Biosensors

DOI: 10.1002/ange.200903951

An In Vivo Fluorescent Sensor Reveals Intracellular Ins(1,3,4,5)P_4 Dynamics in Single Cells**

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The signaling cascades to link extracellular messengers to intracellular Ca^{2+} mobilization are regulated by the second messenger D-*myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3).[1] A direct metabolite of Ins(1,4,5)P_3, D-*myo*-inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4), is also believed to be a pivotal second messenger in cellular signal transduction because of its close relevance to chromatin remodeling,[2] modulation of Ins(1,4,5)P_3 levels,[3] Ca^{2+} mobilization,[4] and immune cell development.[5] Although current interests have focused on the action of Ins(1,3,4,5)P_4, its physiological function remains to be established. The tools that realize the real-time detection of the temporal and spatial dynamics of Ca^{2+} influx[6] and Ins(1,4,5)P_3[7,8] in single cells have accelerated our understanding of their function in cellular signaling events. Because conventional ex situ methods such as HPLC[9] have not provided information on intracellular Ins(1,3,4,5)P_4 mobilization in individual live cells, there is a new demand for a methodology that visualizes the cellular dynamics of the metabolites of Ins(1,4,5)P_3. Herein, we report a fluorescent biosensor that enables real-time monitoring of Ins(1,3,4,5)P_4 mobilization in single mammalian cells.

An optical sensor for Ins(1,3,4,5)P_4 was constructed by utilizing the pleckstrin homology (PH) domain of the general receptor for phosphoinositides 1 (GRP1)[10] that possesses high affinity and selectivity to Ins(1,3,4,5)P_4. Because an Ins(1,4,5)P_3 sensor[7] was successfully obtained from the PH domain of phospholipase C (PLC) δ_1, we conducted a similar structure-based design for the Ins(1,3,4,5)P_4 sensor. Inspection of the three-dimensional structure of the GRP1 PH domain–Ins(1,3,4,5)P_4 complex[11] indicated Val15 and Glu82 as possible fluorophore labeling sites (Figure 1a). All three of the original cysteine residues in the GRP1 PH domain, Cys29, Cys63, and Cys79, were replaced with Ser29, Ala63, and Ser79, respectively, then five arginine residues were introduced at the C terminal of the GRP1 PH domain to improve the solubility. A unique cystein residue, Cys15 or Cys82, was introduced to the resultant mutant followed by labeling with 6-odoacetamidofluorescein to give 15F-IP_4 and 82F-IP_4, respectively (Supporting Information, Figure S1).

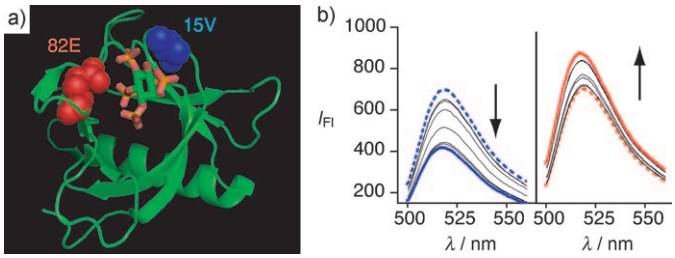

Figure 1. Construction of Ins(1,3,4,5)P_4 sensors. a) Schematic illustration showing the structure of the GRP1 PH domain–Ins(1,3,4,5)P_4 complex.[11] Positions labeled by fluorescein at 15V and 82E are indicated by Corey–Pauling–Koltun (CPK) representation in blue and red, respectively, and Ins(1,3,4,5)P_4 is shown by a wire-frame model. b) Emission spectra (initial: dashed line, final: solid line; I_{FI}: fluorescence intensity) show changes in intensity of the fluorophore-labeled PH domains 15F-IP_4 (left) and 82F-IP_4 (right) in the presence of increasing amounts of Ins(1,3,4,5)P_4.

