Cytosolic inositol 1,4,5-trisphosphate dynamics during intracellular calcium oscillations in living cells

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We developed genetically encoded fluorescent inositol 1,4,5-trisphosphate (IP3) sensors that do not severely interfere with intracellular Ca2+ dynamics and used them to monitor the spatiotemporal dynamics of both cytosolic IP3 and Ca2+ in single HeLa cells after stimulation of exogenously expressed metabotropic glutamate receptor 5a or endogenous histamine receptors. IP3 started to increase at a relatively constant rate before the pacemaker Ca2+ rise, and the subsequent abrupt Ca2+ rise was not accompanied by any acceleration in the rate of increase in IP3. Cytosolic [IP3] did not return to its basal level during the intervals between Ca2+ spikes, and IP3 gradually accumulated in the cytosol with a little or no fluctuations during cytosolic Ca2+ oscillations. These results indicate that the Ca2+-induced regenerative IP3 production is not a driving force of the upstroke of Ca2+ spikes and that the apparent IP3 sensitivity for Ca2+ spike generation progressively decreases during Ca2+ oscillations.

Introduction

An increase in cytosolic [Ca2+] ([Ca2+]c) is a ubiquitous intracellular signal that controls various cellular processes, including fertilization, proliferation, development, learning and memory, contraction, and secretion (Berridge et al., 2000). [Ca2+]c, rises evoked by extracellular stimuli are often observed in the form of pulsatile Ca2+ spikes that result from the transient opening of Ca2+ channels located either in the plasma membrane or on the cytosolic Ca2+ stores (Berridge and Dupont, 1994). The spatial counterpart of Ca2+ spikes are Ca2+ waves, which are produced when an initial localized [Ca2+]c elevation leads to the propagation of the rise in [Ca2+]c throughout the cytoplasm (Lechleiter et al., 1991; Thomas et al., 1991). The frequency of occurrence of Ca2+ spikes is correlated with the stimulus intensity (Woods et al., 1986; Berridge, 1988; Jacob et al., 1988; Prentki et al., 1988), and the time course of an individual Ca2+ spike depends on the type of receptor stimulated but not on the stimulus intensity (Cobbold et al., 1991; Thomas et al., 1991), indicating that the information of both the stimulus species and its intensity is encoded within the temporal pattern of cytosolic Ca2+.

Stimulus-induced cytosolic Ca2+ spikes usually form as a result of an initial slow pacemaker rise in [Ca2+]c, followed by a rapid rise in [Ca2+]c (Jacob et al., 1988; Thomas et al., 1991; Bootman and Berridge, 1996). The rate of the rapid [Ca2+]c rise remains relatively constant regardless of the stimulus intensity (Cobbold et al., 1991; Thomas et al., 1991), suggesting that a regenerative process is involved in the generation of the abrupt upstroke (Thomas et al., 1991). Such regenerative processes require a positive-feedback element (Meyer and Stryer, 1991), and some molecules that are critical to the regenerative process have been proposed. PLC catalyzes the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol from phosphatidylinositol-4,5-bisphosphate and has been hypothesized to act as a positive-feedback element (Meyer and Stryer, 1988; Harootunian et al., 1991) because its activity is stimulated by cytosolic Ca2+...
According to their hypothesis, the positive-feedback regulation of PLC by cytosolic Ca\(^{2+}\) allows generation of IP\(_3\) spikes that result in Ca\(^{2+}\) spikes through activation of the IP\(_3\) receptor (IP\(_3\)R)/IP\(_3\)-gated Ca\(^{2+}\) release channel (Meyer and Stryer, 1988). Another hypothesis is that the positive-feedback regulation is attributable to the intrinsic property of the IP\(_3\)R. The biphasic dependency of channel activity on Ca\(^{2+}\) (Iino, 1990) may create the rapid upstroke of Ca\(^{2+}\) spikes even at constant levels of IP\(_3\) (Wakui et al., 1989; Osipchuk et al., 1990; Wakui et al., 1990; Dupont et al., 1991; De Young and Keizer, 1992; Hajnoczky and Thomas, 1997). These mechanisms for the generation of Ca\(^{2+}\) spikes, however, are not necessarily exclusive, and combined models are also plausible.

Observation of cytosolic IP\(_3\) dynamics during Ca\(^{2+}\) spikes should help us better understand the mechanism responsible for the Ca\(^{2+}\) spike generation (Meyer and Stryer, 1991). The GFP-tagged pleckstrin homology domain (PHD) of PLC-\(\delta_1\), which interacts with phosphatidylinositol 4,5-bisphosphate and/or IP\(_3\), has been used to monitor IP\(_3\) production and its oscillatory translocation from plasma membrane to the cytosol synchronously with Ca\(^{2+}\) oscillation was observed in MDCK cells (Hirose et al., 1999), suggesting that each Ca\(^{2+}\) spike accompanies IP\(_3\) production. However, there is no simple correlation between its translocation from the plasma membrane to the cytosol and actual cytosolic [IP\(_3\)] ([IP\(_3\)], van der Wal et al., 2001; Xu et al., 2003; Irvine, 2004). Fluorescent IP\(_3\) sensors based on the IP\(_3\) binding domain of IP\(_3\)R have recently been developed (Tanimura et al., 2004; Sato et al., 2005; Remus et al., 2006), but no one has ever used them to investigate the mechanism underlying the Ca\(^{2+}\) spike generation in living cells.

In the present study, we developed low IP\(_3\) binding affinity, high signal-to-noise ratio cytosolic IP\(_3\) sensors based on the IP\(_3\) binding domain of mouse type 1 IP\(_3\)R (IP\(_3\)R1) and used them to analyze the mechanism responsible for the generation of Ca\(^{2+}\) spikes. Simultaneous imaging of [Ca\(^{2+}\)] and [IP\(_3\)] in living cells exposed to extracellular stimuli provided us with a novel insight into the mechanism of generation of intracellular Ca\(^{2+}\) spikes.

**Results**

**Development of cytosolic IP\(_3\) sensors based on the IP\(_3\) binding domain of IP\(_3\)R1**

To develop cytosolic IP\(_3\) sensors, we constructed tandem fusion proteins of a variant of yellow fluorescent protein, Venus (Nagai et al., 2002); an IP\(_3\) binding domain of IP\(_3\)R1 (Miyawaki et al., 1991; Yoshikawa et al., 1996; Bosanac et al., 2002); and an enhanced cyan fluorescent protein (ECFP; Fig. 1 A). We used...
amino acid residues 224–569, 224–575, 224–579, 224–584, and 224–604 of mouse IP3R1 as an IP3 binding motif and found that all fusion proteins except the protein composed of residues 224–569 showed IP3-dependent decrease of fluorescence resonance energy transfer (FRET) between Venus and ECFP (Fig. 1 B). The largest FRET change was obtained in the fusion protein composed of residues 224–579 of mouse IP3R1, and the 475-nm (ECFP) to 525-nm (Venus) emission ratio of the fusion protein increased by 25.1 ± 8.0% (n = 3) after the addition of 60 μM IP3 into cell lysates. We designated this fusion protein as IP3R-based IP3 sensor 1 (IRIS-1). The emission ratio of IRIS-1 changed depending on the concentration of IP3 applied, but its apparent IP3 sensitivity (Kd = 549 ± 62 nM; n = 3) was significantly lower than that of fusion proteins composed of residues 224–604 (Kd = 107 ± 41 nM; n = 3), 224–584 (Kd = 105 ± 0 nM; n = 3), and 224–579 (Kd = 95 ± 38 nM; n = 3; Fig. 1 C).

