

Modification of Intracellular Ca^{2+} Dynamics by Laser Inactivation of Inositol 1,4,5-Trisphosphate Receptor Using Membrane-Permeant Probes

Takatoshi Yogo,¹ Kazuya Kikuchi,^{1,3}
Takanari Inoue,¹ Kenzo Hirose,² Masamitsu Iino,²
and Tetsuo Nagano^{1,*}

¹Graduate School of Pharmaceutical Sciences

²Department of Pharmacology

Graduate School of Medicine

The University of Tokyo

7-3-1 Hongo

Bunkyo-ku, Tokyo 113-0033

³PRESTO

Japan Science and Technology Corporation

Kawaguchi, Saitama

Japan

Summary

A membrane-permeant malachite green-conjugated IP_3 analog (MGIP₃/PM) was synthesized as a probe for small molecule-based CALI (smCALI), and its effect on the Ca^{2+} signaling in intact DT40 chicken B cells was examined. In DT40 B cells treated with the smCALI probe, laser irradiation inhibited IP_3 -induced Ca^{2+} oscillations in response to B cell receptor stimulation, demonstrating that IP_3R was acutely inactivated. We then applied smCALI to clarify the mechanism of capacitative Ca^{2+} entry (CCE), in which involvement of IP_3R has been suggested. Despite the inactivation of IP_3R by smCALI, thapsigargin-induced CCE remained unaffected, providing evidence that functional IP_3R is not required for CCE in DT40 cells. These results demonstrate the potency of the smCALI technique for the study of the roles of IP_3R in complex intracellular Ca^{2+} dynamics.

Introduction

Specific inactivation of biomolecules has been one of the most widely used approaches to study the physiological functions of these molecules, and various methods, including the use of pharmacological antagonists, targeted gene disruption, and antibodies, have been used. The activities of most functional biomolecules depend upon the site of expression and the nature of the cellular processes in which they are involved [1–3], so a technique for inactivation of target proteins in a spatiotemporally well controlled manner should be extremely useful. To achieve this purpose, chromophore-assisted laser inactivation (CALI) is an excellent method [4, 5], in which the protein of interest is targeted by an exogenously introduced antibody that has been tagged with a CALI chromophore, usually malachite green, and the chromophore-antibody complex is subsequently irradiated with intense localized laser light. The irradiated chromophore produces radical species that react with the protein to which the antibody is bound, causing its inactivation. Because the radical species are highly

reactive and have very short lifetimes, the antibody-recognized proteins are specifically inactivated. The functions of various molecules, which could not have been analyzed by other currently available methods, have been elucidated by inactivation of target proteins at the appropriate site and time using the CALI technique.

Although CALI has proved very powerful, it has some limitations, which are primarily attributable to the use of antibody for the molecular recognition. It is difficult to label antibodies with chromophores at specific amino acid residues, so that the extent of the damage inflicted on the target protein cannot readily be controlled. Moreover, it is necessary to use an invasive method to introduce antibodies into cells, which may jeopardize the physiological functions and long-term viability of the cells. Further, invasive methods (such as trituration, whole-cell clamp, or microinjection) can be applied to only a few cells at a time and are not applicable to tissues.

To circumvent the limitations associated with conventional CALI, we have set out to develop a new method in which synthetic small molecules are used instead of antibody for target recognition (small molecule-based CALI, or smCALI). We designed and synthesized suitable synthetic small molecular probes for biological application of CALI. For the implementation of smCALI, we chose inositol 1,4,5-trisphosphate receptor (IP_3R) as a target protein. IP_3R is a Ca^{2+} channel localized on the endoplasmic reticulum (ER) membrane, and regulates the cytosolic Ca^{2+} concentration, playing an important role in various physiological functions, such as contraction, secretion, fertilization, synaptic plasticity, and gene expression [6, 7]. Thus, spatiotemporally controlled inactivation of IP_3R using smCALI should be useful for studying of the role of the protein in these functions.

We developed a chromophore-labeled IP_3 analog (carboxymalachite green-aminopropyl-1D-*myo*-inositol-1,4,5-trisphosphate, MGIP₃) and showed that spatially controlled inactivation of IP_3R could be achieved within a few seconds by MGIP₃-mediated smCALI [8–10]. The time required for smCALI is very much shorter than that of antibody-based CALI, which usually requires ~5 min. Furthermore, by focusing a laser beam at the subcellular level in a single PC12 cell, precise control of the area of inactivation could be achieved. In the present study, we derivatized MGIP₃ to a membrane-permeant compound for noninvasive delivery into living cells. This membrane-permeant probe can be easily applied to intact cells or cell populations and smCALI experiments can be conducted under physiological conditions. We describe here the successful application of smCALI in intact cells using the membrane-permeant probe, and we show that smCALI provides a powerful tool to analyze complex intracellular Ca^{2+} dynamics.

