Can We See PIP₃ and Hydrogen Peroxide with a Single Probe?

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Abstract

A genetically encoded sensor for parallel measurements of phosphatidylinositol 3-kinase activity and hydrogen peroxide (H₂O₂) levels (termed PIP-SHOW) was developed. Upon elevation of local phosphatidylinositol 3,4,5-trisphosphate (PIP₃) concentration, the sensor translocates from the cytosol to the plasma membrane, while a ratiometric excitation change rapidly and simultaneously reports changes in the concentration of H₂O₂. The dynamics of PIP₃ and H₂O₂ generation were monitored in platelet-derived growth factor-stimulated fibroblasts and in T-lymphocytes after formation of an immunological synapse. We suggest that PIP-SHOW can serve as a prototype for many fluorescent sensors with combined readouts.

Introduction

Fluorescent microscopy is a powerful method to study cell signaling in vivo. A wide spectrum of fluorescent proteins (FPs), FP-based sensors, and small-molecule chemical dyes allow visualization of many intracellular signaling events. Combinations of various fluorophores with distinct excitation and emission spectra in a single cell enable monitoring of multiple cellular processes simultaneously, usually termed multiparameter imaging (see Supplementary Data; Supplementary Data are available online at www.liebertonline.com/ars).

Phosphorylated forms of phosphoinositide lipids (PIPs) transduce signals via recruiting the PIP-binding domains that vary in their specificity toward the number and position of phosphates of the inositol ring, allowing a fairly precise specificity of downstream signaling activation (8). One important lipid messenger is phosphatidylinositol 3,4,5-trisphosphate (PIP₃). Phosphatidylinositol 3-kinase (PI3K) phosphorylates P(IP₃)₂ to PIP₃, while the lipid phosphatase PTEN reverses the phosphorylation (7). FP fusion with PIP₃-sensitive protein domains allows monitoring of PIP₃ formation by translocation of the fluorescently labeled domain from the cytosol to the plasma membrane (PM) (8).

The global signaling activity of hydrogen peroxide (H₂O₂) is much less commonly addressed (3). H₂O₂ selectively and reversibly oxidizes a small population of cysteines that tend to be deprotonated at physiological pH (9). H₂O₂ production by NADPH oxidase (NOX)/dual oxidase (DUOX) enzymes and generation of PIP₃ by receptor tyrosine kinase activation can be highly cooperative: The NOX subunits p47 and p40 are

Innovation

Combinations of various fluorophores with distinct excitation and emission spectra in a single cell enable monitoring of multiple cellular processes simultaneously, usually termed multiparameter imaging. We successfully combined two different readouts in a single probe: ratiometric for hydrogen peroxide (H₂O₂) and translocation for phosphatidylinositol 3-kinase. The probe simultaneously detects the lipid messenger phosphatidylinositol 3,4,5-trisphosphate (PIP₃) and H₂O₂, showing that the two signals are highly cooperative. The performance of the sensor was tested in two cellular models, including immunological synapse formation of primary human T-cells, where we use it for kinetical measurements of two second messengers simultaneously. Making use of the subcellular localization of PIP₃ at the immune synapse, we are able to analyze H₂O₂ in subcellular domains with good resolution and a very good signal-to-noise ratio. Our data show as a proof of principle that different readouts can be easily combined in a single sensor, increasing a number of measured substances in a multiparameter imaging and minimizing a number of expression constructs.
Results, Discussion, and Future Directions

To generate a dual, PI3K and H2O2 sensor, we fused HyPer with a mutated PH domain (E41K) of Bruton’s tyrosine kinase (BTK). The resulting reporter, named PIP-SHOW (PIP3 and SH Oxidation Watching) (Fig. 1A), emitted fluorescence signals in the expected wavelength range (500–550 nm) when expressed in NIH-3T3 fibroblasts. We then verified if the PIP-SHOW retained the ability of both domains, BTK-PH-E41K and HyPer, to respond to the respective stimuli, H2O2 and PIP3. Addition of H2O2 resulted in the expected change in the probe’s excitation ratio (Fig. 1B) similar to that of HyPer. Incubation of cells with 10 μM of a membrane-permeant photactivatable version of PIP3 (cgPIP3/AM) and subsequent brief illumination with 405 nm light led to partial redistribution of the probe to the PM (Fig. 1C). Hence, both the translocation domain and the HyPer performed correctly.

