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Research Paper

Small molecule-based laser inactivation of inositol 1,4,5-trisphosphate receptor

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Abstract

Background: Chromophore-assisted laser inactivation (CALI) is a powerful method for the study of in situ protein function in cellular processes. By using CALI, it is possible to abrogate the function of a target protein with unprecedented spatial and temporal resolution. However, CALI has some limitations, which restrict wider biological application, owing mainly to the use of antibody for target recognition. To circumvent the limitations, we have developed small molecule-based CALI (smCALI).

Results: The inositol 1,4,5-trisphosphate receptor (IP_3R) was selected as the target protein and a malachite green-conjugated IP_3 analog, $MGIP_3$, was used as a small-molecular probe. We examined the effect of $MGIP_3$-based CALI on Ca^{2+} release via IP_3R using permeabilized smooth muscle cells. When the cells were treated with $MGIP_3$ followed by laser irradiation, the IP_3-induced Ca^{2+} release rate was decreased in a concentration- and irradiation time-dependent manner. The effect was specific for IP_3R, because the Ca^{2+} uptake function of the co-localized sarco/endoplasmic reticulum Ca^{2+}-ATPase was not affected.

Conclusions: IP_3R was specifically inactivated by smCALI using $MGIP_3$. The efficiency of inactivation was calculated to be substantially greater than that of antibody-based CALI. The efficient and specific inactivation of IP_3R would allow us to obtain an insight into spatiotemporal roles of IP_3R in various cell functions. Our results may be considered to be a first step for a wider application of smCALI as a useful method to study spatiotemporal protein functions.

Keywords: Calcium release; Inositol 1,4,5-trisphosphate receptor; Laser inactivation; Small-molecular probe

1. Introduction

Specific inactivation of biomolecules has been one of the most widely used methods to clarify the physiological functions of the molecules. Various methods have been employed, including the use of pharmacological antagonists, gene targeting or antibody against the target molecules. Some of the functional biomolecules change their activities according to expressed locations and to involved cellular processes [1–3], so it is often important to inactivate them in a spatiotemporally controlled manner. It may also be important to inactivate biomolecules at an appropriate developmental stage or in a short period of time so that their chronic loss does not result in embryonic lethality or genetic compensation. Chromophore-assisted laser inactivation (CALI), originally developed in 1988 by Jay, is an excellent method for achieving this purpose [4]. In CALI, chromophore-labeled antibody molecules are introduced into cells, which are then subjected to laser irradiation. Upon absorbing the laser energy, the chromophores mediate generation of radical species [5]. Because the radical species are highly reactive and have a very short lifetime, only the antibody-recognized proteins are specifically inactivated. The functions of various biomolecules, which had been unable to be analyzed by other methods, have been elucidated by inactivation of target proteins at the appropriate site and time using the CALI technique [6].

Although CALI has proved very powerful, it has some limitations, which are primarily attributable to the use of antibody for the molecular recognition. First, the extent of the damage inflicted on the target protein cannot readily

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be controlled, because it is difficult to label antibody molecules with chromophores at specific amino acid residues. Therefore, inactivation would not take place, for example, if the chromophores were conjugated at residues too far away from the antigen binding site [7]. Furthermore, antibody binding to the target protein might be blocked, if the antigen binding site were labeled with the chromophores. Second, it is necessary to use invasive methods to introduce antibody molecules into cells. In most CALI experiments, antibody introduction is conducted by microinjection or trituration. These methods are not universally applicable, and indeed, only a few kinds of cells have been studied.

To overcome the above limitations, we set out to develop a new method in which synthetic small molecules are used instead of antibody for molecular recognition. Various kinds of compounds can be utilized after modification of certain functional groups, so it is easier to control the relative position and distance between the chromophore and the target protein. In addition, membrane-permeable probes can be designed using established methods [8].

For the implementation of small molecule-based CALI (smCALI), we chose the inositol 1,4,5-trisphosphate receptor (IP_3R) as a target protein. IP_3R is a Ca^{2+} release channel found on the endoplasmic reticulum (ER) of virtually all types of cells, and regulates the cytosolic Ca^{2+} concentration, playing an important role in various physiological functions such as secretion, proliferation and muscle contraction [9,10]. Thus, spatiotemporally controlled inactivation of IP_3R using CALI should be useful for the study of the role of the protein in those functions. We have designed and synthesized a chromophore-labeled IP_3 analog (carboxymalachite green-aminopropyl-1D-*myo*-inositol 1,4,5-trisphosphate, $MGIP_3$) in a previous study [11]. Here we show that $MGIP_3$, a synthetic small molecule, can function as an effective probe for smCALI, resulting in specific inactivation of IP_3R upon laser irradiation.