Results and Discussion

MGIP₃/PM Permeates through the Plasma Membrane and Interacts with IP_3R

In previous studies, we designed and synthesized a chromophore-labeled IP_3 analog (MGIP₃, Figure 1) and

*Correspondence: tlong@mol.f.u-tokyo.ac.jp

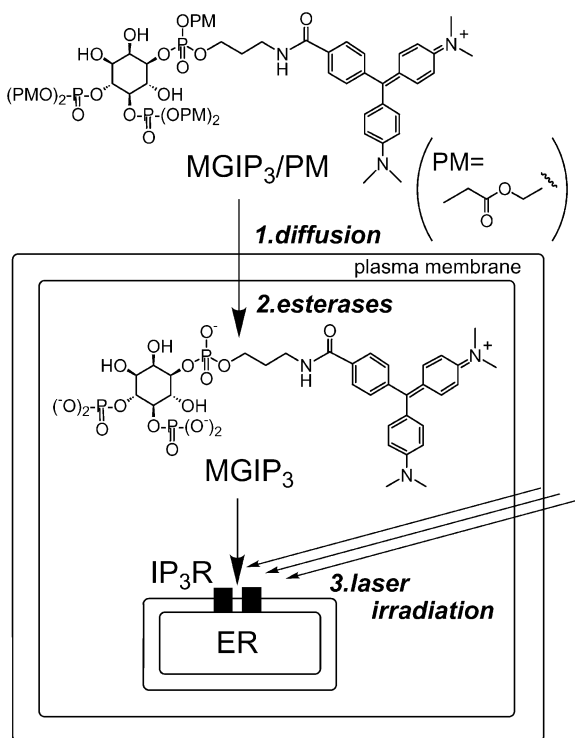


Figure 1. Scheme of smCALI Using a Membrane-Permeant Synthetic Probe, MGIP₃/PM
IP₃R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; PM, propionylloxymethyl group.

showed that MGIP₃ could act as an agonist of IP₃R and function as an effective probe for smCALI of IP₃R [9, 10]. Permeabilization of the cell membrane or whole-cell patch-clamp was required to introduce MGIP₃ into cells, because MGIP₃ cannot permeate through the plasma membrane due to its charge and hydrophilicity. Thus, we derivatized MGIP₃ to a membrane-permeant compound (carboxymalachite green-aminopropyl-1D-myo-inositol-1,4,5-trisphosphate hexakis (propionylloxymethyl) ester, MGIP₃/PM, Figure 1) by masking all the anionic phosphate groups with propionylloxymethyl esters. This compound is expected to diffuse into cells, where it should undergo cleavage by the ubiquitous intracellular esterases to generate the hydrolyzed form (i.e., MGIP₃), which binds to IP₃R (Figure 1).

We examined whether MGIP₃/PM can indeed permeate through the plasma membrane and interact with IP₃R by using DT40 cells expressing type 2 IP₃R (IP₃R-2). MGIP₃ is an agonist of IP₃R and induces Ca²⁺ release via IP₃R in permeabilized DT40 cells. Thus, one would expect to observe an increase in [Ca²⁺]_i, if extracellularly applied MGIP₃/PM dose permeate through the plasma membrane and subsequently undergo hydrolysis to yield MGIP₃. When MGIP₃/PM (100 μM) was added to the extracellular solution of DT40 cells, it induced an increase in [Ca²⁺]_i, indicating that MGIP₃/PM did enter the cells and interact with IP₃R (Figure 2A, black trace; Figure 2B). On the other hand, extracellularly added MGIP₃ (100 μM) or vehicle (0.1% DMSO) induced no detectable [Ca²⁺]_i increase (Figure 2A, green and blue