To further characterize PIP-SHOW, we stimulated NIH-3T3 fibroblasts with platelet-derived growth factor (PDGF) and analyzed the fluorescence signals over time. Addition of PDGF initiated translocation of the probe to the PM reflecting PI3K activation and caused a significant change in the excitation ratio 500/420 nm, indicating generation of H2O2 (Fig. 1B–E). Photouncaging of synthetic PIP3 causes redistribution of the probe to the PM domains. Arrows indicate zones of elevated PIP3. Scale bar = 10 μm. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars). PIP3, phosphatidylinositol 3,4,5-trisphosphate; PM, plasma membrane; BTK, Bruton’s tyrosine kinase.

FIG. 1. PIP-SHOW reports changes in PIP3 and H2O2 levels by two different types of readouts: translocation of the probe and excitation peaks ratio change, respectively. (A) PIP-SHOW consists of two parts, the PIP3-sensitive PH domain of BTK and the H2O2 sensor HyPer. An E41K point mutation was introduced into the PH domain to increase sensitivity. (B) External H2O2 (50 μM) causes change in the sensor excitation ratio, F500/F420, in the cytoplasm of HeLa-Kyoto cell. (C) Photouncaging of synthetic PIP3 causes redistribution of the probe to the PM domains. Arrows indicate zones of elevated PIP3. Scale bar = 10 μm. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars). H2O2, hydrogen peroxide; PIP, phosphatidylinositol phosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PM, plasma membrane; BTK, Bruton’s tyrosine kinase.

FIG. 2. Imaging PIP3 and H2O2 with PIP-SHOW in NIH-3T3 cells stimulated with PDGF. (A) Widefield fluorescence images of PIP-SHOW–expressing NIH-3T3 cells at indicated time points (in minutes) after stimulation of the cells with 10 ng/ml PDGF. Upper row of images represent subcellular distribution of PIP-SHOW ratio reflecting changes in H2O2 level. Middle and lower rows of images show subcellular distribution of PIP-SHOW and changes in fluorescence intensity in each imaging channel. Individual cells are highlighted on the upper left corner ratio panel and numbered 1 to 4. Scale bar = 15 μm. The panel is representative of 20 cells from three experiments. (B–E) Time course of PI3K activation and H2O2 production by the cells 1 to 4 shown on (A). Black lines reflect redistribution of PIP-SHOW to PM, red lines reflect H2O2 level changes in the cells. (F) Pretreatment of the NIH-3T3 cells with wortmannin (500 nM) abolished elevation of both PIP3 and H2O2. Panel represents a typical time course of PI3K and H2O2 production by the cells pretreated with wortmannin. (G) The NOX inhibitor DPI (10 μM) inhibits H2O2 production but does not lead to a drop in PIP3 levels. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars). P3K, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; DPI, diphenyleniodonium; NOX, NADPH oxidase.
H₂O₂ production in more detail, we analyzed their respective dynamics within single cells. Both signals were initially highly cooperative (Fig. 2B–E). Furthermore, Figure 2A–E showed that while both signals rose at similar times, PI3K accumulation reversed with a much slower time course, while H₂O₂ production was strictly transient. This indicates that both signals are not necessarily coupled for prolonged periods of time and highlights the importance of a dual sensor such as PIP-SHOW. This difference in kinetics could result from different sensitivities of the two domains comprising PIP-SHOW. However, this scenario is unlikely given their high sensitivity: BTK-PH-E41K domain was able to detect resting PIP3 in the membrane of nonstimulated T-cells (see below) and the OxyR-RD domain of HyPer has extremely high reaction rates with H₂O₂ (10⁷ M⁻¹ s⁻¹), enabling intracellular H₂O₂ detection even in the presence of peroxiredoxins and glutathione peroxidases. PIP-SHOW does not completely translocate to the PM, and this allows comparison of the excitation ratio between the PM and the cytoplasm. Because PI3K activity acts upstream of NOX/DUOX activation (4), we analyzed the effect of the PI3K inhibitor wortmannin on PI3K activity and H₂O₂ production, after stimulation with PDGF. Figure 2F shows that wortmannin prevented PI3K translocation but also inhibited H₂O₂ production. To further characterize PIP-SHOW, we inhibited NOX enzymes with diphenyleniodonium (DPI). Obviously, DPI led to a rapid drop in PIP-SHOW ratio reflecting decrease in H₂O₂ production (Fig. 2G). Because we did not observe a decrease in PIP3 levels after DPI treatment, a potentially possible feedback loop between PI3K and NOX/DUOX activity via PTEN inhibition can be excluded.

