Femtosecond Laser-Induced Calcium Release in Neural-Type Cells

Nicholas Isaac Smith[a], Shigeki Iwanaga[b], Taro Beppu[b], Katsumasa Fujita[b], Osamu Nakamura[a], Satoshi Kawata[a,b,c]

[a]Department of Frontier Biosciences, Osaka University
[b]Department of Applied Physics, Osaka University
[c]The Institute of Physical and Chemical Research (RIKEN), Japan

ABSTRACT

Here we show that femtosecond laser irradiation can be used to evoke dynamic calcium concentration changes in living cells. The relatively localized interaction that results from the two-photon absorption process allows the release of calcium from intracellular stores in cells in vitro. The self-catalytic response to calcium elevation in a cell can increase the initial release of calcium further so that the entire cell undergoes a rise in cytosolic calcium concentration (i.e. a calcium wave). The calcium stimulation was observed in HeLa (non-excitable) and PC12 (excitable) cells, and could be seen to occur inside a range of power levels between approximately 20 to 80mW. The observation of direct calcium release by femtosecond laser which leads to a calcium wave in the cell has implications for photolytic calcium uncaging experiments since it could be a competing, or even dominant factor in some experiments using caged calcium for the generation of calcium waves.

1. INTRODUCTION

Calcium waves in cells have been studied extensively in order to understand the intricate role that calcium plays as a signaling tool[1]. Calcium is an integral part of a diverse range of signal transduction pathways, and is responsible for or involved in events as fundamental as fertilization, mitosis, apoptosis, nerve signalling, and muscle contraction[2,3,4,5]. The importance of calcium as a signaling molecule in cells has ensured that there has been significant interest in learning how to control the calcium levels in a cell. Either to mimic the effects of natural changes in the calcium concentration in a cell, or to determine how a cell reacts to an abnormal elevation or decrease of calcium, researchers have sought methods to control cellular calcium levels. These methods are generally concerned with how to increase the calcium level in a cell and can therefore be termed “calcium stimulation” methods.

In vitro, cells of interest are cultured in a dish and the most simple method of evoking a change in cellular calcium level is to subject the cell to a certain amount of mechanical stress[6]. This can be done with a mechanical probe, interacting with the cell membrane. The nominal concentration of calcium inside the cell is usually orders of magnitude lower than the extracellular calcium concentration. If the cell, particularly the membrane, is subjected to stress, the membrane permeability can be temporarily affected, and stretch-activated channels in the membrane can briefly open, allowing external calcium to flow with the concentration gradient and increase the calcium level inside the cell. Cells also have a self-catalytic calcium action that allows a small amount of calcium concentration increase to lead to a larger release from the internal stores in the cell[7]. The cytosolic calcium concentration is therefore supplemented by the release of calcium into the cytosol from the cellular calcium stores, usually the endoplasmic or sarcoplasmic reticulum. This calcium-induced calcium release (CICR) is a process in the cell that allows the release of μM order amounts of calcium throughout the entire cell without requiring all of the elevated calcium to have entered the cell from the extracellular solution. When the self-catalytic reaction causes sufficient calcium to be released that the entire cytosolic calcium concentration is significantly elevated, the process is known as a calcium wave. Calcium waves are complex, being modulated in intensity and repetition frequency with a nonlinear interaction with other cellular signaling pathways, and are beyond the scope of this manuscript. Therefore, here we will define any substantial increase in the cytosolic calcium concentration as a calcium wave.

Application of an electrical shock, or spark, to the cell membrane is another common method of evoking a calcium response[8]. The cell membrane normally retains a resting voltage difference of around -50mV between the inner and outer side. It is partly this voltage difference, or membrane potential, that is responsible for preserving the large difference in charged ion concentration between the cytosol and extracellular solution. Additionally, the cell membrane

Nanobiophotonics and Biomedical Applications II, edited by Alexander N. Cartwright, Marek Osinski, Proceedings of SPIE Vol. 5705 (SPIE, Bellingham, WA, 2005) · 1605-7422/05/$15 · doi: 10.1117/12.596923

contains voltage-sensitive channels that allow limited transport of ions across the cell membrane only when the resting membrane potential is changed. Application of an electrical spark to the membrane allows the opening of voltage-sensitive channels and the influx of an amount of calcium from the external solution. This, if sufficient, can lead to a whole-cell calcium rise in a similar manner to mechanical stimulation.