2. Results

2.1. Biological characteristics of $MGIP_3$

We previously designed and synthesized $MGIP_3$ (Fig. 1) [11] as a potential small-molecular probe for CALI. The vicinal phosphates at the 4- and 5-positions of IP_3 were not modified because they are critical for binding to IP_3R [12,13]. The chromophore was conjugated with the phosphate at the 1-position of IP_3, because this phosphate is not critical for binding and because compounds modified at this position are no longer substrates for IP_3-metabolizing enzymes, such as 5-phosphatase and 3-kinase [14]. Malachite green (MG) was chosen as the chromophore, since it has been commonly used for CALI. We examined the effects of $MGIP_3$, the chromophore moiety of $MGIP_3$

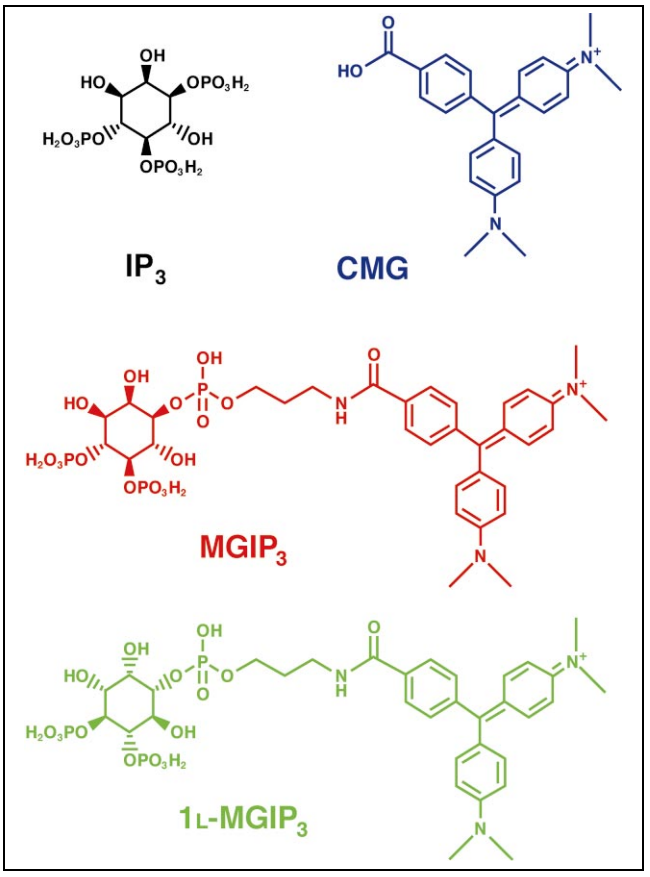

Fig. 1. Structures of chromophore-conjugated IP_3 ($MGIP_3$) and its analogs.

(carboxymalachite green, CMG) or the optical isomer of $MGIP_3$ ($1L$-$MGIP_3$) and IP_3 on IP_3R in smooth muscle cells (Fig. 2). $MGIP_3$ induced Ca^{2+} release in a dose-dependent manner, although the EC_{50} was seven-fold higher than that of IP_3 (Table 1). The Ca^{2+} release activity of $1L$-$MGIP_3$ was >30-fold less than that of $MGIP_3$. This enantioselective ligand recognition by IP_3R is consistent with the known difference between the activities of $1D$-IP_3 and $1L$-IP_3 [15]. CMG had no detectable Ca^{2+} release activity even at a concentration as high as 10 μM.

2.2. Effect of linker structure on the extent of inactivation

In the conventional CALI, antibodies are labeled with malachite green isothiocyanate (MGITC) and the resulting linker forms a thiourea (Fig. 3). In our smCALI, the succinimidyl ester of CMG (CMGSE) was used for conjugation with the amine of an intermediate of $MGIP_3$ (1D-1-*O*-(3-aminopropyl-1-phospho)-*myo*-inositol 4,5-bisphosphate) [11], because MGITC itself did not react efficiently. The conjugation with CMGSE formed an amide bond. In view of the structural difference between thiourea-linked MG and amide-linked MG, it is possible that they have different efficiencies of radical species generation, which might alter the extent of damage to the target protein. We therefore examined the effect of linker structure on