We further characterized IRIS-1 in vitro. The protein was expressed in Sf9 cells and was purified as described in Materials and methods. Fig. 1 D shows emission spectrum of IRIS-1 when excited at 420 nm. Addition of 100 μM IP3 slightly increased the 475-nm (ECFP) emission and decreased the 525-nm (Venus) emission of IRIS-1. We also constructed IRIS-1–Dmut, in which two critical amino acid residues (Thr267 and Lys508) for IP3 binding have been replaced from IRIS-1, and found that the emission spectrum of IRIS-1–Dmut was unaltered by the addition of 100 μM IP3 (Fig. 1 E), indicating that FRET between the flanking fluorescent proteins decreased in response to IP3 binding to the IP3 binding domain of IRIS-1. The emission change of purified IRIS-1 exhibits an IP3 sensitivity (Kd = 437 ± 30 nM; n = 6) that is slightly higher than that measured in COS7 cell lysates (Fig. 1 F). IRIS-1 can discriminate IP3 from its natural metabolites, inositol 1,3,4,5-tetrakisphosphate (IP4) and inositol 1,4-bisphosphate (IP2), with >50 and >400 times the sensitivity, respectively (Fig. 1 F). The IP3 sensitivity of IRIS-1 was not influenced by the addition of 1 μM Ca2+ (Fig. 1 G). IRIS-1 did not reveal severe pH sensitivity, at least in the range examined (Fig. 1 H).

IRIS-1 was uniformly distributed within the cytosol of intact HeLa cells (Fig. 2 A), and ~80% of IRIS-1 was released from the cells after 5 min of treatment with 0.1% saponin (Fig. 2 A and B). ECFP-tagged IP3R1, which is an ER resident protein, from the cells after 5 min of treatment with 0.1% saponin (Fig. 2, A and B). These results indicate that IRIS-1 is mainly localized in the cytosol of HeLa cells. To monitor cytosolic IP3 and Ca2+ dynamics simultaneously, Indo-1 was loaded into IRIS-1–expressing HeLa cells. Fig. 2 D shows FRET changes of IRIS-1 accompanied by Ca2+ transients (Fig. 2 C) elicited by sequential stimulations with 1, 5, and 10 μM of histamine. We did not detect FRET changes in IRIS-1–Dmut–expressing cells even when Ca2+ transients were observed (Fig. 2, E and F), and the expression level of IRIS-1–Dmut was indistinguishable from that of IRIS-1 (Fig. 2 G). FRET changes of IRIS-1 were completely blocked by the addition of 10 μM of PLC inhibitor U73122 but not by its inactive analogue U73343 in (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb. 200512141/DC1). These results indicate that IRIS-1 can monitor [IP3]i changes in living cells.

**IP3 dynamics during Ca2+ oscillations in HeLa cells**

To monitor cytoplasmic IP3 dynamics during Ca2+ oscillations, we introduced both IRIS-1 and metabotropic glutamate receptor 5a (mGluR5a) cDNAs into HeLa cells. The frequency of Ca2+ oscillations mediated by mGluR5a is known to depend on the extent of mGluR5a expression (Nash et al., 2002), and the Ca2+ oscillation frequency varied from 19 to 72 mHz (45.6 ± 16.0 mHz; n = 25) in the cells transfected with mGluR5a cDNA.
alone (no IP3 sensor proteins). IP3 sensors that can bind IP3 may perturb IP3 dynamics in living cells to some extent with their potential to function as an IP3 buffer. IRIS-1-expressing cells, however, exhibited a Ca2+ oscillation frequency (36.9 ± 7.4 mHz; n = 23; Fig. 3, A and B) that was not significantly different from the frequency observed in the IRIS-1–Dmut–expressing cells (32.5 ± 11.2 mHz; n = 17; Fig. 3, A and B; P > 0.05, t test). The decay times of each Ca2+ spike were also indistinguishable between IRIS-1–expressing cells and IRIS-1–Dmut–expressing cells stimulated with 100 μM glutamate (unpublished data). These results indicate that the expression of IRIS-1 did not have a marked influence on Ca2+ dynamics evoked by mGluR5a stimulation. We noticed that the expression of ECFP-fused PHD (C-PHD) and Venus-fused PHD (V-PHD) significantly reduced the Ca2+ oscillation frequency (21.5 ± 10.3 mHz; n = 38; Fig. 3, A and B) in comparison with the cells expressing mGluR5a alone (P < 0.05, t test) or mGluR5a plus IRIS-1–Dmut (P < 0.05, t test), but the expression level of mGluR5a was not reduced in C/V-PHD–expressing cells (Fig. 3 C), indicating that the exogenous expression of PHD perturbs the intracellular Ca2+ dynamics evoked by mGluR5a stimulation.

Fig. 4 A shows the IP3 dynamics monitored by IRIS-1 during Ca2+ oscillations in mGluR5a-expressing HeLa cells stimulated with 100 μM of glutamate. Cytosolic IP3 rapidly increased after the addition of glutamate and was progressively accumulated with 100 μM of glutamate. Cytosolic IP3 rapidly increased after the addition of glutamate (Fig. 4 A) or histamine application (Fig. 4 B). We did not detect IP3 spikes, i.e., rapid upstrokes of [IP3]c, with a constant amplitude followed by a gradual decrease of [IP3]c, to its basal level, even when Ca2+ spikes were triggered by glutamate (Fig. 4 A) or histamine application (Fig. 4 B). However, if the IP3 sensor was saturated with IP3 in the cells and/or the FRET change of IRIS-1 upon IP3 binding was too slow to detect [IP3]c changes, we would have missed the IP3 spikes, so we conducted the following experiments to investigate these possibilities. IRIS-1–expressing HeLa cells were permeabilized with 60 μM β-escin for 3 min and then exposed to solutions containing various concentrations of IP3. IRIS-1 exhibited a maximal response of 28.0% after addition of an