Calcium concentration changes in the cell can also be artificially evoked by the addition of a suitable agonist to the extracellular solution. Addition of ATP, for example, to the extracellular solution can induce calcium activity in PC12 cells and produce a calcium wave that propagates through the cell. This is due to binding of the ATP molecules at cell surface receptors, which then triggers the calcium rise in the cell[10]. The addition of ATP to the extracellular solution to produce a calcium wave is an example of the common process of regulated exocytosis. In HeLa cells, for example, ATP bound to the cell membrane can induce a calcium wave, followed by exocytosis of further amounts of ATP. This allows for the propagation of ATP release and calcium waves over a multiple cells that do not otherwise have a direct connection for cell-to-cell signaling. The use of ATP to stimulate calcium release in cells is therefore a useful tool to study and control regulated exocytosis.

However, the three methods discussed so far are limited to interactions with the cell membrane and do not have a localized effect. In a natural condition, the calcium concentration in cells may respond to a stimulus coming from outside of the cell. It may alternatively occur independently of the extracellular condition. The calcium ion concentration reflects the general cell state, as well as acting as the conduit for the transfer of a variety of information in the cell. As a result, it is of paramount importance to have a method of controlling intracellular signaling, or at least, to have a method of instigating calcium release from within the cell itself. Until now, there has been only one method of inducing calcium release from within the cell itself. The introduction of an amount of caged calcium (e.g. calcium enclosed in photolabile NP-EGTA) into the cytosol allows the release of calcium by the irradiation of a high intensity light source that liberates the caged calcium. The small amount of calcium liberated from the cage molecules allows the creation of a localized calcium elevation in a specific region inside the cell. Uncaging is usually carried out by focused UV light[11], where the uncaging occurs predominantly at the focus, but to a certain extent also occurs throughout the irradiation light cone. It has also been performed by 2 photon absorption inducing the uncaging reaction, using a femtosecond laser[12]. Multiphoton photouncaging retains all the advantages of UV uncaging, with the additional benefits of further localization of the calcium release due to the restriction of nonlinear absorption to the volume around the focal zone. Additionally, in the case where the laser is focused inside the cell, the use of near-infrared wavelengths also reduces the interaction of the laser with the cell membrane.

In our group, however, we have seen that it is possible to directly modify the calcium concentration in cells in vitro[13]. The calcium concentration can be directly modified by the laser-induced release of calcium from intracellular stores, and calcium waves may be evoked in cells by the combination of the laser-induced release and the CICR mechanism. The laser-induced release and subsequent cytosolic calcium elevation can be produced reliably, but was found to exhibit stochastic behaviour, allowing us to define a probability of calcium wave generation but not completely predict the outcome of any single laser-cell calcium stimulation experiment. This is at least in part due to the inhomogeneity of the cell composition between different cells.

2. EXPERIMENTAL SETUP

We used a mode-locked Ti:Sapphire laser (Tsunami, SpectraPhysics) tuned to 780 or 800nm with an output pulsewidth of 80fs (dispersing to around 150fs inside the sample) and a repetition rate of 82MHz. Laser light was introduced to a conventional fluorescence microscope (BX-50WI, Olympus) and focused inside the sample by a water immersion objective lens (60x/0.9NA, Olympus), shown in figure 1. Samples were imaged in epifluorescence using a mercury lamp illumination source and an intensified CCD camera (C2400-35, Hamamatsu). Dichroic filters and an infrared filter were used to prevent Ti:Sapphire illumination from reaching the image intensifier. Individual cells were exposed to laser illumination, for periods of between 0.008 and 0.5 seconds, controlled by a mechanical shutter. Mean laser power was varied up to 80mW, as measured at the focus by a thermal power meter.