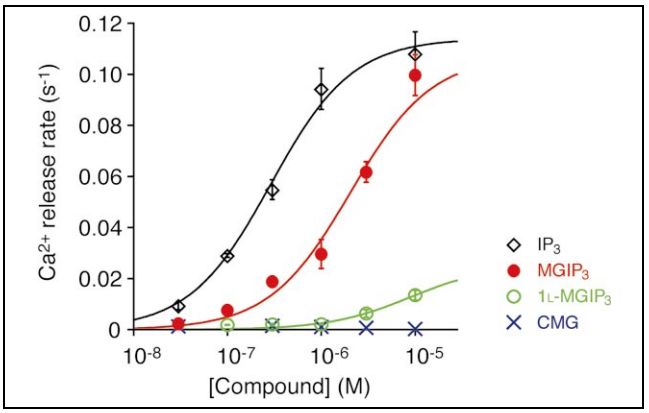

Fig. 2. Ca^{2+} release activity via IP_3R induced by $MGIP_3$ and its analogs. The averages of initial rates of Ca^{2+} release were plotted against the compound concentrations (IP_3, diamonds, $n \geq 3$; $MGIP_3$, filled circles, $n \geq 3$; 1_L-$MGIP_3$, open circles, $n \geq 3$; CMG, crosses, $n \geq 1$). Error bars represent S.E.M.

the extent of inactivation by measuring β-galactosidase activity, which is an established assay for CALI [4,5]. We prepared MGITC-labeled and CMGSE-labeled anti-β-galactosidase antibodies. The average numbers of labeled chromophores per antibody molecule were 8.0 and 7.1, respectively. Using these antibody probes, CALI experiments were performed as previously described [4]. The results showed that there was little difference in the extent of inactivation between MGITC-labeled anti-β-galactosidase antibody (66.2%) and CMGSE-labeled anti-β-galactosidase antibody (56.9%). Thus, $MGIP_3$ was expected to generate radical species as efficiently as MGITC-labeled compounds do upon laser irradiation, and to be a potential probe for CALI of IP_3R.

2.3. *CALI of IP_3R using $MGIP_3$*

After measurement of the IP_3-induced Ca^{2+} release (IICR) rate, the permeabilized smooth muscle cells were

Table 1
Ca^{2+} release activity of various compounds on permeabilized smooth muscle cells

Compound	EC_{50} (nM)
IP_3	290
$MGIP_3$	2 000
1_L-$MGIP_3$	> 60 000
CMG	–

either irradiated or not in the presence or absence of $MGIP_3$. The IICR rate of each specimen was then measured again. A 20% decrease in Ca^{2+} release rate was observed in the untreated specimens, which underwent neither $MGIP_3$ addition nor laser irradiation (Fig. 4b). Such a 20% run-down effect was also observed when only laser irradiation was applied in the absence of $MGIP_3$ or when 1 μM $MGIP_3$ was applied without laser irradiation. In the absence of $MGIP_3$ no difference in the rate of IICR was found with or without laser irradiation. The results suggest that non-specific light-induced damage did not occur. On the other hand, the combination of both 1 μM $MGIP_3$ addition and laser irradiation for 3 min resulted in a significant decrease in the Ca^{2+} release rate (red trace in Fig. 4a and red column in Fig. 4b). Considering the run-down effect, about 50% of IP_3R was inactivated using $MGIP_3$-based CALI.

2.4. *Irradiation time and $MGIP_3$ concentration dependence of CALI*

We examined whether the extent of $MGIP_3$-based laser inactivation of IP_3R depended on the duration of laser irradiation and on the $MGIP_3$ concentration. We performed CALI experiments varying the irradiation time between 0 and 7 min in the presence of 1 μM $MGIP_3$. The 7 min irradiation induced a considerable decrease in Ca^{2+} release rate of the $MGIP_3$-pre-incubated specimen (Fig. 5a). However, no decrease was observed other than