Next, we utilized PIP-SHOW to study lipid and redox signaling events in the first phase of CD4⁺ human T helper (TH) cell activation (Supplementary Data). Transient expression of PIP-SHOW in human TH cells led to its localization within the cytoplasm as well as within the nucleus. However, a fraction of the probe showed membrane localization, indicating of pre-existing PI3K activity (Fig. 3A). Stimulation of TH cells expressing PIP-SHOW with anti-CD3/CD28-coated beads led to establishment of stable contacts, immunological synapses (IS), between TH cells and the beads (Fig. 3A; Supplementary Video S2). This was followed by a massive and immediate redistribution of PIP-SHOW to the IS indicative of locally elevated PIP3 levels. The probe remained in the contact region for the duration of the experiment (up to 1 h). Notably, PI3K activity decreased at the rest of the PM of the activated cell as the ring-like fluorescence pattern disappeared immediately after IS formation (Fig. 3A). Furthermore, IS formation also led to a rapid elevation of H₂O₂ ~ 1 min after translocation of the PIP3 sensor (Fig. 3; Supplementary Video S2). Interestingly, in most of the cells H₂O₂ production was highest in the region adjacent to the central synapse (Fig. 3E), implicating a ring-like localization of NOX/DUOX enzymes around the central IS. DPI quickly attenuated H₂O₂ production (Fig. 3F), suggesting that PM-localized NOX/DUOX are the source of H₂O₂. Similar to NIH-3T3 cells, there was no fast positive feedback loop between PI3K and NOX/DUOX activities, as we did not observe a decrease in PIP3 upon addition of DPI (Fig. 3F). However, it is possible that the oxidant-induced decrease in phosphatase activity is only slowly reversible and acts in a long-term memory-like fashion. This might be the reason that a decrease in PIP3 concentration was not detected in the time course of this experiment. Inhibition of PI3K by wortmannin, however, led to a rapid decrease in both PI3K activity and H₂O₂ production (Fig. 3G) as expected.

We demonstrated that the novel dual-parameter sensor PIP-SHOW is well suited for simultaneous monitoring of PIP3 and H₂O₂ levels and can thereby serve as a prototype for indicators with combined readouts. The concept of a translocating domain fused to a ratiometric sensor is widely applicable to other combinations of intracellular signaling parameters as well. The second component of PIP-SHOW, HyPer, was designed and used to report H₂O₂ levels (2). Because HyPer is a protein of moderate size (~2 green fluorescent protein [GFP] molecules), it can be fused with subcellular localization tags. As HyPer is derived from a bacterially encoded protein, it is unlikely to interfere with other proteins and signaling pathways in mammalian cells. An important advantage of HyPer and therefore PIP-SHOW is the ratiometric response to H₂O₂, which is independent on the relative amount of the sensor, its redistribution, cell movement, or shape change. Indeed, in the theoretical case of intensiometric readout for H₂O₂, it would be impossible to discriminate between PI3K and near-membrane H₂O₂ during PI3K activation and PIP-SHOW translocation. Thus, the ratiometric nature of HyPer is necessary to allow the simultaneous quantification of H₂O₂ during PI3K activity. In addition, we also showed that PIP-SHOW is combinable with GFP-based sensors, allowing synchronous measurements of an additional third parameter (Supplementary Fig. S1; Supplementary Data).