Figure 3. Effects of exogenously expressed proteins on Ca2+ oscillation frequency in mGluR5a-expressing HeLa cells. (A) Emission ratio change of Indo-1 signals in cells stimulated with 100 μM of glutamate (horizontal bars). (B) Histograms of Ca2+ oscillation frequency in mGluR5a-expressing cells stimulated with 100 μM of glutamate. (C) Western blot analysis of cell lysates prepared from HeLa cells transfected with mGluR5a alone (lane 2), mGluR5a plus C/V-PHD (lane 3), mGluR5a plus IRIS-1 (lane 4), or mGluR5a plus IRIS-1–Dmut (lane 5). Nontransfected cells were used as a control (lane 1). An anti-mGluR5 antibody was used. Molecular mass markers are shown on the left (×10^{-7}).
excess amount of IP3 under the conditions used (Fig. S1 C). The maximal response observed was greater than that observed in intact cells stimulated with 100 μM glutamate (~20%; Fig. 4 A) and 3 μM histamine (~7%; Fig. 4 B), indicating that the dynamic range of changes in the IRIS-1 signals is greater than the changes in [IP3]c evoked by the stimuli when the resting level of [IP3]c is sufficiently low (<100 nM). The possibility of saturation of the IRIS-1 signals was further investigated with IRIS-1.2, a low-affinity mutant of IRIS-1 in which lysine 249 is replaced by glutamine (K249Q). IRIS-1.2 exhibited approximately sevenfold lower affinity for IP3 than IRIS-1 (Fig. S1 D). FRET signals observed with IRIS-1.2 revealed temporal patterns similar to those observed with IRIS-1 during Ca2+ oscillations evoked by mGluR5a stimulation (Fig. S1, E and F). The IRIS-1.2 signals rapidly rose after the addition of glutamate, and they remained at the elevated level and underwent fluctuations (but not baseline spikes) during Ca2+ oscillations. These results indicate that the IRIS-1 signals in intact cells were not saturated when the mGluR5a-expressing HeLa cells were stimulated with 100 μM glutamate. The rate of changes in IRIS-1 signals was evaluated directly by measurements of the fluorescent changes of IRIS-1 after rapid mixing with IP3 using stopped-flow fluorescence spectrometry (see the supplemental text and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200512141/DC1). The analysis of the kinetics of the fluorescence changes indicates that there are two conformations of IRIS-1 with a different FRET efficiency and only IRIS-1 with a low FRET efficiency is able to bind to IP3 (see the supplemental text). The kinetic parameters were estimated from both the kinetics data (Fig. S2 B) and the equilibrium data (Fig. S2 C) and were used to calculate the FRET changes during and after the addition of a brief pulse of IP3. The resting level of [IP3]c was configured to be 40 nM based on measurements in Xenopus laevis oocytes (Luzzi et al., 1998). Fig. S2 D shows the dynamics of the fraction of IRIS-1 with low FRET efficiency evoked by a 1-s pulse of various concentrations of IP3 (from 200 nM to 12.8 μM). The fraction of IRIS-1 with low FRET efficiency increased more than twofold 1 s after the onset of the IP3 pulses and returned to its basal level within 3 s after the IP3 pulses with all concentrations applied (Fig. S2 D). The frequency of the Ca2+ oscillations in the IRIS-1–expressing cells stimulated with 100 μM glutamate was 36.9 ± 7.4 mHz (Fig. 3 B), and the mean duration of the interspike interval was 27.1 s. If [IP3]c actually returns to its basal level during the period of each Ca2+ spike, IRIS-1 would be able to monitor it. We therefore concluded that IRIS-1 possesses a sufficient temporal property to detect [IP3]c changes during Ca2+ oscillations evoked in HeLa cells.

**Relationship between [IP3]c and [Ca2+]c during the rising phase of Ca2+ spikes**

Spatiotemporal profiles of both [Ca2+]c and cytosolic [IP3]c at the onset of initial Ca2+ spikes in HeLa cells after mGluR5a stimulation were monitored with the fast acquisition equipment (see Materials and methods). Images were acquired every 246 ms.
Fig. 5 shows the spatiotemporal patterns of [Ca\textsuperscript{2+}] and [IP\textsubscript{3}], when the first Ca\textsuperscript{2+} spike occurred. The rapid [Ca\textsuperscript{2+}] rise occurred in HeLa cells stimulated with 100 μM glutamate with little or no pacemaker Ca\textsuperscript{2+} rise (Fig. 5 B). No clear initiation site of the [Ca\textsuperscript{2+}] rise was detected in the cell shown (Fig. 5 A). The [IP\textsubscript{3}] rise was found to precede the abrupt [Ca\textsuperscript{2+}] rise (Fig. 5, B and C) in 48 of 52 cells, and [IP\textsubscript{3}] gradually rose almost homogeneously in the cells stimulated (Fig. 5 A). The onset of the [IP\textsubscript{3}] rise, which was identified as an increase greater than twice the SD of the baseline signals, preceded the onset of the [Ca\textsuperscript{2+}] rise by 1.11 ± 1.75 s (n = 52). The rate of [IP\textsubscript{3}] rise did not accelerate during the rising phase of the Ca\textsuperscript{2+} spike (Fig. 5, B and C, between c and e). If the regenerative IP\textsubscript{3} production mediated by PLC drives the rising phase of Ca\textsuperscript{2+} spikes, the rate of [IP\textsubscript{3}] rise should peak when the rate of [Ca\textsuperscript{2+}] rise is at its maximum. To test this possibility, the fluorescent signals of both Indo-1 and IRIS-1 were differentiated and aligned at the time when the rate of [Ca\textsuperscript{2+}] rise reached its maximum (Fig. 5, D and E). As shown in Fig. 5 E, the rate of [IP\textsubscript{3}] rise did not peak when the rate of [Ca\textsuperscript{2+}] increase was at its maximum, indicating that the steep rise in [Ca\textsuperscript{2+}] occurred without any acceleration of the [IP\textsubscript{3}] rise. A similar relationship was observed between [Ca\textsuperscript{2+}] and [IP\textsubscript{3}] during the rising phases of the subsequent Ca\textsuperscript{2+} spikes (Fig. 6). As reported in histamine-stimulated HeLa cells (Bootman and Berridge, 1996), the shape of the first and the subsequent Ca\textsuperscript{2+} spikes was different in mGluR5a-expressing HeLa cells stimulated with glutamate. The subsequent spikes had more extended pacemaker activity, and the rate of the [Ca\textsuperscript{2+}] rise after the pacemaker activity of the subsequent spikes was slower than that of the first spike (Fig. 6 A). As shown in Fig. 6, [IP\textsubscript{3}] started to increase before the onset of the pacemaker [Ca\textsuperscript{2+}] rise of the second, third, and fourth Ca\textsuperscript{2+} spikes (filled and open arrowheads), and the [IP\textsubscript{3}] increase did not accelerate during the period of the rapid [Ca\textsuperscript{2+}] rise of these Ca\textsuperscript{2+} spikes (between the thin vertical lines). The number of cells that showed [IP\textsubscript{3}] rises preceding [Ca\textsuperscript{2+}] increases and the mean interval between the onset of the [IP\textsubscript{3}] rise and the onset of the [Ca\textsuperscript{2+}] rise are summarized in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200512141/DC1). These results suggest that regenerative IP\textsubscript{3} production does
not drive the abrupt Ca\textsuperscript{2+} increase of either the first or the subsequent Ca\textsuperscript{2+} spikes in mGluR5a-stimulated HeLa cells.