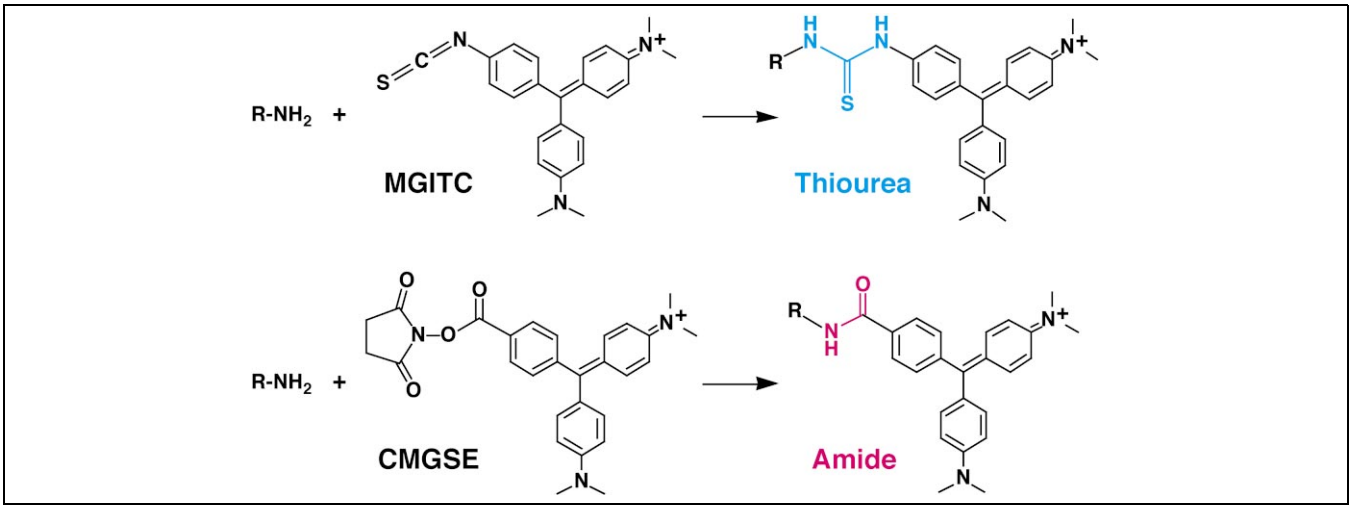

Fig. 3. Structural difference between thiourea-linked MG and amide-linked MG.

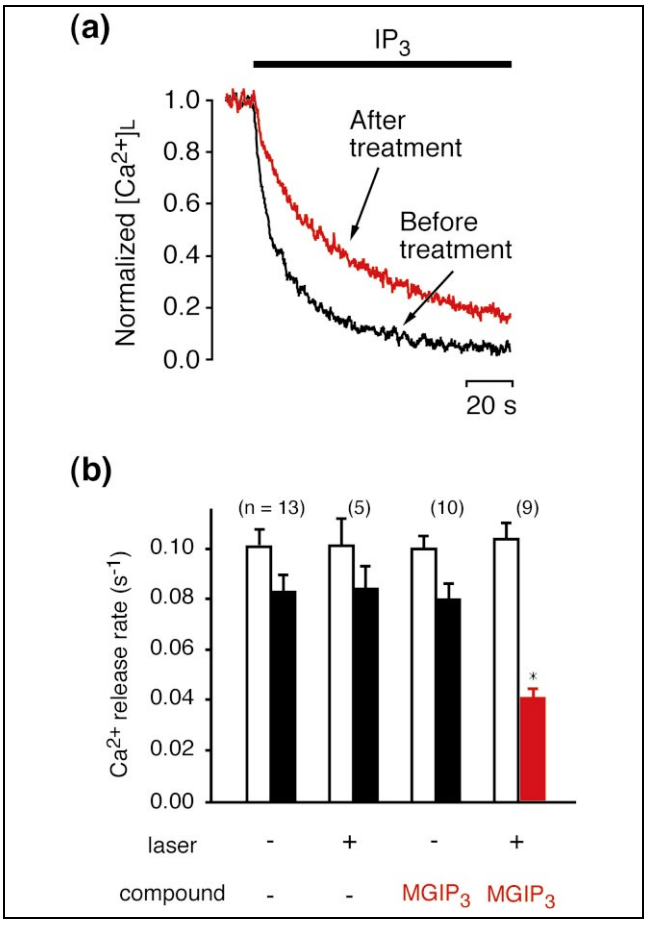

Fig. 4. IP_3-induced Ca^{2+} release is inactivated by CALI using $MGIP_3$. **(a)** Time courses of IICR before (black trace) and after (red trace) treatment with 1 μM $MGIP_3$ and 635 nm laser irradiation for 3 min. Luminal Ca^{2+} concentration ($[Ca^{2+}]_L$) was monitored. Application of 1 μM IP_3 is indicated by the horizontal bar. **(b)** Initial rates of IICR before (open columns) and after (filled columns) the indicated treatments (mean ± S.E.M., the number of experiments is indicated at each column). *Significant differences were found only when the Ca^{2+} release rate of the double-treated group (red column) was compared with the other groups after treatment ($P < 0.0001$, analysis of variance (ANOVA) test and Student's unpaired t-test). The differences among the other three Ca^{2+} release rates after treatment were not statistically significant ($P > 0.6$, ANOVA test). No significant difference was found among the Ca^{2+} release rates before treatment ($P > 0.9$, ANOVA test).