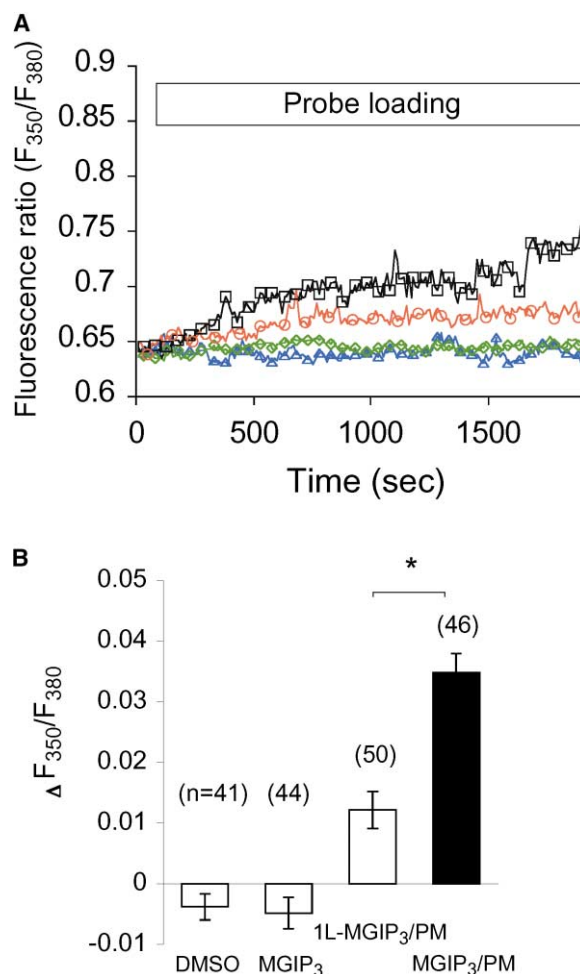


Figure 2. [Ca²⁺]_i Increase Induced by 100 μM MGIP₃/PM in Intact DT40 Cells

(A) [Ca²⁺]_i increase induced by 100 μM MGIP₃/PM, 100 μM 1L-MGIP₃/PM, 100 μM MGIP₃, and vehicle (0.1% DMSO). Test compounds were added to an extracellular solution of Fura-2-loaded DT40 cells as indicated by the box. All traces show Ca²⁺ responses in single DT40 cells. 100 μM MGIP₃/PM (black square) induced a much greater [Ca²⁺]_i increase than 100 μM 1L-MGIP₃/PM (red circle). 100 μM MGIP₃ (green diamond) or vehicle (blue triangle) induced no detectable [Ca²⁺]_i increase.

(B) [Ca²⁺]_i increase induced by 100 μM MGIP₃/PM and 100 μM 1L-MGIP₃/PM. 100 μM MGIP₃/PM induced a significantly larger increase of the fluorescence ratio than 100 μM 1L-MGIP₃/PM within 1800 s after extracellular addition of each compound (*p < 0.0001). These data were acquired in two independent experiments. The number of analyzed cells is indicated on top of each column. Results are the mean ± SEM.

traces; Figure 2B), indicating that masking of the phosphate groups is essential for membrane permeation. As a control experiment, we used the optical isomer of MGIP₃/PM (1L-MGIP₃/PM), which has the same photochemical nature as that of MGIP₃/PM, though its hydrolyzed form (i.e., 1L-MGIP₃) has a much weaker agonistic effect on IP₃R [9, 10]. 1L-MGIP₃/PM (100 μM) was much less effective in increasing [Ca²⁺]_i than MGIP₃/PM and a significant difference in their Ca²⁺ releasing activity was observed (Figures 2A, red versus black traces; Figure 2B, p < 0.0001). In all experiments, cells

showed the same responses to B cell receptor stimulation. We also examined the concentration of MGIP₃ in the cell by HPLC. After uptake of MGIP₃/PM from the extracellular solution by DT40 cells, cells were washed and disrupted by sonication; then the intracellular solution was analyzed by HPLC. The result suggested that the concentration of MGIP₃ was at most 10 μM in the cell when MGIP₃/PM was extracellularly applied at a concentration of 100 μM (data not shown). This range of MGIP₃ concentration is the same as that used in the previous study [10], in which the specificity of CALI was established. Furthermore, DT40 cells lacking all three type of IP₃R [11] exhibited no detectable [Ca²⁺]_i increase in response to extracellular addition of 100 μM MGIP₃/PM (data not shown). These results indicate that MGIP₃/PM can permeate through the plasma membrane and interact with IP₃R after intracellular hydrolysis.

Although MGIP₃/PM has an inherent agonistic effect, at low concentrations MGIP₃/PM induced no detectable [Ca²⁺]_i increase. Indeed, there was no significant difference between the increase of fluorescence ratio induced by 4 μM MGIP₃/PM and that by 4 μM 1L-MGIP₃/PM (−0.009 ± 0.006 and −0.013 ± 0.006, respectively, *p* = 0.6825). Thus, we used 4 μM MGIP₃/PM for smCALI so that the agonistic effect of the ester could be neglected.

MGIP₃/PM-Mediated smCALI Inactivates IP₃R in Intact DT40 Cells

Stimulating the B cell antigen receptor (BCR) induces production of IP₃ via phospholipase C (PLC)-γ2, followed by repetitive cyclic increases in [Ca²⁺]_i (Ca²⁺ oscillation) in DT40 cells [12]. In the previous study, we showed that MGIP₃-mediated smCALI inhibited IP₃-induced Ca²⁺ release in permeabilized DT40 cells [10]. We then examined whether laser beam irradiation focused onto intact single DT40 cells loaded with MGIP₃/PM induces protein inactivation, by measuring the IP₃-induced Ca²⁺ oscillation. Fura-2-loaded DT40 cells were loaded with 4 μM MGIP₃/PM for 60 min, and then we routinely allowed an incubation period of ≥30 min to ensure complete hydrolysis of MGIP₃/PM after wash-out of excess ester. MGIP₃ in the cell is expected to be stable because it has been shown that inositol 1,4,5- trisphosphate derivatives modified at 1-phosphate are not substrates for 5-phosphatase or 3-kinase [13]. Subsequently, several MGIP₃/PM-loaded cells were successively irradiated with the laser for 60 s each (Figure 3A), followed by induction of Ca²⁺ oscillation by BCR stimulation with anti-BCR antibody M4 (1 μg ml^{−1}) [12]. Ca²⁺ oscillation was strongly inhibited in irradiated cells (Figure 3B). The average number of Ca²⁺ oscillations within 500 s after BCR stimulation was decreased by 50% in irradiated cells compared with nonirradiated cells (Figures 3C and 3D, *p* < 0.0001). MGIP₃/PM-mediated smCALI also decreased the average peak amplitude of Ca²⁺ oscillations: 2.04 ± 0.10 in irradiated cells and 2.41 ± 0.07 in nonirradiated cells (*p* < 0.005).