The cells used in this procedure were cultured HeLa (cancerous epithelial) and PC12 cells. HeLa cells were immersed in a solution which contained $CaCl_2$ (1mM), NaCl (145mM), KCl (4mM), $MgCl_2$ (1mM), glucose (10mM) and N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES, buffer, pH =7.4, 10mM), and Fluo-4AM (fluorescent Ca^{2+} indicator, Molecular Probes, 0.018mM) for 30 minutes in order to infuse the samples with the Ca^{2+} indicator. The solution was then replaced by the equivalent solution without Ca^{2+} indicator to prevent extracellular fluorescence emission. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal

calf serum, 2mM glutamine, 100 units/ml penicillin and 100µg/ml streptomycin in an humidified atmosphere (5% CO_2) at 37 degrees Celsius. Nerve Growth Factor (NGF) was added to the DMEM solution to a final concentration of 50µmol/l. Cells were loaded with fluo-4 by incubation with 20 µM membrane-permeable fluo-4 acetoxymethyl ester (Molecular Probes) for 30 min at room temperature (22°C). After loading of the membrane permeant fluorophore, the fluo-4AM solution was removed, the cell cultures were again washed, and the PBS was replaced by immersion in the following solution (145mM NaCl, 4mM KCl, 1mM MgCl2, 1mM CaCl2, 10mM glucose, and 10mM HEPES buffer, pH 7.4, without fluo-4AM).

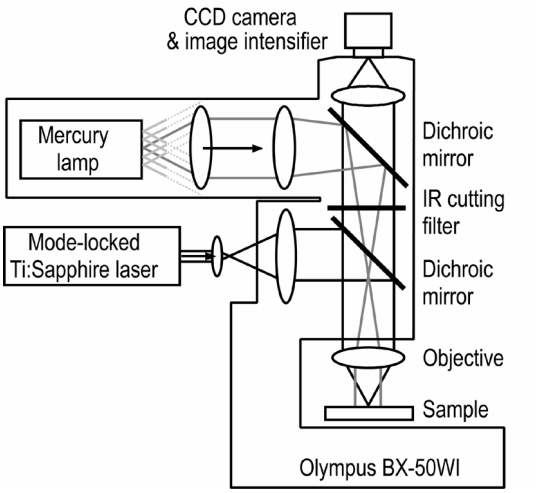

Figure 1: Optical setup for laser irradiation and fluorescence imaging

3. RESULTS

Figure 2 shows the laser-induced generation of calcium waves in HeLa cells where the mean illumination power was 37mW and the cell was exposed for 0.5 seconds. The laser focus is positioned 2µm in height above the lower membrane of the cell, and is approximately halfway between the upper and lower membranes. The axial length of the diffraction-limited spot for the 0.9NA water-immersion lens with 800nm illumination is around 1 µm (FWHM)[14]. The effective two-photon point-spread function is the single photon absorption point-spread function squared[15], which reduces the effective focal spot axial length to approximately 700nm (FHWM). A distance of 2µm between the focus and the lower membrane should be large enough to prevent direct interaction between the light and cell membrane. In any case, a larger distance is impractical since the overall thickness of the cells varies from cell to cell.

The intensity in figure 2 represents fluorescence emission from the calcium indicator, corresponding to unbound calcium concentration in the cell. After irradiation, first the local area around the focal zone, and then the entire target cell becomes bright, due to the increase in cytosolic calcium concentration. The adjacent cells respond in turn to the rise in calcium in the original target cell through intercellular signalling based on ATP release from the cell membrane and into the extracellular solution where it can diffuse to adjacent cells. In this manner the calcium wave can propagate through multiple cultured cells[16], as shown in the final three frames of the image sequence.

The laser-induced calcium response was also observed in PC12 (neural-type) cells. PC12 cells are a mutant form of neuron, derived from rat pheochromocytoma[17] that are not strictly neurons, but exhibit the behavior of neural cells depending on the presence or absence of nerve growth factor (NGF). The ability to control the morphology and function of PC12 cells by modifying the chemical environment is one of the reasons they are often used in experiments on cell signaling[18]. Figure 3 shows the laser-induced calcium rise in a target PC12 cell, induced by 50mW average power irradiation for 8ms.