**Ca\textsuperscript{2+}-dependent IP\textsubscript{3} production in HeLa cells**

Ca\textsuperscript{2+}-dependent stimulation of PLC activity has been proposed to be a positive-feedback element that causes the steep rising phase of Ca\textsuperscript{2+} spikes (Meyer and Stryer, 1988). As shown in Figs. 5 and 6, however, the rapid increase in [Ca\textsuperscript{2+}], in the rising phase of Ca\textsuperscript{2+} spikes was unaccompanied by any acceleration of the [IP\textsubscript{3}]\textsubscript{i} rise. To evaluate the component of the Ca\textsuperscript{2+}-dependent IP\textsubscript{3} production in HeLa cells, [IP\textsubscript{3}]\textsubscript{i} was monitored in the cells in which [Ca\textsuperscript{2+}]\textsubscript{i} increased without activation of G protein-coupled receptors. HeLa cells were treated with 1 μM thapsigargin for 5 min to deplete intracellular stores and then exposed to 2 mM Ca\textsuperscript{2+}. In these cells, a [IP\textsubscript{3}]\textsubscript{i} increase that exceeded twice the SD of the baseline signal was detected with a much greater delay (66.6 ± 13.6 s; n = 9) than the onset of the [Ca\textsuperscript{2+}]\textsubscript{i} increase mediated by capacitative Ca\textsuperscript{2+} entry (Fig. 7 A). IP\textsubscript{3} signals were well fitted with a monoexponential function having a time constant of 85.7 ± 25.1 s (n = 10). Histamine stimulation alone in the absence of extracellular Ca\textsuperscript{2+} induced a rapid but small increase in [IP\textsubscript{3}]\textsubscript{i} (Fig. 7 B). When thapsigargin-treated cells were stimulated with 3 μM histamine in the presence of 2 mM extracellular Ca\textsuperscript{2+}, [IP\textsubscript{3}]\textsubscript{i} increased in a complex pattern consisting of a rapid transient increase (Fig. 7 B, arrowheads) followed by a slow sustained increase (Fig. 7 C). The amplitude of the initial rapid transient was far below the maximal IP\textsubscript{3} concentration observed at the end of the stimulation (Fig. 7 C). The rapid transient increase in [IP\textsubscript{3}]\textsubscript{i} was never observed in the thapsigargin-treated cells stimulated with histamine alone (n = 14; Fig. 7 B) or with extracellular Ca\textsuperscript{2+} alone (n = 19; Fig. 7 A). There were no differences in rate of Ca\textsuperscript{2+} increase and maximal Ca\textsuperscript{2+} concentration reached between the cells stimulated with extracellular Ca\textsuperscript{2+} alone (Fig. 7 A) and the cells stimulated with histamine in the presence of extracellular Ca\textsuperscript{2+} (Fig. 7 C). Similar temporal patterns of [Ca\textsuperscript{2+}]\textsubscript{i} were obtained with Fura-4F (K\textsubscript{D} = 770 nM; unpublished data), indicating that Indo-1 was not saturated under the conditions used. These results indicate...
that (1) the cytosolic Ca\(^{2+}\) increase itself induces IP\(_3\) production in HeLa cells, but the process is relatively slow compared with agonist stimulation–evoked IP\(_3\) production, and (2) receptor activation and the [Ca\(^{2+}\)]\(_i\) increase synergistically induce the rapid, transient IP\(_3\) production in HeLa cells, but the amplitude of the IP\(_3\) transient is relatively small even when [Ca\(^{2+}\)]\(_i\) is persistently elevated.

### Discussion

We developed cytosolic IP\(_3\) sensors based on the IP\(_3\) binding domain of mouse IP\(_3\),R1 and used them to analyze the mechanism that generates [Ca\(^{2+}\)]\(_i\) oscillations. Nonexcitable human HeLa carcinoma cells were chosen to measure cytosolic IP\(_3\) dynamics because they have been used by several groups as a model system to study hormone-evoked Ca\(^{2+}\) signals. We had already developed the high-affinity IP\(_3\) binding protein based on the IP\(_3\) binding domain of mouse IP\(_3\),R1, the IP\(_3\) sponge, to chelate cytosolic IP\(_3\) (Uchiyama et al., 2002), and found that introduction of the IP\(_3\) sponge into living cells significantly inhibits or completely suppresses stimuli-evoked intracellular Ca\(^{2+}\) increases (Iwasaki et al., 2002; Uchiyama et al., 2002). The challenge was to develop low IP\(_3\) binding affinity IP\(_3\) sensors with a sufficient signal-to-noise ratio to monitor the physiological dynamics of cytosolic IP\(_3\) without serious impairment of Ca\(^{2+}\) signals. We created >300 recombinant proteins and found that IRIS-1 exhibited a 25.1 ± 8.0% FRET ratio change upon the addition of an excess amount of IP\(_3\) with a K\(_d\) value of 549 ± 62 nM in cell lysates (Fig. 1) and did not significantly alter the Ca\(^{2+}\) oscillation frequency or rate of decrease in individual Ca\(^{2+}\) spikes in mGluR5a-stimulated HeLa cells (Fig. 3). We used mGluR5a as a surface receptor that is linked with phosphoinositide hydrolysis, as the receptor has been used to investigate the mechanism of generation of Ca\(^{2+}\) oscillations (Kawabata et al., 1996) and to measure IP\(_3\) dynamics with GFP-tagged PHD (Nash et al., 2002). We used IRIS-1 to simultaneously monitor the spatiotemporal dynamics of both [Ca\(^{2+}\)]\(_i\) and [IP\(_3\)]\(_c\), in single living cells and investigated the mechanism underlying the generation of Ca\(^{2+}\) oscillations. In mGluR5a-stimulated HeLa cells, [IP\(_3\)]\(_c\), started to increase at a relatively constant rate before the pacemaker [Ca\(^{2+}\)]\(_i\), rise and the subsequent abrupt [Ca\(^{2+}\)]\(_i\), rise did not accelerate the rate of [IP\(_3\)]\(_c\), rise (Figs. 5 and 6). [IP\(_3\)]\(_c\) did not return to its basal level during the interval between Ca\(^{2+}\) spikes and, as a result, IP\(_3\) gradually accumulated in the cytosol with little (Fig. 4 A and Fig. 6) or no detectable (Fig. 4 B) fluctuations when repeated Ca\(^{2+}\) spikes occurred in the continuous presence of the stimulus. Each Ca\(^{2+}\) spike seemed to be triggered at a different concentration of [IP\(_3\)]\(_c\), (Fig. 4 C and Fig. 6), contradicting the findings by other investigators that Ca\(^{2+}\) spikes are triggered at a critical [Ca\(^{2+}\)]\(_i\) (Iino et al., 1993). Our results indicate that (1) Ca\(^{2+}\)-induced regenerative IP\(_3\) production is not a driving force of the abrupt upstroke of Ca\(^{2+}\) spikes, (2) the apparent IP\(_3\) sensitivity for generation of Ca\(^{2+}\) spikes progressively decreases during Ca\(^{2+}\) oscillations, and (3) the clearance of IP\(_3\) in the cytosol of living cells is slower than the decrease of cytosolic Ca\(^{2+}\).