Laser irradiation alone in the absence of MGIP₃/PM had no significant effect on the average number of Ca²⁺ oscillations (Figure 3D, *p* > 0.82). We then examined whether or not the laser-induced inhibitory effect on Ca²⁺ oscillation is also observed in 1L-MGIP₃/PM-

loaded cells. No effect of laser irradiation on the average number of Ca²⁺ oscillations was observed in cells loaded with 4 μM 1L-MGIP₃/PM (Figure 3D, *p* > 0.36). These results indicate that there is no nonspecific laser damage to IP₃R, and that the inhibition of Ca²⁺ oscillation was the result of IP₃R inactivation by MGIP₃/PM-mediated smCALI.

MGIP₃-mediated smCALI has been shown to inhibit IP₃-induced Ca²⁺ release [10]. Therefore, it seems likely that Ca²⁺ oscillation was inhibited as a result of the inhibition of IP₃-induced Ca²⁺ release. Ca²⁺ oscillation is a genuine physiological phenomenon, and it is not appropriate to analyze it by using methods that disrupt the intact cell structure, for example, using detergents or microinjection. SmCALI provides the opportunity to analyze Ca²⁺ signaling under physiological conditions.

It was not clear quantitatively to what extent the IP₃R was inactivated by our procedure, but the oscillation frequency, an important factor of Ca²⁺ signaling, was greatly reduced, except for a few initial spikes, as a result of IP₃R inactivation.

Absence of Involvement of IP₃R in Capacitative Calcium Entry

After release of Ca²⁺ from intracellular stores via IP₃R, Ca²⁺ entry across the plasma membrane follows in many cell types [14]. This Ca²⁺ signaling is called capacitative Ca²⁺ entry (CCE). CCE is thought to form an important component of Ca²⁺ signaling. Although IP₃R has been implicated in CCE, the molecular mechanism of CCE remains elusive [15–17]. To study whether IP₃R is involved in CCE, CCE was examined in cells in which IP₃R had been inactivated by smCALI. Inactivation of IP₃R in DT40 cells expressing IP₃R-2 by smCALI was carried under the same conditions as the above experiments. Ca²⁺ stores were then depleted by applying 1 μM thapsigargin (TG), an inhibitor of sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) [18], in the absence of extracellular Ca²⁺. Subsequent addition of 2 mM Ca²⁺ evoked an increase in [Ca²⁺]_i due to CCE (Figure 4A). The amplitude of the CCE-mediated increase in [Ca²⁺]_i was not significantly different between irradiated and nonirradiated cells (*p* > 0.86, Figure 4B), suggesting that the activation of IP₃R is not essential for TG-induced CCE in DT40 cells under these experimental conditions.

It has been suggested that CCE is regulated by the IP₃R [15]. However, it has been difficult to acutely and specifically inactivate IP₃R. Most of the conventional antagonists of IP₃R have direct inhibitory effects on CCE [17, 19]. Targeted disruption of the IP₃R genes is a powerful method, but may not be free from possible long-term compensatory reactions. Recent findings using mutant DT40 cells, in which all subtypes of IP₃R were deleted, strongly suggested that IP₃R is not involved in CCE, because the cells showed typical CCE or *I*_{crac} (calcium release-activated calcium current) identical to that observed in wild-type cells [11, 16, 20, 21]. However, it was suggested that ryanodine receptors would work as a compensatory mechanism in the mutant DT40 cells [22]. Taking the above circumstances into consideration, smCALI appears to be an excellent method to analyze the functions of IP₃R in CCEs, because acute