To evaluate the Ins(1,3,4,5)P_4 binding, changes in the fluorescence emission of these fluorophore-labeled PH domains (200 nM) were monitored by addition of Ins(1,3,4,5)P_4. The fluorescence intensity of 15F-IP_4 eventually decreased to 0.6-fold of the initial intensity (Figure 1b, left). In contrast, 82F-IP_4 showed a 1.3-fold enhancement of the fluorescence intensity in response to increasing concentrations of Ins(1,3,4,5)P_4 (Figure 1b, right). Binding curves for the sensors determined by fluorescence titration were fitted to the standard binding isotherm, which revealed the dissociation constants (K_D) for the Ins(1,3,4,5)P_4 complexes of 15F-IP_4 and 82F-IP_4 to be 130 and 150 nM, respectively (Supporting Information, Figure S2). The fluorescence emission properties of both sensors were not affected significantly in the presence of Ca^{2+} or Mg^{2+} ions at cellular concentration ranges (Supporting Information, Table S1).

The selectivity of the Ins(1,3,4,5)P_4 sensor against other phosphoinositide derivatives was evaluated and compared with that of the parent PH domain (Table 1 and Table S2 in

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[**] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (Japan) to T.M. (Nos. 19021023, 20241051). We thank Yoshitsugu Uriu and Dr. Masatora Fukuda for discussions. Ins(1,3,4,5)P_4 = D-*myo*-inositol 1,3,4,5-tetrakisphosphate.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200903951.

Table 1: Dissociation constants (K_D [μM]) of Ins(1,3,4,5)P_4 sensors and wild-type GRP1 PH domain for complexes with inositol phosphate derivatives.

Ligand	15F-IP_4[a]	82F-IP_4[a]	GRP1 PH domain[b]
Ins(1,3,4)P_3	>30[c]	0.40±0.17[e]	>2.3
Ins(1,4,5)P_3	68±12	>30[c]	>4.5
Ins(1,3,4,5)P_4	0.13±0.01	0.15±0.02	0.027
Ins(1,3,4,5,6)P_5	1.0±0.15[d]	>30[c]	0.33
PIP_3	0.20±0.03	2.6±0.50[e]	0.49

[a] Measured in 10 mM phosphate buffer (pH 8.0) containing 50 mM NaCl and 0.005% Tween 20 at 25°C. [b] From reference [12]. [c] The fluorescence intensity was not altered upon addition of up to 30 μM of these nonspecific ligands. [d] The fluorescence intensity of 15F-IP_4 was enhanced by adding Ins(1,3,4,5,6)P_5. [e] The fluorescence intensity of 82F-IP_4 was slightly suppressed by adding Ins(1,3,4)P_3 or PIP_3.

the Supporting Information). Both Ins(1,3,4,5)P_4 sensors exhibited the strongest affinity to Ins(1,3,4,5)P_4. The affinity of both 15F-IP_4 and 82F-IP_4 to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a membranous Ins(1,3,4,5)P_4 derivative with lipid moiety, was lower than that for Ins(1,3,4,5)P_4. Interestingly, the fluorescence intensity of 82F-IP_4, which increased upon binding to the specific ligand Ins(1,3,4,5)P_4, slightly decreased or was virtually unchanged upon adding any other nonspecific ligands. The fluorescence intensity of 15F-IP_4 was suppressed upon binding to Ins(1,3,4,5)P_4, but was enhanced by the addition of ligands such as Ins(1,3,4,5,6)P_5, with a phosphate at the sixth position (Table 1 and Table S2 in the Supporting Information). The Ins(1,3,4,5)P_4-dependent response of 15F-IP_4 was still observed in the presence of 10 μM Ins(1,3,4,5,6)P_5 with the appropriate affinity to monitor intracellular Ins(1,3,4,5)P_4 dynamics (Supporting Information, Figure S3). Such emission characteristics of the sensors would be quite favorable to specifically detect Ins(1,3,4,5)P_4 in the cell.