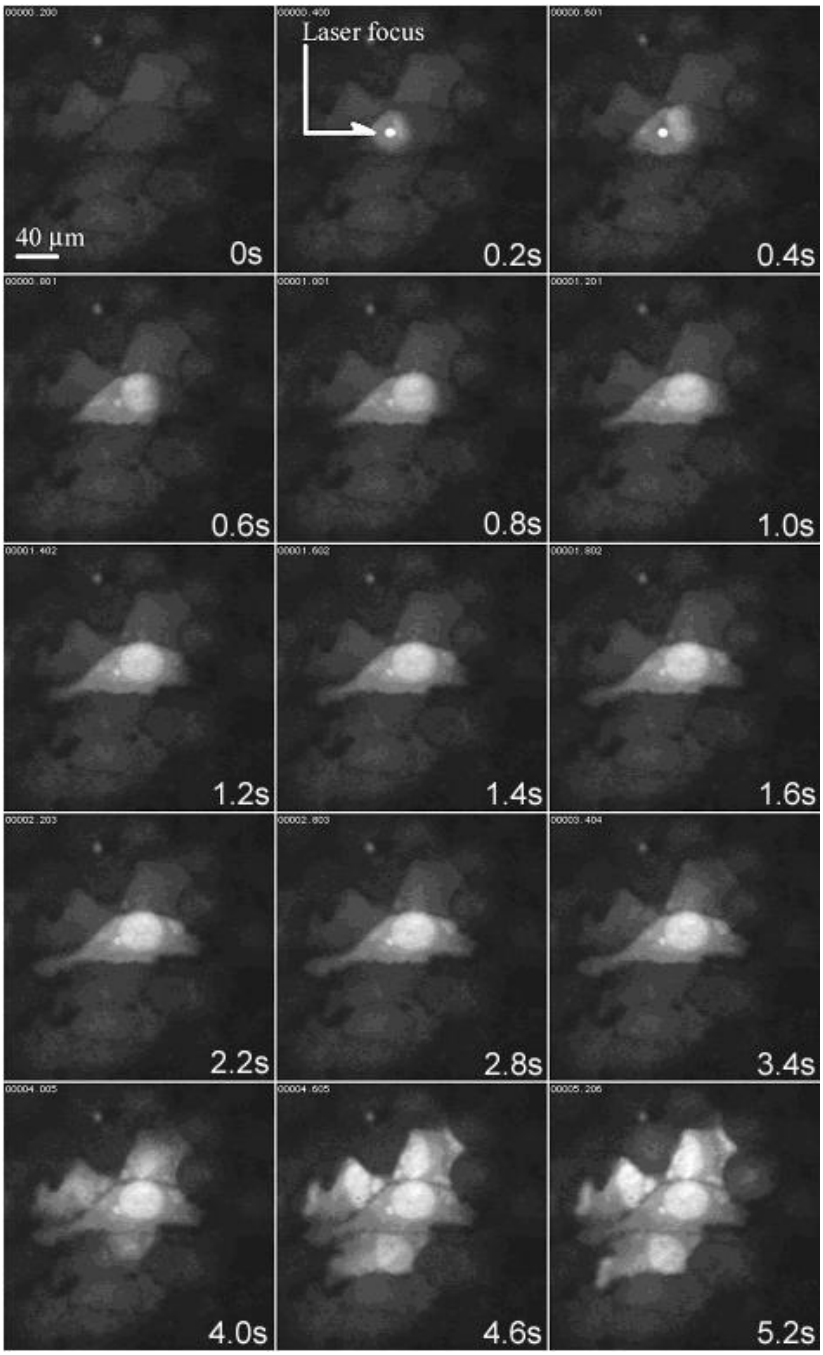

Figure 2: Fluorescence image sequence showing the laser-induced rise in calcium concentration in a group of cultured HeLa cells. The calcium rise is first seen to rise in and around the laser focal spot after irradiation for 0.5 seconds by a 37mW average power, 800nm femtosecond laser. Later frames show a calcium rise throughout the target cell, which later propagates to adjacent cells. The timescale (note the change in step side midway through the sequence) is noted on each frame.