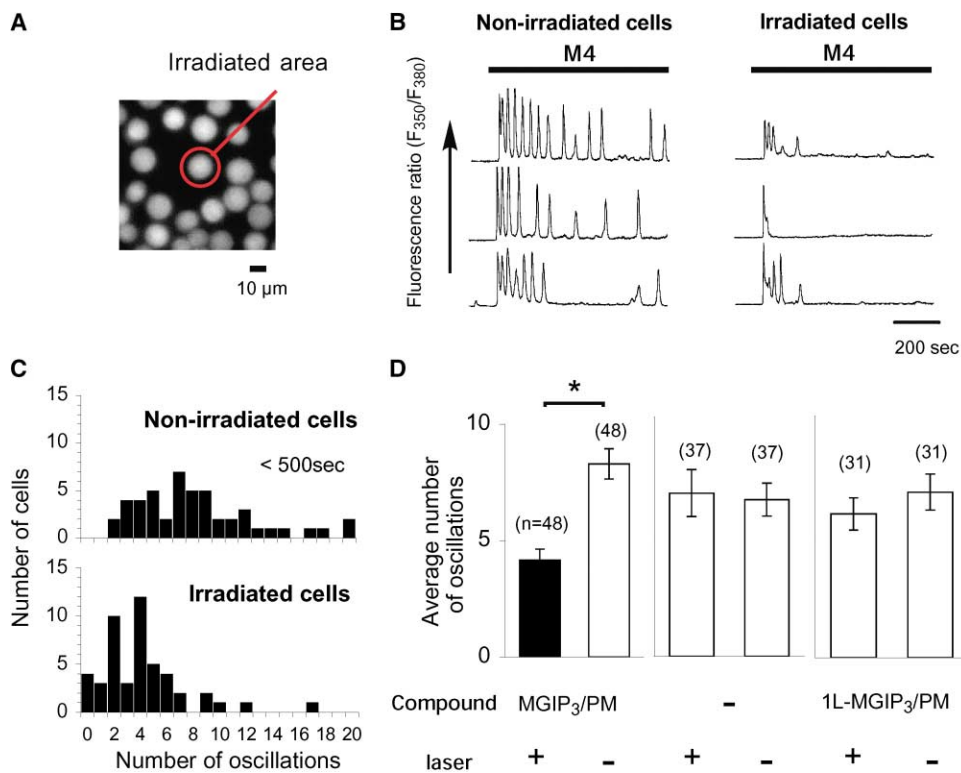


Figure 3. Inhibitory Effect of MGIP₃/PM-Mediated smCALI on Ca²⁺ Oscillations in Intact DT40 Cells

(A) Fluorescence image of DT40 cells excited at 350 nm after loading Fura-2AM. The red circle indicates the laser spot for irradiation. (B) Ca²⁺ responses upon ligation of BCR with anti-BCR antibody (1 μg ml⁻¹) after smCALI with 4 μM MGIP₃/PM. Traces show several representative responses in irradiated or nonirradiated cells. Antibody was applied as indicated by horizontal bars above the traces. (C) Histogram showing the number of Ca²⁺ oscillations within 500 s of BCR stimulation in irradiated or nonirradiated cells (48 cells in each condition). (D) Average number of Ca²⁺ oscillations in response to BCR stimulation after the indicated treatment. A significant difference was found between the average numbers of Ca²⁺ oscillations under irradiated and nonirradiated conditions with MGIP₃/PM (*p < 0.0001). The number of analyzed cells is indicated on top of each column. These data were acquired in six independent experiments. Results are the mean ± SEM.

inactivation of IP₃R can be carried out under physiological conditions. Other studies have also shown that acute inhibition of IP₃R by heparin (a potent IP₃R antagonist) failed to inhibit *I*_{crac} [20, 21, 23]. Our results provided evidence that IP₃R is not involved in CCE in DT40 cells. Further application of smCALI should clarify the function of IP₃R in CCE in other cell types.

In the present study we showed that smCALI can overcome the main limitation of the conventional CALI technique, in which invasive methods have to be used to introduce antibodies into cells. Indeed, we have succeeded in examining the effect of smCALI on Ca²⁺ oscillations. The role of IP₃R in CCE could be analyzed by smCALI without any long-term compensatory effect. Spatiotemporally controlled genetic modification (conditional knockout) [24] has emerged as a powerful molecular inactivation method. However, it requires a much longer time for molecular inactivation than smCALI. Thus, smCALI provides a unique method to analyze Ca²⁺ signaling under physiological conditions in various cell types. Another feature of MGIP₃-mediated smCALI is the catalytic inactivation mechanism [10], i.e., the small-molecular probe (MGIP₃) may rapidly associate with and rapidly dissociate from its target (IP₃Rs), and repetitive

excitation of malachite green causes cumulative inactivation of IP₃Rs. Due to the catalytic nature of the inactivation, even a low probe concentration may cause sufficient inactivation. Indeed, we showed that 4 μM MGIP₃/PM caused sufficient inactivation of IP₃Rs without any agonistic effect. Thus, the smCALI technique provides high spatiotemporal resolution in molecular inactivation, and should be applicable to studies of various physiological functions, especially in polarized cells such as neurons.