The Ins(1,3,4,5)P_4 sensor was next taken into HeLa cells by means of electroporation using a submicromolar solution of the sensor (see the Supporting Information). The sensor-loaded cells and the co-stained cells with nucleus-specific Hoechst 33342 showed an even distribution of fluorescence in the cells by confocal microscopy analysis (Figure 2a and Figure S4 in the Supporting Information), which indicated that the sensors would detect the total fluctuation of Ins(1,3,4,5)P_4 in the whole cell. The sensitivity of fluorescence changes would be largely affected by the biosensors in the cytosol, because the receptor-mediated production of Ins(1,4,5)P_3 and subsequent conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 occur mainly in the cytosol.

In assessing the efficiency of 15F-IP_4 and 82F-IP_4 as in vivo Ins(1,3,4,5)P_4 sensors, we analyzed changes in the fluorescence images of the sensor-loaded HeLa cells at an emission wavelength of 510 nm by exciting at 480 nm at ambient temperature. Ins(1,3,4,5)P_4-induced emission changes of the sensor in the cellular environment were observed by permeabilizing the cells with digitonin under controlled Ins(1,3,4,5)P_4 concentration (Figure 2b). The Ins(1,3,4,5)P_4 sensors responded to the increase of intracellular Ins(1,3,4,5)P_4 concentration ($[Ins(1,3,4,5)P_4]_i$) and exhibited K_D values in the cell (68 and 120 nM for 15F-IP_4 and 82F-IP_4, respectively) comparable to those observed in vitro (Table 1). Because the enhancement of $[Ins(1,3,4,5)P_4]_i$ has been reported to be nominally 0–3 μM from resting to the stimulated state of the cell,[5a,13] both Ins(1,3,4,5)P_4 sensors are suitable for monitoring the $[Ins(1,3,4,5)P_4]_i$ fluctuation in living cells.

In HeLa cells, binding of histamine or adenosine triphosphate (ATP) to H1 or ATP receptors, respectively, activates PLC to produce Ins(1,4,5)P_3, thus inducing Ca^{2+} release from the internal Ca^{2+} store.[14,15] It is generally accepted that inositol trisphosphate 3-kinase (IP3K) activated by Ca^{2+} ions phosphorylates Ins(1,4,5)P_3 to produce Ins(1,3,4,5)P_4.[16] To evaluate the potential of 15F-IP_4 and 82F-IP_4 as real-time in