the run-down effect in the non-irradiated specimen even with 7 min of 1 μM $MGIP_3$ incubation. The IICR of the irradiated specimen decreased exponentially depending on the irradiation time, with a $t_{1/2}$ of 4 min. The $MGIP_3$ concentration was then varied between 0 and 1 μM with the irradiation time fixed at 3 min. As shown in Fig. 5b, there was also a concentration dependence of the extent of inactivation, and the plots were well fitted by a bimolecular interaction model. These results suggest that the mechanism of $MGIP_3$-based CALI is very simple: only when $MGIP_3$ is bound to IP_3R does the inactivation occur upon laser irradiation. At $MGIP_3$ concentrations exceeding 3 μM, we could not completely wash out $MGIP_3$ from the specimens, due probably to the hydrophobic nature of $MGIP_3$. Thus, the concentration was fixed at 1 μM in the following experiments.

2.5. *$MGIP_3$-based CALI is specific*

CALI experiments were performed using CMG or 1L-$MGIP_3$. There was no inactivation other than the run-down effect when these compounds were used in place of $MGIP_3$ (Fig. 6). It is one of the advantages of smCALI, as shown here, that the specificity of inactivation of the target protein can be demonstrated by using the optical isomer as a control probe. Because neither CMG nor 1L-$MGIP_3$, but only $MGIP_3$ was able to cause laser-mediated inactivation, the binding of $MGIP_3$ to IP_3R was assumed to be essential for the CALI of IP_3R. To confirm this

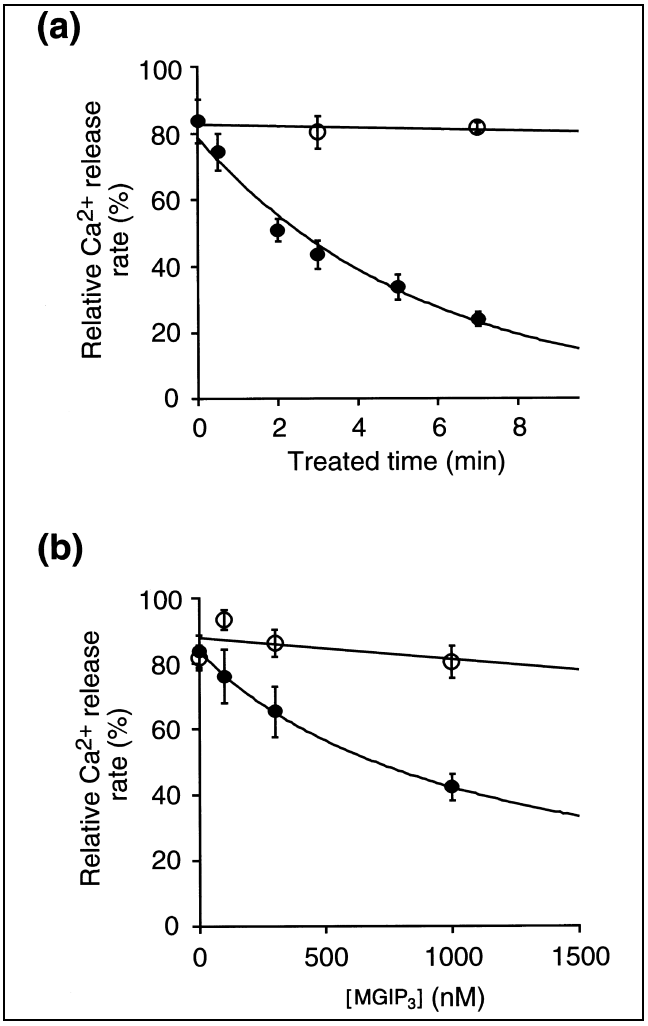

Fig. 5. Dependence of CALI on the period of laser irradiation and the concentration of $MGIP_3$. **(a)** Specimens were subjected to 635 nm laser irradiation in the presence of 1 μM $MGIP_3$ for the indicated period of time, and the ratio of the Ca^{2+} release rate after the treatment to that obtained before the treatment was plotted (filled circles, mean ± S.E.M., $n \geq 4$). In control experiments specimens were subjected to the same solution change without laser irradiation (open circles, mean ± S.E.M., $n \geq 4$). **(b)** Various concentrations of $MGIP_3$ were applied to the specimen, and the relative Ca^{2+} release rates with (filled circles) or without (open circles) 3 min laser irradiation are shown (mean ± S.E.M., $n \geq 5$).