Significance

Biological application of conventional CALI has been hampered mainly by the necessity to use invasive methods to introduce antibodies into the cells for target recognition. In the present study, we successfully carried out smCALI in intact cells by using membrane-permeant synthetic small molecules. Some variations of small molecule-based CALI have been developed. For example, arsenic chromophore derivatives designed to specifically bind to a tetracysteine tag attached to a target protein induce target inactivation under light illumination (FIAsH-FALI) [25, 26]. Although

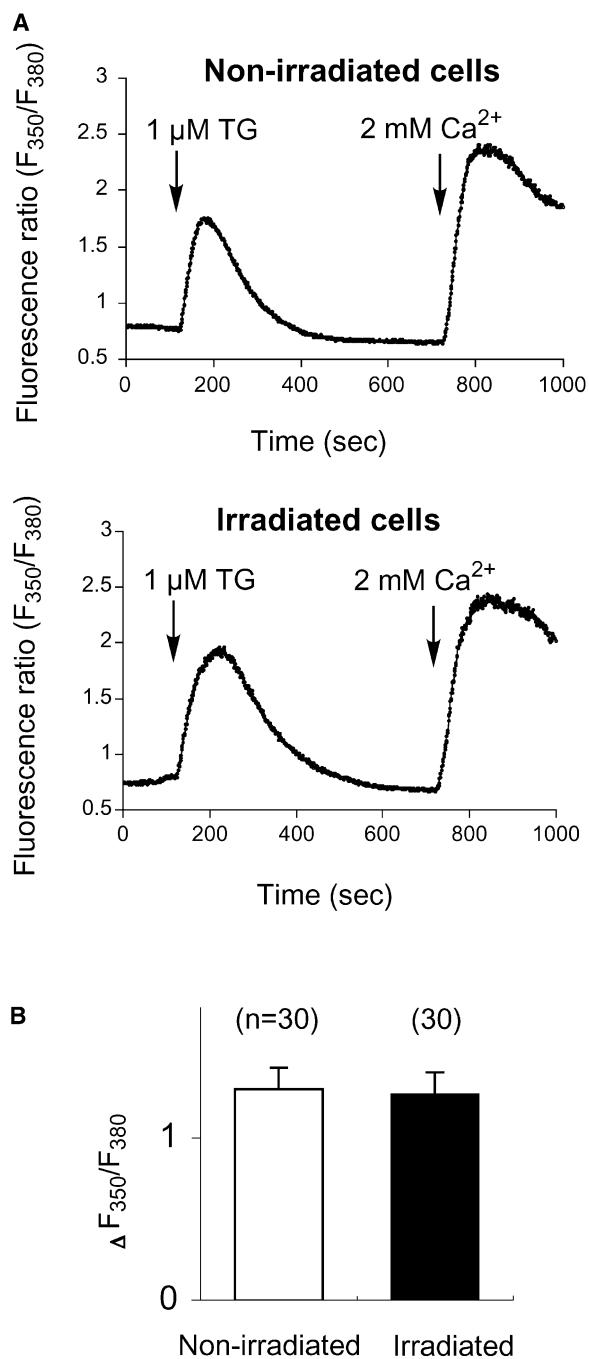


Figure 4. The Effect of MGIP₃/PM-Mediated smCALI on Thapsigargin-Induced Capacitative Calcium Entry in DT40 Cells

(A) Intracellular Ca²⁺ release and Ca²⁺ influx were measured in TG-stimulated irradiated and nonirradiated cells. After washing of the cells with Ca²⁺-free solution, 1 μM TG was used to deplete the Ca²⁺ store, followed by addition of 2 mM Ca²⁺.

(B) Increase of fluorescence ratio induced by addition of 2 mM Ca²⁺ after TG treatment of irradiated and nonirradiated cells. No significant difference was found between irradiated and nonirradiated cells ($p > 0.86$). The number of analyzed cells is indicated on top of each column. These data were acquired in three independent experiments. Results are the mean \pm SEM.

this method is powerful, toxicity and nonspecific damage [27] can be problematic. In our smCALI strategy, we showed that there is no apparent nonspecific damage to IP₃R, presumably due to the catalytic nature of the inactivation and high efficiency. We demonstrated that intracellular Ca²⁺ dynamics can be modified by smCALI of IP₃R under physiological conditions. Since IP₃R is an important regulator of intracellular Ca²⁺ dynamics, spatiotemporally specific inactivation of IP₃R by smCALI using MGIP₃/PM may provide a useful method for clarifying the complex Ca²⁺ signaling mechanisms in intact living cells. smCALI should also be applicable to targets other than IP₃R, if appropriate malachite green-conjugated ligands are synthesized.