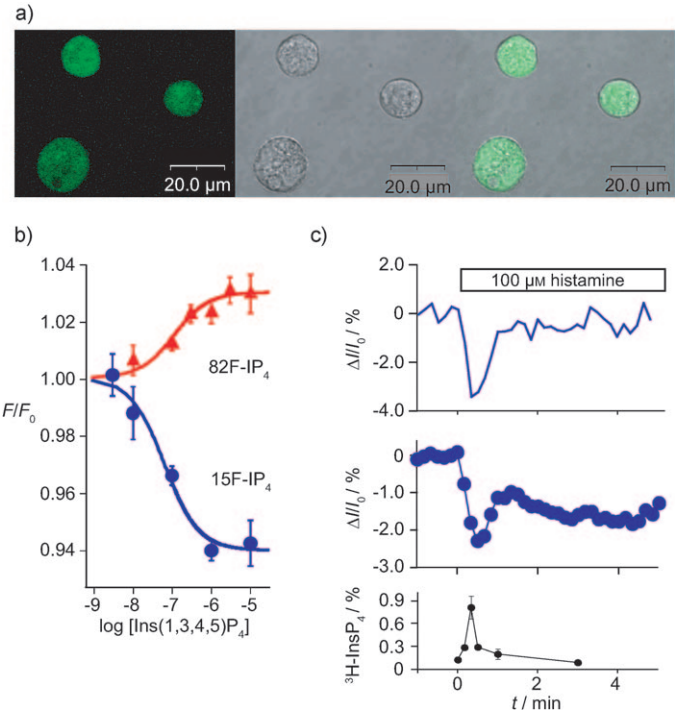

Figure 2. Ins(1,3,4,5)P_4 sensors in HeLa cells. a) Confocal microscopic observation of 15F-IP_4 in HeLa cells. Left: fluorescence image; middle: differential interference contrast (DIC) image of the same cells; right: merged image. b) Binding curves for cells loaded with 82F-IP_4 (red) and 15F-IP_4 (blue) were determined by fluorescence titrations with various concentrations of Ins(1,3,4,5)P_4. The experimental data points of relative intensity at 510 nm (F/F_0), where F_0 is the initial fluorescence intensity of the cell and F is the fluorescence intensity in the presence of the indicated amount of Ins(1,3,4,5)P_4, are plotted against the Ins(1,3,4,5)P_4 concentration (M). c) Time course of the production of Ins(1,3,4,5)P_4 observed by temporal changes of 15F-IP_4 fluorescence in a single cell (top) and in averaged cells (middle, nine cells), and analyzed by ^{3}H-labeled cells (bottom) under 100 μM histamine stimulation.

vivo Ins(1,3,4,5)P_4 sensors, we monitored the time courses of their fluorescence changes upon agonist stimulation of HeLa cells. A typical trace observed for single-cell analysis under histamine stimulation (100 μM) by 15F-IP_4 is shown in Figure 2c (top). An averaged profile of the temporal changes

of the fluorescence (nine cells) displayed an initial suppression of fluorescence intensity in 20 s and subsequent gradual increase of the fluorescence intensity over 60 s (Figure 2c, middle). The observed decrease in the fluorescence intensity of 15F-IP_4-loaded cells was in accordance with the behavior of 15F-IP_4 in vitro. Moreover, the response of 15F-IP_4 was inhibited dose-dependently by the co-incorporation of fluorophore-unlabeled GRP1 PH domain, thus indicating the specific recognition of Ins(1,3,4,5)P_4 by the sensor (Supporting Information, Figure S5). The histamine stimulation resulted in a transient enhancement of the fluorescence signal of 82F-IP_4-incorporated cells, as expected from the in vitro emission property (Supporting Information, Figure S6). Addition of histamine, ATP, or Ca^{2+} up to 100 μM did not affect the fluorescence intensity of either of the two sensors in vitro (Supporting Information, Figure S7).

The production of Ins(1,3,4,5)P_4 upon agonist stimulation in HeLa cells was confirmed by HPLC analyses of HeLa cells containing ^{3}H-labeled inositol derivatives. As reported previously,[14] the ^{3}H-labeling experiments revealed the production of Ins(1,4,5)P_3 within 10 s after stimulation (Supporting Information, Figure S8), followed by Ins(1,3,4,5)P_4 production in 20 s (Figure 2c, bottom) as an averaged profile of cells ($\approx 5 \times 10^6$), roughly consistent with the averaged time course of Ins(1,3,4,5)P_4 production in each cell visualized by the Ins(1,3,4,5)P_4 sensor (Figure 2c, middle). The temporal pattern for the Ca^{2+} dynamics of 15F-IP_4- or 82F-IP_4-loaded cells was quite similar to that of intact HeLa cells as monitored by the Ca^{2+} indicator Fura-2,[6] even after incubation for more than 24 h (Supporting Information, Figure S9). These observations imply that the individual temporal fluctuation of the emission from the Ins(1,3,4,5)P_4 sensor in the single living cell reflects the change of $[Ins(1,3,4,5)P_4]_i$ upon agonist stimulation.

The histamine-dose-dependent behavior of the sensor-loaded cells was investigated to further establish whether the observed fluorescence responses actually correspond to the changes of $[Ins(1,3,4,5)P_4]_i$. The degree by which the fluorescence intensity of 15F-IP_4 changed, calculated from the averaged trace of cells ($\Delta I/I_0$), increased in a dose-dependent manner under histamine stimulation (Figure 3a). A similar behavior was observed for the 15F-IP_4-loaded cells stimulated with ATP (Figure 3b). Furthermore, the observed response of 15F-IP_4-loaded cells to histamine was suppressed by pyrilamine, a histamine antagonist,[14] in a concentration-dependent manner with complete inhibition at around 100 nM (Figure 3c). This effect was also in good accordance with the result of ^{3}H experiments that showed the inhibition of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production by pyrilamine (Supporting Information, Figure S10). A similar experiment using U73122, an inhibitor of PLC,[17] also resulted in the repression of the Ins(1,3,4,5)P_4 sensor responses in a dose-dependent manner, and showed saturation at around 1 μM U73122 (Figure 3d). Complementary results were obtained for each of these experiments using 82F-IP_4-loaded cells (Supporting Information, Figure S11).