conclusion, the binding of $MGIP_3$ to IP_3R was competitively inhibited by addition of a high concentration of IP_3 (100 μM) to a $MGIP_3$-containing solution. In the presence of IP_3, the laser irradiation failed to cause an $MGIP_3$-induced inhibitory effect (Fig. 6). These results strongly suggest that the specific binding of $MGIP_3$ to IP_3R is essential for the laser-mediated inactivation of IP_3R.

2.6. *Effect of CALI on Ca^{2+} loading*

The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is co-localized with IP_3R on the same ER membrane [16], and works for the Ca^{2+} uptake of the Ca^{2+} store [17]. We therefore studied if $MGIP_3$-based CALI had any effect on the Ca^{2+} uptake capacity of the Ca^{2+} store. Using permeabilized smooth muscle cells, we measured and compared the Ca^{2+} loading rates before and after the various treatments (see Materials and methods). There was no significant change in the Ca^{2+} loading rate (Fig. 7), even after laser irradiation in the presence of $MGIP_3$. These results indicate that $MGIP_3$-based CALI did not induce non-specific damage to non-targeted proteins, even if they were present on the same intracellular organelle.

3. Discussion

In the present study, we demonstrated for the first time that CALI can be successfully performed using a synthetic small-molecular probe instead of a conventional chromophore-labeled antibody. We examined the effect of $MGIP_3$-based CALI on the Ca^{2+} release activity in permeabilized smooth muscle cells. The treatment with $MGIP_3$ followed by laser irradiation decreased the Ca^{2+} release rate in a concentration- and irradiation time-dependent manner. The effect was specific for IP_3R, because the Ca^{2+} pump activity of SERCA, which is co-localized in the same intracellular organelle with IP_3R, was not affected.

We compared the inactivation efficiency of smCALI with that of antibody-based CALI. The recent study of antibody-based CALI of IP_3R by Takei et al. [18] may be an appropriate case for comparison. Those authors labeled a monoclonal anti-IP_3R antibody (4C11) with MG, introduced it into chick DRG neurons by trituration for the CALI experiments, and found that IICR in growth cones has a crucial role in the control of nerve growth. Prior to the experiments in DRG neurons, in vitro CALI assay using microsomal fraction was conducted for evaluation of the potency of MG-labeled 4C11 as a probe for CALI. In this assay, MG-labeled 4C11 required 5 mJ laser power at 10 Hz for 10 min within an area of 1.5 mm diameter to inactivate about 50% of IICR. In our case, to obtain the same extent of $MGIP_3$-mediated IICR inactivation, 3 min irradiation with 15 mJ laser light at 10 Hz was required within an area of 3 mm diameter (Fig. 5a). Calculation of the total energy applied to each sample shows that $MGIP_3$-based inactivation requires four times less energy than 4C11-mediated inactivation (3.8 and 17.0 J/mm^2 for $MGIP_3$ and MG-labeled 4C11, respectively). If we consider the number of chromophores labeled per antibody molecule (six to eight in typical CALI experiments) [4], the efficiency of $MGIP_3$-based CALI at the single chromophore level seems to be six to eight times greater than that of antibody-based CALI, on top of the four-fold difference in the laser energy requirement. This simple comparison suggests that $MGIP_3$ works with substantially