#### Positive-feedback element that drives the steep rising phase of Ca\(^{2+}\) spikes

Meyer and Stryer (1988) proposed that cytosolic Ca\(^{2+}\) and IP\(_3\) are a pair of reciprocally coupled messengers based on evidence that IP\(_3\) induces cytosolic Ca\(^{2+}\) increase by triggering Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores and that Ca\(^{2+}\) enhances IP\(_3\) production by activating PLC. Their model predicts that the cooperative opening of IP\(_3\)R by the binding of multiple IP\(_3\) molecules and positive-feedback regulation of PLC by cytosolic Ca\(^{2+}\) lead to the bistability of the system (Meyer and Stryer, 1988) and that IP\(_3\) spikes in synchrony with Ca\(^{2+}\) spikes (we use “spike” to mean a threshold phenomenon characterized by an explosive rise and constant amplitude independent of stimulus intensity; Meyer and Stryer, 1988, 1991). We used the cytosolic IP\(_3\) sensor IRIS-1 to directly test the cross-coupling hypothesis in living HeLa cells and found that no IP\(_3\) spikes were detected during Ca\(^{2+}\) oscillations, according to the aforementioned definition, because the IRIS-1 signals did not exhibit an explosive rise during the rising phase of Ca\(^{2+}\) spikes (Figs. 5 and 6), and even when fluctuations were detected the absolute value of the peak level of fluctuations in IRIS-1 signals was not constant during Ca\(^{2+}\) oscillations evoked by mGluR5a stimulation (Figs. 4 and 6). The dynamic range of the IRIS-1 signals seems to be wider than the dynamic range of [IP\(_3\)]\(_c\), changes evoked by extracellular stimuli (Fig. S1 C), and the sevenfold lower IP\(_3\) binding affinity mutant of IRIS-1, IRIS-1.2, also failed to detect IP\(_3\) spikes (Fig. S1 E). The reaction of IRIS-1 is thought to be fast enough to detect [IP\(_3\)]\(_c\), changes during Ca\(^{2+}\) oscillations in mGluR5a-expressing HeLa cells, which exhibited 36.9 ± 7.4 mHz of Ca\(^{2+}\) oscillations (Fig. S2 D). Thus, the failure to detect IP\(_3\) spikes was not due to inability because of either the dynamic range or time resolution of IRIS-1. Fast acquisition of both Ca\(^{2+}\) and IP\(_3\) signals revealed that [IP\(_3\)]\(_c\), started to increase before the pacemaker rise in [Ca\(^{2+}\)]\(_i\), and the abrupt increase in [Ca\(^{2+}\)]\(_i\), which forms the rising phase of Ca\(^{2+}\) spikes, was not accompanied by a detectable acceleration in the [IP\(_3\)]\(_c\), increase (Figs. 5 and 6). The cytosolic Ca\(^{2+}\) increases actually induced IP\(_3\) production in HeLa cells without G protein–coupled receptor activation (Fig. 7 A), but it was a relatively slow process compared with the IP\(_3\) production evoked by histamine (Fig. 7 B). Receptor activation and [Ca\(^{2+}\)]\(_i\), increases have a synergistic effect on IP\(_3\) production, but it was not sufficiently strong to generate IP\(_3\) spikes even when [Ca\(^{2+}\)]\(_i\), was persistently elevated (Fig. 7 C). Although we cannot completely exclude the possibility that the local rapid rise in [IP\(_3\)]\(_c\), contributes to the generation of the rising phase of Ca\(^{2+}\) spikes, we used relatively small regions (Fig. 5 A), not the entire cytosol, to analyze the relationship between [Ca\(^{2+}\)]\(_i\), and [IP\(_3\)]\(_c\), and found that any acceleration in the [IP\(_3\)]\(_c\), increase was not detected in the region in which [Ca\(^{2+}\)]\(_i\), rose abruptly (Fig. 5, C and E). We therefore concluded that IP\(_3\) spikes do not occur during Ca\(^{2+}\) oscillations in HeLa cells and that the positive-feedback regulation of PLC by cytosolic Ca\(^{2+}\) is not a prime mechanism for the generation of the upstroke of Ca\(^{2+}\) spikes.

What is the positive-feedback element that drives the rising phase of Ca\(^{2+}\) spikes? One plausible mechanism is Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mediated by IP\(_3\)R. IP\(_3\)R is activated
by cytosolic Ca\(^{2+}\), and positive-feedback regulation by cytosolic Ca\(^{2+}\) enables the Ca\(^{2+}\) released by one receptor to excite its neighbors, thereby igniting a regenerative Ca\(^{2+}\) wave. However, we did not test the CICR hypothesis in the present study. The CICR hypothesis needs to be evaluated more directly under native conditions in future studies.