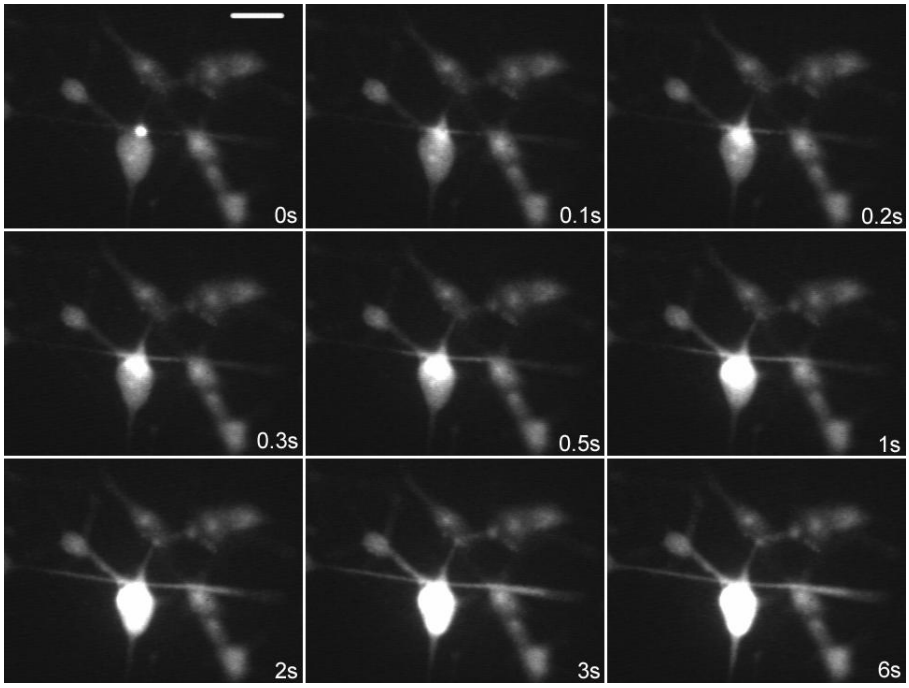

Figure 3: Fluorescence image sequence showing the laser-induced rise in calcium concentration in a cultured PC12 cells. The laser focus was positioned at the edge of the soma, in close proximity to the surrounding neurites. The calcium rise occurs first in the soma and is seen to propagate along both directions of the axon lying horizontally across the image. The irradiation exposure time was 8ms by a 50mW average power, 780nm femtosecond laser. The timescale (note the change in step side midway through the sequence) is noted on each frame. The white scalebar length is 20μm.

The mechanism by which the initial rise in calcium concentration occurs is complicated due to the fact that several different processes may be simultaneously taking place in the irradiated focal zone. Multiphoton absorption is well-known for the characteristics of restricting the nonlinear interaction to a small zone the size of the focal spot. However, if the power is high enough, various nonlinear effects can easily propagate out of the focal zone. For example, it is possible to induce calcium waves by mechanical pressure that can itself be produced by the laser. Focused ultrashort pulsed laser illumination of sufficient power can readily cause a shockwave to expand at supersonic speeds from the focal point.[19,20] This shockwave may act on sensitive zones within the cell which control calcium signaling. Another process is the direct photodisruption of regions that store calcium (the endoplasmic reticulum), causing the release of calcium into the cell body. The largest of the cellular calcium stores is the endoplasmic reticulum, containing calcium ions of around 100μM concentration. Compared to the resting cytosolic concentration of approximately 1μM or even lower, a temporary leak of the endoplasmic membrane could allow enough calcium leakage to trigger a whole-cell calcium wave. There are also a number of complex channels, in the cell membrane that control ion exchange in and out of the cell. Disruption of these channels or creation of a temporary hole in the membrane itself would cause extracellular calcium to enter the cell. However, in the case where calcium was omitted from the extracellular solution and 1mM EGTA was added to remove residual calcium, the observed wave response was not significantly affected. This provides strong evidence that the observed concentration rise in the target cell is largely due to internal interaction between the focused laser irradiation and the cell stores.

The power range for the laser-induced calcium wave was found to be approximately 20 to 80mW average laser power, as measured at the focal spot, with an exposure time of between 8ms and 0.5s. For example, 40mW average power for an exposure time of 125ms was enough to produce a calcium wave in 50% of cells where the laser was focused in the cytoplasm, outside the nucleus, and approximately halfway between the upper and lower membrane. It is somewhat surprising that the cell can withstand irradiation power levels approaching 80mW average power since the corresponding peak power is around 6.1kW and the peak power density is approximately 2.6TW/cm^2. At these peak power levels, significant morphological changes in cells have been observed[21]. Such changes were not, in general, observed during these experiments, so long as the laser focus is positioned sufficiently far from the membrane, and was

not moved during the exposure. More precise testing of cell viability with respect to laser power and calcium elevation levels (which can itself cause cell damage) is currently underway.