We validated PIP-SHOW using two cellular systems in which both PI3K and NOX/DUOX (reactive oxygen species [ROS]) were already shown to be important determinants of...
IMAGING PIP₃ AND H₂O₂ WITH ONE SENSOR

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several signaling pathways. Our results provide relevant information about the effectiveness of the sensor but also new information about the cooperative dynamics of the two signaling systems. While PDGF stimulated fibroblasts are a well-documented experimental model to study lipid and redox signaling, not much was known about the role of ROS and their interaction with PI3K and, respectively, PIP3 in human TH cells.

In both cellular systems, H2O2 and PIP3 demonstrated a high degree of synchronicity, particularly in the initial phase after stimulation (see Figs. 2 and 3). In fibroblasts, however, the decrease in H2O2 was accompanied by decrease in PIP3 only in a fraction of the cells. In polarized T cells, on the other hand, the PI3K activity rapidly redistributed and increased at the IS and decreased in the rest of the PM. The H2O2 distribution demonstrated a higher degree of heterogeneity: whereas in most of the cells highest H2O2 levels were detected at the IS, in some H2O2 was more evenly distributed (Fig. 3E). Nevertheless, in most cases the source of H2O2 was visibly associated with either the PM or, more specifically, the IS. Interestingly, in contrast to fibroblasts, PI3K activity in the stimulated TH cells started before H2O2 production was detectable with HyPer (Figs. 2 and 3). More physiological studies are needed to determine the functional role of local ROS microdomains close to the IS not only in TH but also in other immune cells.

PIP3 concentration at the PM is a result of the concerted action of the PI3-kinases and lipid phosphatases. The latter contain, similar to protein tyrosine phosphatases, thiolates in the active site, rendering them redox sensitive. H2O2 was shown to oxidize the lipid phosphatase PTEN, enabling positive feedback loop in the phosphorylation cascade. We would therefore expect to see a decrease in PIP3 concentration upon elimination of H2O2. The kinetics of PIP-SHOW disulphide reduction should be similar to the reduction of the phasemases. To our surprise, in both experimental models we did not see an effect of NOX inhibition on the PIP3 content. While the HyPer signal (H2O2) decreased rapidly upon addition of DPI, the localization of the PH domain did not change, indicating that PIP3 concentrations did not decrease significantly. A possible explanation is that in strongly stimulated cells, PI3K activity is dominating over phosphatases activity in such a way that inhibition of the phosphatases (at least lipid phosphatases) by H2O2 does not shift the equilibrium significantly. Another interpretation might be an increased sensitivity of the probe to PIP3 due to the E41K mutation that prevents retrograde translocation of the sensor. However, this explanation appears unlikely because inhibition of PI3K by wortmannin led to rapid redistribution of the probe from PM to cytoplasm, implicating a high lipid phosphatase activity.

In summary, we successfully combined two different readouts in a single probe. In fact, a variety of translocation-based sensor domains are suitable to be fused with any ratiometric indicator regardless whether it is single fluorophore or a Forster resonance energy transfer pair. Ratiometric sensors can serve as the fluorescence tags instead of conventional FPs, and they can provide additional read-out parameters, such as 2nd messenger concentrations in time-lapse imaging experiments. Obvious other benefits are that only a small part of the usable spectra is occupied and additional colors may be used for other sensors in a multiparameter imaging setup. Most importantly, only one expression construct is needed for the dual-parameter readout, which makes the relative quantification and the interpretation of the results much easier.