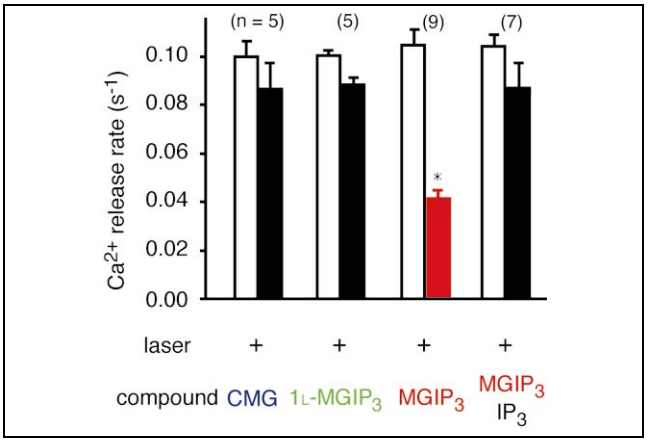

Fig. 6. Specificity of $MGIP_3$-based CALI. CMG, 1_L-$MGIP_3$ or $MGIP_3$ in the presence of 100 μM IP_3 was added to the specimens followed by laser irradiation. The data of $MGIP_3$ in Fig. 4b are also shown (red column). The concentration of the compounds was 1 μM and the laser irradiation time was 3 min throughout these experiments. Initial rates of Ca^{2+} release of each specimen before and after the indicated treatments are shown (mean ± S.E.M., the number of experiments is indicated at each column). *Significant differences were found only when the Ca^{2+} release rate of the $MGIP_3$-treated group (red column) was compared with the other groups after treatment ($P < 0.0005$, ANOVA test and Student's unpaired t-test). The differences among the other three Ca^{2+} release rates after treatment were not statistically significant ($P > 0.8$, ANOVA test). No significant difference was found among the Ca^{2+} release rates before treatment ($P > 0.8$, ANOVA test).

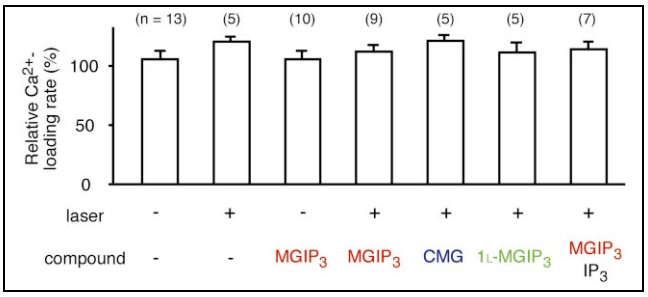

Fig. 7. Effect of $MGIP_3$-based CALI on the activity of SERCA. The Ca^{2+} loading rate of the permeabilized smooth muscle cells as an indicator of SERCA pump activity was evaluated from the slope of the normalized time course of Mg-ATP^{2-}-induced Ca^{2+} loading. The Ca^{2+} loading rates of each specimen before and after the indicated treatments were measured, and the relative Ca^{2+} loading rates are shown (mean ± S.E.M., the number of experiments is indicated at each column). No significant difference was found among all the relative Ca^{2+} loading rates ($P > 0.2$, ANOVA test)

greater efficiency than MG-labeled 4C11, although there could be errors due to subtle differences in the experimental conditions.

Why was $MGIP_3$ able to cause such efficient and specific inactivation of IP_3R upon laser irradiation? We estimated the distance from MG to the binding site of IP_3R to answer this question. The intramolecular distance from the central C atom of MG to the P atom of phosphate at the 5-position of $MGIP_3$, which interacts strongly with the binding site of IP_3R [12], is ~17 Å according to semi-empirical PM3 [19] calculation. Since the half-maximal damage is restricted to a distance of 15 Å from MG [5], $MGIP_3$ should cause effective damage at most ~32 Å away from the IP_3 binding site. Taking into consideration the reports that IP_3R has surface dimensions of 150–250 Å on each side with four-fold symmetry [20,21], only a small region of IP_3R should be inactivated. This is consistent with the conclusion that $MGIP_3$ caused specific and efficient inactivation of IP_3R in the present experiments. It is one of the key advantages of smCALI that we can place a chromophore in close proximity to the target protein. In contrast, it is difficult to label specific amino groups near the antigen binding site of an antibody with chromophores, because antibodies are large molecules per se (150 kDa and 85 Å long for IgG) [7].

The smCALI technique may be applied more generally because a variety of proteins could be targeted by choosing specific small molecules for recognition. Since carboxyl group-activated malachite green (CMGSE or MGITC) can be readily conjugated to any small molecules in a mild condition through an amino group, it would be possible to yield small-molecular probes by high-throughput synthesis. Although small molecules without an amino group must be modified before conjugation with MG, it is often straightforward to modify the synthetic pathway of such molecules to yield derivatives suitable for the conjugation. Furthermore, small-molecular probes may be modified so that their properties are more advantageous for biological experiments. For example, $MGIP_3$ could be introduced into live cells by masking all the anionic phosphate groups with, say, propyloxymethyl groups to form esters [8]. Such flexibility of molecular design and appropriate chemical modification should readily provide diverse small-molecular probes suitable for specific experimental needs. Thus, smCALI should be widely applicable for the elucidation of the functions of many proteins in a spatiotemporally specific manner.