Experimental Procedures

Synthesis of MGIP₃/PM

MGIP₃ was synthesized as reported previously [8]. To synthesize MGIP₃/PM, MGIP₃ (1.0 eq.) was mixed vigorously with CH₃CN and *N,N*-diisopropylethylamine (DIEA) (2.0 eq.). The mixture was then dried under vacuum. This procedure was repeated at least three times until a homogenous solution was obtained after adding CH₃CN/DIEA. After a final round of drying, the green solid was suspended in CH₃CN and DIEA (5.0 eq.). Bromomethyl propionate (5.0 eq.) [28] was added to this solution. The mixture was stirred for 24 hr, then further bromomethyl propionate (2.5 eq.) and DIEA (2.5 eq.) were added and the reaction was continued for another 24 hr. The solvent and excess reagents were evaporated under vacuum. The remaining mixture was purified by C₁₈ reversed-phase HPLC. ¹H-NMR (CD₃OD): δ 1.02 (m, 15H), 1.80 (m, 2H), 1.93 (br, 2H), 2.32 (m, 10H), 3.30~5.50 (m, 10H), 3.60 (s, 12H), 5.61 (br, 10H), 7.30 (m, 2H), 7.58 (m, 4H), 7.78 (m, 6H). 1L-MGIP₃/PM was also synthesized in the same manner.

Cell Culture

DT40 chicken B lymphoma cells were cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS), 1% chicken serum, penicillin (100 U/ml), streptomycin (100 U/ml), and 2 mM glutamine. In some experiments, genetically engineered DT40 cells that express only type 2 IP₃R or that lack all three type of IP₃R were used [11, 12].

Ca²⁺ Imaging

Cells were attached to poly-L-lysine and collagen-coated coverslips and loaded with 1 μM Fura-2AM (Molecular Probes) for 30 min in a physiological salt solution (PSS: 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 5.6 mM glucose, pH 7.4) containing 0.1% bovine serum albumin (BSA). An Olympus IX71 inverted microscope, equipped with a cooled CCD camera (Photometrics) and a polychromatic illumination system (T.I.L.L. Photonics) was used to capture the fluorescence images generated by alternate excitations at 350 and 380 nm every 1 or 10 s. Intracellular Ca²⁺ concentration was measured as the ratio of the fluorescence intensity between the pair of frames (F_{350}/F_{380}). The test compounds were predissolved in PSS (containing 0.1% DMSO).

Laser Irradiation for smCALI

A nitrogen-driven pulsed dye laser (wavelength 635 nm, pulse width <4 ns, and pulse energy \sim 30 μJ at 20 Hz; Laser Science Inc.;VSL-337ND-S and DUO-220) was spatially filtered using a pair of objective lenses. A circular beam with a required spot size was introduced into a water-immersion objective (60 \times , NA 0.90, Olympus) attached to the inverted fluorescence microscope. The laser beam was focused onto a single DT40 cell. The diameters of the laser spot and a single DT40 cell were 20 and \sim 15 μm , respectively. The laser pulse energy at the focal plane was \sim 7 μJ . Laser irradiation was carried out as follows. The probe was added to the extracellular solution of Fura-2-loaded DT40 cells, and after 60 min, the excess ester was washed away with PSS. After \geq 30 min, several cells were successively irradiated for 60 s. For the analysis of nonirradiated

cells surrounding irradiated cells, we randomly selected cells within a frame.

Statistical Analyses

Statistical analysis was performed using Student's unpaired t-test.

Acknowledgments

This work was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grants 11794026, 12470475, and 12557217 to T.N., 13558078, 14045210, and 15681012 to K.K.), by the Mitsubishi Foundation, and by the Research Foundation for Opt-Science and Technology.

