et al. (Brown et al., 1999) measured a 2P action cross-section of 0.013 GM (Goppert-Meyer, 10^{-50} $\text{cm}^4 \cdot \text{s}/\text{photon}$) for DM-nitrophen and were unable to measure any 2P photolysis from NP-EGTA. These findings permit an estimate of 0.001 GM as the upper bound for the 2P action cross section of NP-EGTA. In their initial study on 2P photolysis of caged Ca^{2+} in cardiac myocytes, Lipp and Niggli (Lipp and Niggli, 1996) typically operated a laser with a repetition rate of 80 MHz ($f = 80 \times 10^6 \text{ s}^{-1}$) and a pulse width of 75 fs ($\tau = 75 \times 10^{-15}$ s) at a time-average power of 50 mW. If we assume that 2P excitation occurs in a diffraction-limited spot with radius 180 nm (DelPrincipe et al., 1999b), and 80% of the laser light is attenuated by the optics, the time-average intensity $[I]$ at the sample is $0.2(50 \times 10^{-3})/[\pi(1.8 \times 10^{-5})^2] = 9.82$ MW/cm^2 . We therefore calculate a 2P photolysis rate constant of:

$$k_{2P} = 0.59 (0.001 \times 10^{-50}) (9.82 \times 10^6)^2 / \left\{ (80 \times 10^6) (75 \times 10^{-15}) \left[(6.626 \times 10^{-34}) (3 \times 10^8) / (705 \times 10^{-9}) \right]^2 \right\} = 1.19 \times 10^3 \text{ s}^{-1}$$

These calculations therefore imply that over 5 ns, a single UV pulse applied with our system will be roughly 3.2 million times more likely to induce liberation of Ca^{2+} from NP-EGTA than a 2P pulse. From this it follows that a 16 ms 2P pulse train will be as effective as the 5 ns UV pulse, as noted in the text.

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Figure 1. Schematic of Flash Photolysis System

Extremely brief (5 ns) flash photolysis pulses from a frequency-tripled Nd-YAG laser (355 nm) are coupled into a fused silica optical fiber and delivered to the specimen through the microscope optics. The “Photolysis Module” is interfaced with a laser scanning confocal microscope (Zeiss LSM 510) so that cellular fluorescence can be recorded during photolysis. Precise electrical triggering allows laser flashes to be delivered at specified times relative to laser scanning.

Figure 2. Three dimensional distribution of UV laser power

Caged fluorescein was dissolved in a lacquer and allowed to dry. (A) After a series of low-power, 5 ns laser pulses uncaged the fluorescein, a 3 dimensional Z-stack was acquired to determine the spatial distribution of laser power. Photolysis occurred in a region approximately 3 μm in diameter, and in the axial (Z) dimension, photolysis was maximal in the plane of focus. (B) Changing the microscope objective through which the UV light was focused altered the diameter of the uncaging spot in the plane of focus. Photolysis is more localized when light is delivered through a 100× objective (blue line) than when a 63× objective (red line) is used. (C) Plots of average fluorescence in the center of the field of view versus distance along the optical axis for three different optical fiber positions. Linear translation of the fiber's exit aperture with a custom-built micromanipulator varied the location at which maximal uncaging occurred relative to the microscope plane of focus (position zero).

Figure 3. Photorelease of Ca^{2+} in a cardiac cell

Pseudo-color representations of $[\text{Ca}^{2+}]_i$ within a region of a quiescent rat ventricular myocyte loaded with NP-EGTA and fluo-3. Frames of fluorescence within the region displayed were obtained at 31 ms/frame, and a single 5 ns UV pulse (42 μJ) was delivered during the trial. Images obtained before (left), immediately after (middle), and roughly 75 ms after the flash (right) illustrate that the UV pulse caused a transient elevation of $[\text{Ca}^{2+}]_i$ in a region $\sim 8 \mu\text{m}$ in diameter. In this experiment, UV light was delivered to the cell through the 63 $\times$ objective (water immersion, N.A. 1.2).

Figure 4. Repeatability of Ca^{2+} photorelease and dependence on laser power

Line scan images (1.92 ms/line) of changes in $[\text{Ca}^{2+}]_i$ induced by flash photolysis of caged Ca^{2+} in cells pretreated with ryanodine and thapsigargin to block SR release. **(A)** Nearly identical amounts of Ca^{2+} are uncaged by two consecutive flashes (74 μJ ; 2 seconds apart). **(B)** Ca^{2+} uncaging increases with increasing flash energy. The dashed line was drawn by eye.

Figure 5. Localized triggering of CICR with flash photolysis

Four laser flashes (61 μJ; 10 seconds apart) were delivered to a resting rat cardiac myocyte loaded with NP-EGTA. The four identical flashes induced different changes in cellular $[Ca^{2+}]_i$, as seen most clearly in the superimposed F/F_0 versus time traces displayed at bottom left. The variability observed indicates that the Ca^{2+} liberated by the consecutive flashes caused differing amounts of SR Ca^{2+} release. An estimate of the SR Ca^{2+} release triggered by each flash was obtained by assuming that the dimmest response, that to flash 1, represented only photorelease of Ca^{2+} . This space-time image of fluorescence was then subtracted from the others to obtain images that reflected only SR Ca^{2+} release. This procedure yielded a signal with a spatiotemporal profile very similar to that of a Ca^{2+} spark (bottom right). The white bar on the image delineates the 1.84 μm region over which the signal was averaged to obtain the “spark-like” local Ca^{2+} transient shown.

Figure 6. Triggering of regenerative Ca^{2+} waves with localized flash photolysis

(A) Line scan of the effect of a single flash (61 μJ) delivered to a cell that had been quiescent for several minutes. Black and red lines show, respectively, F/F_0 averaged over 7 pixels at the location of the flash and at a distal location, as marked by the black and red bars to the left of the image. (B) After stimulating the cell electrically for 1 minute at 1 Hz to increase SR Ca^{2+} load, a single flash of the same energy induced a larger increase in F/F_0 . (C) Another flash delivered under the same conditions as in (B) triggered a regenerative Ca^{2+} wave.