Notes

Materials used

H2O2, DPI, EGF, and PDGF-BB were purchased from Sigma. Dulbecco-phosphate-buffered saline, Dulbecco’s modified Eagle’s medium (DMEM), Opti–minimal essential medium (MEM), MEM, fetal calf serum (FCS), and FuGene6 transfection reagent were from Invitrogen. Glass-bottomed dishes were from MatTek. NIH-3T3 cells were from ATCC. HeLa-Kyoto cell line was provided by EMBL. Encyclo polymerase chain reaction (PCR) kit and HyPer expression vectors were from Evrogen. Restriction endonucleases were from SibEnzyme.

DNA constructs

To make PIP-SHOW, the PH-domain coding region of BTK was amplified from pEGFP-BtkPH encoding vector using the primers 5′-ATCCGCTAGCATGCGCCGATGGTCTCGGAGA-3′ and 5′-CGGTGGATCCCCGTTCACAAATTGGCACCCA-3′. The PCR product was digested with Nhel and BamHI and cloned into pHyper-dMito vector (Evrogen) in place of the red FP. It has been shown that E41K mutation increases the affinity of the BTK-PH to PIP3 (1). To introduce E41K mutation, site-directed mutagenesis of Btk PH-domain was applied using the PCR overlap extension procedure with the primers 5′-CTCTTACTATAAGTACGACTTGA-3′ and 5′-TCAAGTCTACATTATAGTAGGAG-3′. All constructs were confirmed by sequence analysis.

Cell culture and transfection

NIH-3T3 cells were cultured in DMEM supplemented with 10% FCS at 37°C in an atmosphere containing 95% air and 5% CO2. Cells were split every 2nd day and seeded on glass bottom dishes. Twenty-four hours later cells were transfected by the mixture of vector DNA and FuGene6 transfection reagent according to the manufacturer recommendations. Human TH cells were isolated from leukocyte reduction filters from healthy blood donors. First, peripheral blood mononuclear cells (PBMCs) were purified by a density gradient centrifugation at 450 g for 30 min at room temperature (Ficol-Paque PLUS, Amersham Biosciences), while the remaining red blood cells were removed using a lysis buffer (155 mM NaCl, 10 mM KHCO3, and 0.1 mM EDTA [pH 7.3]). PBMCs were re-suspended in PBS buffer containing 0.5% BSA, and CD4+ and CD8+ cells were negatively isolated using CD4+ and CD8+ positive isolation kit (Invitrogen). Naive TH cells were transduced with 4+ NIH-3T3 cells were negatively isolated using CD4+ negative isolation kit (Invitrogen). Naive TH cells were transduced with 4+ NIH-3T3 cells were negatively isolated using CD4+ negative isolation kit (Invitrogen). Naive TH cells were transduced with 4+ NIH-3T3 cells were negatively isolated using CD4+ negative isolation kit (Invitrogen). Naive TH cells were transduced with 4+ NIH-3T3 cells were negatively isolated using CD4+ negative isolation kit (Invitrogen). Naive TH cells were transduced with 4+ NIH-3T3 cells were negatively isolated using CD4+ negative isolation kit (Invitrogen). Naive TH cells were transduced with 4+ NIH-3T3 cells were negatively isolated using CD4+ negative isolation kit (Invitrogen). Naive TH cells were transduced with 4+ NIH-3T3 cells were negatively isolated using CD4+ negative isolation kit (Invitrogen). Naive TH cells were transduced with 4+
an and a photometric evolve: 512 EMCCD camera at binning 1 × 1. Filters were a CFP, YFP dualband pinkel set (55HE, Zeiss). Fluorescence was recorded using excitation at 420 and 505 nm and emission at 515 nm. NIH-3T3 cells were imaged using Leica 6000 widefield microscope equipped with an HCX PL APO 1.4 NA oil objective and an environmental chamber. Fluorescence was excited sequentially via 427/10 and 504/12 band-pass excitation filters. Emission of the probe was collected every 10 or 30 s using a 525/50 bandpass emission filter. After three to five images were acquired, 10 ng/mL PDGF was added.