4. Significance

To circumvent the limitations of conventional antibody-based CALI, we have developed small molecule-based CALI (smCALI). Use of small molecules for target recognition is advantageous in the following points. (1) It is possible to gain control of the relative position and the distance between the chromophore and target protein, which critically determines the efficiency and the nature of the inactivation. (2) It is possible to synthesize membrane-permeable probes for non-invasive delivery into cells. Thus, our strategy to challenge biological conundrums using small molecules may be promising for wide-range application of CALI. In this study we targeted IP_3R, which regulates intracellular Ca^{2+} dynamics and thereby plays an important role in various physiological functions. The use of the present probe would allow us to obtain an insight into spatiotemporal roles of IP_3R in various cell functions, especially in polarized cells like neurons.

5. Materials and methods

5.1. Materials

β-Escin and anti-β-galactosidase monoclonal antibody were purchased from Sigma, mag-fura-2 AM and MGITC from Molecular Probes, *o*-nitrophenyl-β-D-galactopyranoside and β-galactosidase from Wako Chemicals (Osaka, Japan) and IP_3 from Dojin-do (Kumamoto, Japan). All other materials were purchased either from Sigma or from Wako Chemicals. The synthesis of $MGIP_3$, 1L-$MGIP_3$, CMGSE and CMG has been reported elsewhere [11].

5.2. β-Galactosidase assay

The β-galactosidase assay was conducted as previously described [4].

5.3. Preparation of permeabilized smooth muscle cells

Ca^{2+} release via IP_3R was measured using permeabilized smooth muscle cells as previously described [16,22]. Briefly, thin smooth muscle bundles (2–3 mm in length, 200–250 μm in width and 50–60 μm in thickness) were dissected from the portal vein of guinea pigs and were tied to thin stainless steel wires. The specimens were first incubated for 3.5 h at 35°C with 40 μM mag-fura-2 AM in physiological salt solution (150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.6 mM glucose and 5 mM HEPES; pH 7.4) containing 0.1% bovine serum albumin. Then, the sample was permeabilized by incubation with 30–50 μM β-escin in relaxing solution (116 mM potassium methanesulfonate, 3.31 mM ATP, 0.554 mM magnesium methanesulfonate, 1 mM EGTA and 20 mM PIPES; pH 7.0). The specimens thus contained the Ca^{2+} indicator within the intracellular organelles and real-time monitoring of the luminal Ca^{2+} concentration ($[Ca^{2+}]_L$) was possible.

5.4. Measurement of Ca^{2+} release rate and Ca^{2+} loading rate

The specimens were inserted into a small glass capillary (4 mm in length, 400 μm in internal diameter) and were attached to the cuvette holder of a fluorescence spectrophotometer (CAF-110,

from Jasco, Tokyo, Japan). The fluorescence intensity of magfura-2 was measured with dual-wavelength excitation at 340 and 375 nm and the ratio of the fluorescence intensities was used as an indicator of $[Ca^{2+}]_L$. One end of the glass capillary was connected to the various solutions and the other to two peristaltic pumps so that the solutions around the specimen could be changed. The solution within the capillary was sequentially changed to load and release Ca^{2+} from the Ca^{2+} store of the specimen. The slope of the normalized time course of the Mg-ATP^{2-}-induced increase in $[Ca^{2+}]_L$ was used as an indicator of the pump activity of SERCA. The initial part of the normalized time course of IP_3- or other test compound-induced Ca^{2+} release was fitted by a single exponential function, e^{-rt}, where r is the rate constant, which we used as an index of the activity of IP_3R.

5.5. Inactivation of IP_3R by CALI

The rate of IICR from the specimens was measured at an IP_3 concentration of either 100 nM or 1 μM. The capillary with the specimen inside was transferred to and fixed on an ice-cooled metal board and then was irradiated for various periods of time using a pulsed Nd:YAG-driven dye laser (wavelength 635 nm, Surelite Laser and Surelite Optical Parametric Oscillator, Continuum, Santa Clara, CA, USA) with spot size 3 mm, pulse width 2–4 ns, and pulse energy 14–16 mJ at 10 Hz. The laser energy at this level does not cause obvious damage to cellular components [6]. The solution around the specimen was constantly changed by pipetting during the laser irradiation. In the control experiments, only the solution was changed without irradiation. After the irradiation, the capillary was re-transferred to the cuvette and the rate of IICR was measured again at the same IP_3 concentration as in the initial measurement. Although only the data of 1 μM IICR rate are given in the present study, essentially the same results were obtained when the rate of IICR at 100 nM IP_3 was measured before and after CALI.

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