In addition, the treatment of the sensor-loaded cells with 1 μM ionomycin, a Ca^{2+} ionophore, induced the fluorescence response of both sensors (Supporting Information, Figure S12). Since ionomycin itself evokes Ins(1,4,5)P_3 production[18] and IP3K activities are known to be enhanced by Ca^{2+},[16] the result most likely reflects the production of Ins(1,3,4,5)P_4 by these Ca^{2+}-activated enzymes. Transfection of small interfering RNA (siRNA) against all three IP3K isozymes resulted in the reduction of messenger RNA (mRNA) production of IP3K, as confirmed by reverse transcription polymerase chain reaction (RT-PCR; Supporting Information, Figure S13). When these siRNA-treated cells were loaded with 15F-IP_4, the cells showed a significant suppression of the fluorescence response of 15F-IP_4 (Supporting Information, Figures S14–S16). These results clearly indicate that the Ins(1,3,4,5)P_4 biosensor specifically detects the real-time changes of $[Ins(1,3,4,5)P_4]_i$ in HeLa cells.

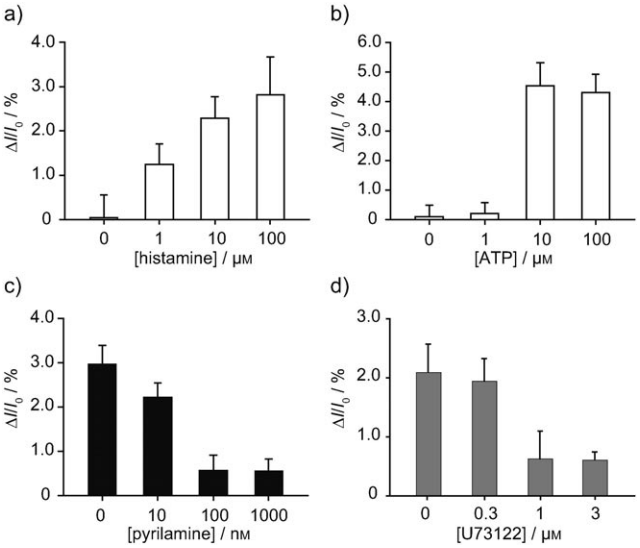

Figure 3. Agonist- and inhibitor-dose-dependent behaviors of Ins(1,3,4,5)P_4 sensors. a) Histamine-dose-dependent fluorescence responses of 15F-IP_4-loaded cells. b) ATP-dose-dependent fluorescence responses of 15F-IP_4-loaded cells. c) Dose-dependent inhibition of 100 μM histamine-induced fluorescence responses by pyrilamine in 15F-IP_4-loaded cells. d) Dose-dependent inhibition of 100 μM histamine-induced fluorescence responses by U73122 in 15F-IP_4-loaded cells. $\Delta I/I_0$/% is shown as the absolute value in this figure.

In conclusion, fluorescent sensors based on the GRP1 PH domain exhibited appropriate affinity and specificity to Ins(1,3,4,5)P_4 and distinct fluorescence responses upon target binding in single cells. Expression of the genetically encoded biosensors sometimes perturbs the intracellular dynamics of target ligands.[8] The Ins(1,3,4,5)P_4 sensor is homogeneously introduced into the cell without greatly affecting the molecular geography of inositol phosphates by controlling the loading conditions, and would serve as a tool to unveil a vital but yet unknown physiological function of Ins(1,3,4,5)P_4.

Received: July 18, 2009
Revised: September 26, 2009
Published online: November 7, 2009

Keywords: biosensors · fluorescent probes · inositol polyphosphates · protein engineering · signal transduction

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