\[ cgP(3,4,5)P_3/AM \text{ treatment and uncaging} \]

\[ cgP(3,4,5)P_3/AM \text{ (6) was dissolved in DMSO at stock concentration of 10 mM. Just before applying to cells it was mixed with 10\% pluronic/DMSO solution (Invitrogen) in 1:1 ratio to facilitate cell entry. } \]

\[ cgP(3,4,5)P_3/AM \text{ was used on cells in a concentration of 10 mM. Uncaging and imaging were performed on Carl Zeiss LSM 510 META confocal microscope, equipped with environment control box (37°C, 0\% CO_2), using HCX PL APO 1.4 NA oil objective. Cells were pre- } \]

\[ \text{unciliated with } cgP(3,4,5)P_3/AM \text{ 2 h before uncaging in the imaging medium. For uncaging, cells were illuminated with 405 nm laser (7\%) every 10 s. Imaging of PIP-SHOW was done using 488 nm laser excitation (5\%) with 10 s time resolution. } \]

**Time series processing**

Time series were analyzed using Imagej software. For H_2O_2 dynamics calculation, stacks corresponding to 420 and 500 nm excitation peaks of PIP-SHOW were converted to 32 bit after background subtraction. 420 nm stack was thresholded to remove pixel values from background (Not-a-Number function). A 500 nm stack was divided by the corresponding 420 nm stack frame by frame. The resulting stack was depicted in pseudocolors using a “ratio” lookup table. Time course of PIP-SHOW fluorescence was calculated for regions of interest (ROI) inside the imaged cell. The anti-CD3/CD28-coated beads used for T cell stimulation are fluorescent in both 420 nm and medium-intensity pixels shows dynamics of PI3K activity in the IS. However, in case of PIP-SHOW the brightness of the probe in either channel depends also on H_2O_2. The influence of H_2O_2 can be removed by dividing each frame in the stack by the mean value (in the same frame) of the ROI drawn in the cytoplasm of the cell. To enable this procedure, we designed a simple Imagej plugin named “Divide by ROI” (Supplementary Data).
### Abbreviations Used (Cont.)

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<td>PCR</td>
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<td>PDGF</td>
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<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<td>PIP</td>
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<td>PIP3</td>
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**Supplementary Data**

*Multiparameter imaging*

Combinations of various fluorophores with distinct excitation and emission spectra in a single cell enable monitoring of multiple cellular processes simultaneously, usually termed “multiparameter imaging” (MI) (5, 20). Typical readouts of the fluorescent probes are (i) changes in emission brightness (14, 22); (ii) ratiometric changes between two excitation or emission peaks (1, 2, 8, 12), especially when Forster resonance energy transfer probes are involved (4, 5, 10, 13, 23); and (iii) translocation of the probe from one to another cellular compartment (20, 24). The combination of these modalities should provide even more opportunities for MI in living cells. So far, only very few examples of dual parameter fluorescent sensors have been reported (4).