4. CONCLUSION

Femtosecond laser irradiation can directly influence calcium concentration changes in living cells. The technique described here can be used as a new method to instigate calcium waves in cell signaling experiments. It is also possible that, since this experiment is similar to 2 photon calcium uncaging experiments, that the direct effect of laser-induced calcium release is present in other experiments that are designed to work by uncaging. The amount of laser-induced calcium release is shown here to be enough to trigger a whole-cell calcium response, and is therefore likely to be comparable with the photoinduced release of calcium in uncaging experiments (although the power level here is higher than that generally used in uncaging experiments). The calcium stimulation was observed in HeLa (non-excitable) and PC12 (excitable) cells, and could be seen to occur inside a range of power levels between approximately 20 to 80mW. While experiments at the higher end of the power range did sometimes produce observable physical damage in the cell (particularly if the focal spot was too close to the outer membrane), most of the cells where calcium waves were evoked did not exhibit significant damage.

REFERENCES

1. M.J. Berridge, M.D. Bootman and P. Lipp, *Nature* **395**, 645-648, 1998.
2. L.F. Jaffe, Dev. Biol. **99**, 265-276, 1983.
3. C.R. Rose and A. Konnerth, *Neuron* **31**, 519-522, 2001.
4. S.B. Kater, and L.R. Mills, *J. Neurosci*. **11**, 891-899, 1991.
5. X. Gu and N.C Spitzer, *Nature* **375**, 784-787, 1995.
6. K. Paemeleire, P. Martin, S. Coleman, K. Fogarty, W. Carrington, L. Leybaert, L. Tuft, W.H. Evans, and M. Sanderson, *Mol. Biol. Cell.* **11**, 1815-1827, 2000.
7. M.J. Berridge, P. Lipp, and M.D. Bootman, *Nature Rev. Mol. Cell. Biol.* **1**, 11-21, 2000.
8. T. Fauquier, N.C. Guérineau, R.A. McKinney, K. Bauer, and P. Mollard, *Proc. Natl. Acad. Sci. USA* **98**, 8891-8896, 2001.
9. R. Jacob, J.E. Merritt, J.E. Hallam, and T.J. Rink, *Nature* **335**, 40-45, 1998.
10. V.A. Barry and T.R. Cheek, *J. Cell Sci.* **107**, 451-462, 1994.
11. V. Parpura and P.G. Haydon, *Proc. Natl. Acad. Sci. USA* **97**, 8629-8634, 2000.
12. E.B. Brown, J.B. Shear, S.R. Adams, R.Y. Tsien, and W.W. Webb, *Biophys. J.* **76**, 489-499, 1999.
13. N.I. Smith, K. Fujita, T. Kaneko, K. Katoh, O. Nakamura, S. Kawata, and T. Takamatsu, *Appl. Phys. Lett.* **79**, 1208-1210, 2001.
14. T. Wilson, *Confocal Microscopy*, Academic, London, 1990.
15. O. Nakamura, *Optik (Stuttgart)* **93**, 39-42, 1993.
16. Y. Osipchuk and M. Cahalan, *Nature* **359**, 241-244, 1992.
17. L A Greene, A. S. Tischler, *Proc. Natl. Acad. Sci. USA* **73** 2424-2428, 1976.
18 D. Vaudry, P.J.S. Stork, P. Lazarovici, L.E. Eiden, *Science* **296**, 1648-1649, 2002.
19. A. Vogel, J. Noack, K. Nahen, D. Theisen, S. Busch, U. Parlitz, D.X. Hammer, G.D. Noojin, B.A. Rockwell, and R. Birngruber, *Appl. Phys. B.* **68**, 271-280, 1999.
20. A. Vogel, J. Noack, G. Hüttmann, and G. Paltauf, *Proc. SPIE* **4633A**, 1-15, 2002.
21. K. König, I. Riemann, P. Fischer, and K.-J. Halbhuber, *Cell. Mol. Biol.* **45**, 195-201, 1999.