Mechanisms of initiation of the steep rising phase of Ca\(^{2+}\) spikes

We found that [IP\(_3\)]\(_c\) started to increase before the pacemaker rise in [Ca\(^{2+}\)]\(_c\), and that the subsequent steep rise in [Ca\(^{2+}\)]\(_c\) was unaccompanied by any acceleration in the [IP\(_3\)]\(_c\) increase (Figs. 5 and 6). The individual abrupt [Ca\(^{2+}\)]\(_c\) rises of at least the first eight Ca\(^{2+}\) spikes were initiated at different concentrations of [IP\(_3\)]\(_c\) (Fig. 4 C), and the Ca\(^{2+}\) spikes of mGlur5a-expressing cells stimulated with 100 \(\mu\)M glutamate and cells stimulated with 3 \(\mu\)M histamine were triggered at different IP\(_3\) concentrations (Fig. 4 C). These results indicate that a constant threshold [IP\(_3\)]\(_c\) is not involved in the initiation of the steep Ca\(^{2+}\) rise in HeLa cells. What is the actual trigger for generation of the rapid upstroke of Ca\(^{2+}\) spikes? Numerous observations have shown that elevation of [IP\(_3\)]\(_c\) to submaximal levels causes only a transient, partial release of Ca\(^{2+}\), whereas further increments of [IP\(_3\)]\(_c\) evoke an additional transient Ca\(^{2+}\) release (Muallem et al., 1989; Taylor and Potter, 1990; Bootman et al., 1992; Ferris et al., 1992; Hirota et al., 1995; Yao et al., 1995). Subsequent elevations of [IP\(_3\)]\(_c\) yielded the same degree of Ca\(^{2+}\) release as the first [IP\(_3\)]\(_c\) elevation, and the retention of responsiveness has been called “incremental detection” (Meyer and Stryer, 1990). However, because we did not detect rapid increments in [IP\(_3\)]\(_c\) when the abrupt [Ca\(^{2+}\)]\(_c\) rises were triggered (Fig. 5, D and E), IP\(_3\)-sensitive Ca\(^{2+}\) stores did not respond to the rate of [IP\(_3\)]\(_c\) increase in the cells examined. A time-dependent decrease in the IP\(_3\) binding affinity of IP\(_3\)R-gated Ca\(^{2+}\) release channels and/or multiple Ca\(^{2+}\) stores with different levels of IP\(_3\) sensitivity could account for this phenomenon. Ca\(^{2+}\) influx across plasma membranes might be involved in the initiation of repetitive spikes (Sneyd et al., 2004). Further analysis is required for the elucidation of the Ca\(^{2+}\) spike initiation mechanism.

IP\(_3\) metabolism in living cells

The mechanism underlying the generation of Ca\(^{2+}\) oscillations has been extensively investigated using various theoretical models. What kind of uncertainty is involved in such analyses? To address this issue, we tried to reproduce the experimental measurements of both IP\(_3\) and Ca\(^{2+}\) dynamics using a model based on previously reported models that incorporate the biphasic cytosolic Ca\(^{2+}\) dependency of IP\(_3\)R and Ca\(^{2+}\) feedback on the production of IP\(_3\) (Meyer and Stryer, 1991; De Young and Keizer, 1992; Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200512141/DC1). The model exhibited sharp Ca\(^{2+}\) oscillations accompanied by IP\(_3\) oscillations (Fig. S3, A and B) with the original parameters (Table S2; De Young and Keizer, 1992). When we compared the calculated trace of cytosolic IP\(_3\) with the experimental measurements (Fig. 4), the [IP\(_3\)]\(_c\) change predicted by the model was obviously too fast. We found that an ~60-fold decrease in the rate of IP\(_3\) production (Fig. S3 E and Table S2) and a 30-fold decrease in the rate of IP\(_3\) degradation (Fig. S3 F and Table S2) produced reasonable [IP\(_3\)]\(_c\) changes (Fig. S3 C) accompanied by repetitive Ca\(^{2+}\) spikes (Fig. S3 D). The calculated Ca\(^{2+}\) dynamics (Fig. S3 D), however, were significantly different from the experimentally observed Ca\(^{2+}\) dynamics (Fig. 4) in terms of frequency and latency. Because the apparent IP\(_3\) sensitivity for the generation of Ca\(^{2+}\) spikes progressively decreases during Ca\(^{2+}\) oscillations (Fig. 4 C), this property must be incorporated into the IP\(_3\)R model. It is clear that the actual IP\(_3\) metabolism in living cells is slower than previously estimated (Meyer and Stryer, 1991; De Young and Keizer, 1992). A similar conclusion was obtained from the estimation of the lifetime of IP\(_3\) in N1E-115 neuroblastoma cells (Wang et al., 1995).

Allbrüttn et al. (1992) proposed that Ca\(^{2+}\) is a local messenger, whereas IP\(_3\) is a global messenger based on their diffusion constants estimated in isolated cytosol. We propose that Ca\(^{2+}\) is a fast messenger and IP\(_3\) a slow messenger, based on the evidence that the concentration change of cytosolic IP\(_3\) is slower than that of Ca\(^{2+}\) in living cells. IP\(_3\)R functions as a pulse generator in the signal transduction cascade that translates the information from the slow IP\(_3\) signal to the fast Ca\(^{2+}\) signal. Elucidation of the encoding algorithm of this signal conversion is the next challenge in terms of better understanding the molecular basis of Ca\(^{2+}\) signaling.

Materials and methods

Materials

An anti-GFP antibody and an anti-mGlur5 antibody were purchased from MBL International Corporation and Upstate Biotechnology, respectively.

Gene construction

The IP\(_3\) binding core cDNA was obtained by PCR from pbact-ST-neoB-C1 (Furuichi et al., 1989; Miyawaki et al., 1990) and ECFP cDNAs were fused to the 5’ and the 3’ end, respectively, of the IP\(_3\) binding domain to produce IRIS proteins. Mutations of T267A and K508Q or K249Q were introduced as described elsewhere (Sawano and Miyawaki, 2000) to create an IP\(_3\) binding-deficient mutant and a low IP\(_3\) binding-affinity mutant, respectively. IP\(_3\) sensor cDNAs were cloned in BamHI and Xhol sites of pcDNA3.1 zeo (+) (Invitrogen) that contains 6× His flag tag (Fig. 1 A) in NheI to HindIII site for the expression in mammalian cells. The multicloning site of pcDNA3.1 zeo (+) was amplified by PCR and cloned into Sall site of pfastBacI (Invitrogen). IRIS-1 and IRIS-1-Dmut were digested by Nhel and Xhol from pcDNA3.1 zeo (+) and inserted to the modified pfastBacI for the expression in SF9 cells. Venus or ECFP cDNA was fused to 5’ end of cDNA corresponding to amino acid residues 11–140 of rat PLC\(_{a}\)c1 to create the expression in SF9 cells (provided by M. Hattori, Nagoya City University, Aichi, Japan) and CPHD, respectively. The resulting cDNAs were cloned in pcDNA3.1 zeo (+). Rat mGlur5a cDNA inserted into pME18S (Kawabata et al., 1996) was a gift from S. Nakanishi (Osaka Bioscience Institute, Osaka, Japan).

Expression, purification, and characterization in vitro

In vitro characterization of IRIS proteins was performed by using lysates prepared from COS7 or SF9 cells expressing IRIS proteins and IRIS proteins purified from SF9 cells. COS7 cells expressing IRIS proteins were lysed with the solution containing 10 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.5% NP-40. His-tagged IRIS-1 and IRIS-1-Dmut proteins were expressed by SF9 baculovirus-expressing system (Invitrogen) and were purified as follows. SF9 cells were suspended in homogenization solution containing 10 mM Hepes, pH 7.8, 100 mM NaCl, 5 mM 2-mercaptoethanol, 0.1% NP-40, and protease inhibitors (0.1 mM PMSF, 10 \(\mu\)M Leupeptin, 10 \(\mu\)M Pepstatin A, and 10 \(\mu\)M E60) and were homogenized at 1,000 rpm for 10 strokes at 4°C. Homogenates were applied to ProBond resin (Invitrogen), and His-tagged proteins were eluted with 300 mM imidazole. Proteins were then dialyzed for 30 min against the solution containing 10 mM Hepes, pH 7.4, and 100 mM KCl.