*Combining red fluorescent readout with PIP-SHOW imaging*

MI by PIP-SHOW does not use the entire available visible spectra for life cell imaging. Therefore, we combined measurements of PIP-SHOW with a translocation probe (C2-mRFP) to detect changes in intracellular calcium ions [Ca$^{2+}$]$_i$ (15, 25). C2 domain binds phosphatidylserine upon elevation of [Ca$^{2+}$]$_i$ translocating from the cytoplasm to the plasma membrane (PM). Therefore, by monitoring C2-mRFP

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**SUPPLEMENTARY FIG. S1.** Kymogram of simultaneous imaging of PIP$_3$, H$_2$O$_2$ and Ca$^{2+}$ in NIH-3T3 cells expressing PIP-SHOW and C2-mRFP. (A) A line across the cell was chosen to build kymogram from the time stack. Scale bar = 10 μm. (B) Kymograms of PIP$_3$ (PIP-SHOW), H$_2$O$_2$ (F500/F420), and Ca$^{2+}$ (C2-mRFP) dynamics along the line in time. ROIs were chosen as vertical lines of 20-pixel thickness to measure profiles of fluorescence changes with time. PIP$_3$ and H$_2$O$_2$ were measured in the ROI that includes plasma membrane. Ca$^{2+}$ dynamics was measured in the ROI that includes cytoplasm (C2-mRFP drop upon Ca$^{2+}$ elevation). (C) Changes in PIP$_3$, H$_2$O$_2$, and Ca$^{2+}$ along the selected ROIs in time. H$_2$O$_2$, hydrogen peroxide; ROI, regions of interest; PIP, phosphatidylinositol phosphate.
SUPPLEMENTARY VIDEO S1. PIP-SHOW reports PI3K activity and H₂O₂ levels change by two different types of readouts: translocation of the probe and excitation peaks ratio change. See legend of Figure 2 in the main text for details. PI3K, phosphatidylinositol 3-kinase; H₂O₂, hydrogen peroxide; PIP, phosphatidylinositol phosphate.

SUPPLEMENTARY VIDEO S2. PIP-SHOW detects PI3K activity and H₂O₂ production in the human T₁H cell upon IS formation. See legend of Figure 3 in the main text for details. IS, immunological synapse; T₁H, T helper.

translocation, it was possible to track [Ca²⁺]i dynamics. We co-expressed PIP-SHOW and C2-mRFP in NIH-3T3 fibroblasts and stimulated the cells with platelet-derived growth factor. We were able to monitor three parameters (hydrogen peroxide [H₂O₂], phosphatidylinositol 3,4,5-trisphosphate [PIP₃], and cytosolic [Ca²⁺]i) in parallel (Supplementary Fig. S1). Translocation of C2-mRFP showed rapid and transient elevation of [Ca²⁺]i. By analyzing PIP-SHOW translocation and F500/F420 ratio, we were able to demonstrate that elevations of PIP₃ and H₂O₂ occur at a similar time scale compared to [Ca²⁺]i increases. However, both PIP₃ and H₂O₂ signals decayed much slower than [Ca²⁺]i.

Lipid and redox signaling in TCR activation

PIP₃ signaling plays a key role in T cell activation (16, 21, 26), and TCR stimulation leads to generation of reactive oxygen species (ROS) (9, 18). T helper (T₁H) cells are activated through the interaction of their TCRS, together with costimulatory receptors (i.e., CD28), with the major histocompatibility complex on the surface of antigen-presenting cells, thereby forming an immunological synapse (IS) (11). In in vitro studies, IS formation can be mimicked by incubation of cells with beads coated with anti-CD3/anti-CD28 antibodies (17). It has been shown that IS formation leads to a strong activation of phosphatidylinositol 3-kinase (PI3K) and PIP₃ elevation both at the IS and throughout rest of PM (6, 7). Although formation of ROS has been shown to appear downstream of TCR signaling, real-time dynamics, quantification, and localization of H₂O₂ during IS formation are very difficult to assess. Previous work demonstrated ROS production by T₁H cells stimulated with soluble agonists using the fluorescent redox-sensitive dye DCF (9). However, DCF derivatives have many disadvantages, such as photoinduced ROS production and lack of specificity toward different species of ROS (3, 19). Moreover, stimulation with soluble TCR cross-linkers does not lead to T cell polarization and IS formation. We therefore studied dynamics of both PI3K and H₂O₂ levels change in IS formation.

References

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