Received: January 18, 2004

Revised: May 6, 2004

Accepted: May 11, 2004

Published: August 20, 2004

References

- Schmucker, D., Su, A.L., Beermann, A., Jäckle, H., and Jay, D.G. (1994). Chromophore-assisted laser inactivation of patched protein switches cell fate in the larval visual-system of *Drosophila*. *Proc. Natl. Acad. Sci. USA* *91*, 2664–2668.
- Vernos, I., Raats, J., Hirano, T., Heasman, J., Karsenti, E., and Wylie, C. (1995). Xklp1, a chromosomal *Xenopus* kinesin-like protein essential for spindle organization and chromosome positioning. *Cell* *81*, 117–127.
- Kiselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L., and Muallem, S. (1998). Functional interaction between InsP_3 receptors and store-operated Htrp3 channels. *Nature* *396*, 478–482.
- Jay, D.G. (1988). Selective destruction of protein function by chromophore-assisted laser inactivation. *Proc. Natl. Acad. Sci. USA* *85*, 5454–5458.
- Jay, D.G., and Sakurai, T. (1999). Chromophore-assisted laser inactivation (CALI) to elucidate cellular mechanisms of cancer. *Biochim. Biophys. Acta* *1424*, M39–M48.
- Berridge, M.J. (1993). Inositol trisphosphate and calcium signaling. *Nature* *367*, 315–325.
- Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* *4*, 517–529.
- Inoue, T., Kikuchi, K., Hirose, K., Iino, M., and Nagano, T. (1999). Synthesis and evaluation of 1-position-modified inositol 1,4,5-trisphosphate analogs. *Bioorg. Med. Chem. Lett.* *9*, 1697–1702.
- Inoue, T., Kikuchi, K., Hirose, K., Iino, M., and Nagano, T. (2001). Small molecule-based laser inactivation of inositol 1,4,5-trisphosphate receptor. *Chem. Biol.* *8*, 9–15.
- Inoue, T., Kikuchi, K., Hirose, K., Iino, M., and Nagano, T. (2003). Spatiotemporal laser inactivation of inositol 1,4,5-trisphosphate receptors using synthetic small-molecule probes. *Chem. Biol.* *10*, 503–509.
- Sugawara, H., Kurosaki, M., Takata, M., and Kurosaki, T. (1997). Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. *EMBO J.* *16*, 3078–3088.
- Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T., and Iino, M. (1999). Encoding of Ca^{2+} signals by differential expression of IP_3 receptor subtypes. *EMBO J.* *18*, 1303–1308.
- Prestwich, G.D., Marecek, J.F., Mourey, R.J., Theibert, A.B., Ferris, C.D., Danoff, S.K., and Snyder, S.H. (1991). Tethered IP_3 . Synthesis and biochemical applications of the 1-O-(3-aminopropyl) ester of inositol 1,4,5-trisphosphate. *J. Am. Chem. Soc.* *113*, 1822–1825.
- Venkatachalam, K., van Rossum, D.B., Patterson, R.L., Ma, H.T., and Gill, D.L. (2002). The cellular and molecular basis of store-operated calcium entry. *Nat. Cell Biol.* *4*, E263–E272.
- Ma, H.T., Patterson, R.L., van Rossum, D.B., Birnbaumer, L., Mikoshiba, K., and Gill, D.L. (2000). Requirement of the inositol trisphosphate receptor for activation of store-operated Ca^{2+} channels. *Science* *287*, 1647–1651.
- Ma, H.T., Venkatachalam, K., Li, H.S., Montell, C., Kurosaki, T., Patterson, R.L., and Gill, D.L. (2001). Assessment of the role of the inositol 1,4,5-trisphosphate receptor in the activation of transient receptor potential channels and store-operated Ca^{2+} entry channels. *J. Biol. Chem.* *276*, 18888–18896.
- Bootman, M.D., Collins, T.J., Mackenzie, L., Roderick, H.L., Berridge, M.J., and Peppiatt, C.M. (2002). 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP_3 -induced Ca^{2+} release. *FASEB J.* *16*, 1145–1150.
- Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., and Dawson, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific-inhibition of the endoplasmic-reticulum Ca^{2+} -ATPase. *Proc. Natl. Acad. Sci. USA* *87*, 2466–2470.
- Bultynck, G., Sienaert, I., Parys, J.B., Callewaert, G., De Smedt, H., Boens, N., Dehaen, W., and Missiaen, L. (2003). Pharmacology of inositol trisphosphate receptors. *Pflugers Arch.* *445*, 629–642.
- Broad, L.M., Braun, F.J., Lievreumont, J.P., Bird, G.S.J., Kurosaki, T., and Putney, J.W. (2001). Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry. *J. Biol. Chem.* *276*, 15945–15952.
- Prakriya, M., and Lewis, R.S. (2001). Potentiation and inhibition of Ca^{2+} release-activated Ca^{2+} channels by 2-aminoethyl-diphenyl borate (2-APB) occurs independently of IP_3 receptors. *J. Physiol. (London)* *536*, 3–19.
- Kiselyov, K., Shin, D.M., Shcheynikov, N., Kurosaki, T., and Muallem, S. (2001). Regulation of Ca^{2+} -release-activated Ca^{2+} current (I_{CRAC}) by ryanodine receptors in inositol 1,4,5-trisphosphate-receptor-deficient DT40 cells. *Biochem. J.* *360*, 17–22.
- Fierro, L., and Parekh, A.B. (1999). On the characterisation of the mechanism underlying passive activation of the Ca^{2+} release-activated Ca^{2+} current I_{CRAC} in rat basophilic leukaemia cells. *J. Physiol. (London)* *520*, 407–416.
- Tsien, J.Z., Chen, D.F., Gerber, D., Tom, C., Mercer, E.H., Anderson, D.J., Mayford, M., Kandel, E.R., and Tonegawa, S. (1996). Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* *87*, 1317–1326.
- Marek, K.W., and Davis, G.W. (2002). Transgenically encoded protein photoinactivation (FIAH-FALI): acute inactivation of synaptotagmin I. *Neuron* *36*, 805–813.
- Poskanzer, K.E., Marek, K.W., Sweeney, S.T., and Davis, G.W. (2003). Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. *Nature* *426*, 559–563.
- Tour, O., Meijer, R.M., Zacharias, D.A., Adams, S.R., and Tsien, R.Y. (2003). Genetically targeted chromophore-assisted light inactivation. *Nat. Biotechnol.* *21*, 1505–1508.
- Li, W.H., Schultz, C., Llopis, J., and Tsien, R.Y. (1997). Membrane-permeant esters of inositol polyphosphates, chemical syntheses and biological applications. *Tetrahedron* *53*, 12017–12040.