IP\(_3\) DYNAMICS DURING CA\(^{2+}\) SPIKES • MATSUURA ET AL.
The diacylated proteins were loaded to HitTrap heparin columns and eluted with the high KCl solution containing 10 mM Hepes, pH 7.4, and 250 mM KCl. Proteins were diluted by adding two volumes of the solution containing 10 mM Hepes, pH 7.4, and 100 mM KCl and centrifuged with centrifugal filter devices (30,000 MWCO; Amicon Ultra 15 [Millipore]). For the evaluation of the rate of changes in IRIS-1 signals, IRIS-1-expressing SPF cells were suspended in 10 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.01% NP-40, and proteasome inhibitors and were homogenized at 1,000 rpm for 10 strokes at 4°C. The homogenates were centrifuged at 20,000 g for 30 min, and the supernatants were used for stopped-flow fluorescent measurements. The number of IRIS-1 molecules in the supernatants was quantified by the equilibrium IP3 binding analysis using [3H]IP3 as described previously (Iwai et al., 2005). IRIS signals were measured with a spectrofluorometer (FP-750; Jasco).

Imaging
IRIS cDNAs inserted in pcDNA3.1 zeo+ were transfected into HeLa cells with transfection reagents (TransIT; Mirus). After 12–36 h, cells were used for imaging. After loading the cells with 5 μM Indo-1 AM (DOJINDO), imaging was performed under the constant flow (2 mL/min) of the balanced salt solution containing 20 mM Hepes, pH 7.4, 115 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, and 10 mM glucose as an imaging medium at 37°C. Imaging was performed at 37°C through an inverted microscope (IX71 or IX81; Olympus) with a cooled charge-coupled device camera (ORCA-EKR; Hamamatsu Photonics) and a 40× (NA 1.35) objective. A 425–445-nm excitation filter and pair of 460–470- and 500–510-nm emission filters and a 425–445-nm excitation and pair of 460–510-nm (cyan) and 525–565-nm (yellow) emission filters were used for fluorochrome (Indo-1 and IRIS, respectively). Beam-splitter mirrors (400 and 450 nm) were alternately inserted into the light path for Indo-1 and IRIS, respectively. Images were acquired at 0.25 Hz, with an exposure time of 100 or 150 ms. For the fast acquisition (4.07 Hz) of fluorescent images of IRIS-1 and Indo-1, an emission splitter (W-view; Hamamatsu Photonics) was used with a fast light source excchanger (DG-4; Sutter Instrument Co.). Imaging was performed with the custom software TI Workbench (written by T. Inoue). Off-line analysis was performed with TI Workbench combined with Igor Pro software (WaveMetrics). Spectral analysis of Ca2+ oscillations was performed as described previously (Uhlen, 2004).

Stopped-flow fluorescent measurement
The change in the intensity of Venus fluorescence (525 ± 20 nm) of IRIS-1 after the addition of various concentrations of IP3 was monitored using the FR-750 spectrofluorometer with a stopped-flow rapid mixing accessory (RX2000; Applied Photophysics). The IRIS-1-containing supernatants (final 2.5 μM of IRIS-1) were mixed with 2.5-fold-excess volume of 10 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.01% NP-40 containing various concentrations of IP3 at 25°C, and the change in the intensity of Venus fluorescence of IRIS-1 excited at 440 ± 20 nm was monitored at 1,000 Hz. At least 10 traces were averaged and were used for the nonlinear regression analysis with Igor Pro software. The IP3 dependency of FRET changes of IRIS-1 at equilibrium was monitored using the same batches of the supernatants as used for the kinetic measurements with a 1-cm cuvette and the FR-750 spectrofluorometer.

Online supplemental material
Fig. S1 shows effects of PLX inhibitors on emission changes of IRIS-1 elicited by 10 μM of histamine and evaluation of IRIS-1 signals observed in living HeLa cells. Fig. S2 shows the rate of reaction of IRIS-1. Fig. S3 shows simulation of IP3 and Ca2+ dynamics. Table S1 shows numbers of cells that showed [IP3]r, rises preceding [Ca2+]i, and the mean intervals between the onset of [IP3]r, rises, and the onset of [Ca2+]i rises. Table S2 shows the parameters used to calculate IP3 and Ca2+ dynamics. The supplemental text shows the evolution of the effect of the rate of FRET changes of IRIS-1 upon IP3 binding. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200512141/DC1.

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References


Supplemental materials and methods

Evaluation of the effect of the rate of FRET change of IRIS-1 upon IP₃ binding

The change of the intensity of Venus fluorescence (525 ± 20 nm) of IRIS-1 excited at 440 ± 20 nm after the addition of various concentrations of IP₃ was monitored using a stopped-flow fluorescence spectrometry (Fig. S2 A). We found a good fit between all of the traces except the control experiments (without IP₃) and a double exponential function (Fig. S2 A). Because the time constant of the slow component (1.2–16.7 s) was almost constant irrespective of [IP₃] applied (unpublished data) and was close to the time constant (2.9 s) of a single exponential function fitted to the fluorescent intensity change observed without the addition of IP₃ (Fig. S2 A), the fast component alone was used for the evaluation of the reaction rate of IRIS-1. Fig. S2 B shows the relationship between the inverse time constants of the fast component and [IP₃] applied. The inverse time constant was changed depending on [IP₃] in a nonlinear hyperbolic manner, indicating that the IP₃ binding is not a rate-limiting step for the FRET change of IRIS-1 and that conformational changes of the IRIS-1 molecule may be involved in the reaction. We therefore applied the following model for the evaluation of the reaction mechanism of IRIS-1:

\[
[I₃] + [IRIS] \xrightarrow{k_{on}} [IP₃ \cdot IRIS] \xrightarrow{k_f} [IP₃ \cdot IRIS*]
\]  

(1),

where [IP₃] is the concentration of IP₃, [IRIS] is the concentration of IP₃ unbound IRIS-1 with high FRET efficiency, [IP₃ \cdot IRIS] is the concentration of IP₃ bound IRIS-1 with high FRET efficiency, [IP₃ \cdot IRIS*] is the concentration of IP₃ bound IRIS-1 with low FRET efficiency, \( k_{on} \) is the association rate constant, \( k_{off} \) is the dissociation rate constant, \( k_f \) is the rate constant of the forward conformational change, and \( k_r \) is the rate constant of the reverse conformational change. In this model, a conformational change accompanied with FRET change occurs after IP₃ binding, and the relationship between the fraction of [IP₃ \cdot IRIS*] and [IP₃] at equilibrium is described as follows:

\[
\frac{[IP₃ \cdot IRIS*]}{[IRIS]_{total}} = \frac{1}{K_1K_2/[IP₃] + K_2 + 1}
\]  

(2)

\[
[IRIS]_{total} = [IRIS] + [IP₃ \cdot IRIS] + [IP₃ \cdot IRIS*]
\]  

(3),

where [IRIS]ₜ𝑜𝑡𝑎𝑙 is the total concentration of IRIS-1, \( K_1 \) is \( k_{off}/k_{on} \) (the equilibrium constant of the interaction between IP₃ and IRIS-1), and \( K_2 \) is \( k/k_f \) (the equilibrium constant of the conformational change of IP₃ bound IRIS-1). The equation provides reasonable fits with the parameters, \( K_1 = 7.26 \times 10^{-5} \) (M) and \( K_2 = 0.00269 \), and the experimental data measured at equilibrium (Fig. S2 C, solid line). We then tried to find the rate constants that fit with the apparent inverse time constants observed (Fig. S2 B) in the following equations:

\[
\frac{d[IP₃ \cdot IRIS*]}{dt} = k_f [IP₃ \cdot IRIS] - k_r [IP₃ \cdot IRIS*]
\]  

(4),

\[
\frac{d[IP₃ \cdot IRIS]}{dt} = k_{on} [IP₃][IRIS] - k_{off} [IP₃ \cdot IRIS] - k_f [IP₃ \cdot IRIS] + k_r [IP₃ \cdot IRIS*]
\]  

(5),

\[
\frac{d[IP₃]}{dt} = - k_{on} [IP₃][IRIS] + k_{off} [IP₃ \cdot IRIS]
\]  

(6), and

\[
\frac{d[IRIS]}{dt} = k_{on} [IP₃][IRIS] - k_{off} [IP₃ \cdot IRIS]
\]  

(7).

However, we did not find the parameters that satisfy the data shown in both Fig. S2 B and C because the estimated value of the equilibrium binding constant, \( K_1 \), from the data shown in Fig. S2 C is too high when it is compared with the apparent IP₃ sensitivity of the inverse time constants of changes in the Venus fluorescence \( EC_{50} < 1 \times 10^{-7} \) M; Fig. S2 B). We therefore used the other model,
Figure S1. Effects of PLC inhibitors on emission changes of IRIS-1 and evaluation of IRIS-1 signals observed in living HeLa cells. (A and B) Cells were pretreated with 10 µM of PLC inhibitor U73122 (A) or its inactive analog U73343 (B) for 5 min and then with 10 µM of histamine. Three different color plots represent data from three cells in the same viewing field. (C) Relationship between IRIS-1 signals and [IP\(_3\)] in permeabilized HeLa cells. Cells were permeabilized with 60 µM β-escin for 3 min, and bath solutions containing various concentrations of IP\(_3\) were perfused at a flow rate of 4 ml/min. Steady-state values of IRIS-1 were plotted. Error bars correspond to the standard deviation of at least six measurements. (D) IP\(_3\) sensitivity of IRIS-1 (open circles) and IRIS-1.2 (closed circles) in COS-7 lysates. The emission change of IRIS-1.2 exhibits an IP\(_3\) sensitivity with a K\(_{d}\) value of 4.0 µM. Error bars correspond to the standard deviation (n = 3). (E and F) Cells expressing mGluR5a were stimulated with 100 µM of glutamate (horizontal bars). Signals of IRIS-1.2 (E) and Indo-1 (F) are shown. Images were acquired every 4 s. Similar results were observed in 8 out of 25 cells.

Table S1. Numbers of cells that showed [IP\(_3\)] rises preceding [Ca\(^{2+}\)] increases and the average intervals between the onset of [IP\(_3\)] rises and the onset of [Ca\(^{2+}\)] rises

<table>
<thead>
<tr>
<th>Spike number</th>
<th>Cell number</th>
<th>Interval (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>31 (33)</td>
<td>3.59 ± 2.89</td>
</tr>
<tr>
<td>3</td>
<td>20 (24)</td>
<td>2.94 ± 3.08</td>
</tr>
<tr>
<td>4</td>
<td>14 (19)</td>
<td>1.56 ± 2.44</td>
</tr>
<tr>
<td>5</td>
<td>13 (16)</td>
<td>1.96 ± 2.43</td>
</tr>
<tr>
<td>6</td>
<td>9 (13)</td>
<td>1.18 ± 2.86</td>
</tr>
<tr>
<td>7</td>
<td>8 (10)</td>
<td>1.92 ± 2.08</td>
</tr>
<tr>
<td>8</td>
<td>7 (9)</td>
<td>1.47 ± 2.41</td>
</tr>
</tbody>
</table>

Total numbers of cell analyzed are shown in parenthesis. Intervals are shows as mean ± SD. Positive values indicate [IP\(_3\)] rises preceding [Ca\(^{2+}\)] rises.
Figure S2. The rate of reaction of IRIS-1. (A) Kinetics of Venus fluorescence intensity of IRIS-1 after the rapid mixing of 10 µM (dark blue), 10 nM (light blue), and 0 IP$_3$ (red). IP$_3$ was added at time 0. Double-exponential functions and a single-exponential function are shown as red smooth lines and a blue smooth line, respectively. (B) Relationship between the inverse time constant of the fast component of Venus fluorescence changes of IRIS-1 and [IP$_3$]. The data were obtained from three independent experiments. Error bars correspond to the standard deviation. (C) Relationship between equilibrium FRET changes of IRIS-1 and [IP$_3$]. The data were obtained from three independent experiments. Error bars correspond to the standard deviation. (D) Changes in the fraction of IRIS-1 with a low FRET efficiency (IRIS* and IP$_3$-IRIS*) in response to addition of a 1-s IP$_3$ pulse (horizontal bar). Various concentrations of IP$_3$ were used to calculate the fractional change, and all the calculated traces (0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 µM IP$_3$, from bottom to top) were superimposed. Basal [IP$_3$] was 40 nM. For more details, see the supplemental Materials and methods.

Table S2. Parameters used to calculate IP$_3$ and Ca$^{2+}$ dynamics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_1$</td>
<td>0.185</td>
<td>(ER vol)/(cytosolic vol)</td>
</tr>
<tr>
<td>$v_1$</td>
<td>6 s$^{-1}$</td>
<td>Max Ca$^{2+}$ channel flux</td>
</tr>
<tr>
<td>$v_2$</td>
<td>0.11 s$^{-1}$</td>
<td>Ca$^{2+}$ leak flux constant</td>
</tr>
<tr>
<td>$v_3$</td>
<td>0.9 µM$^{-1}$ s$^{-1}$</td>
<td>Max Ca$^{2+}$ uptake</td>
</tr>
<tr>
<td>$v_4$</td>
<td>2.8 s$^{-1}$</td>
<td>0.046 s$^{-1}$</td>
</tr>
<tr>
<td>$k_3$</td>
<td>0.1 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>$k_4$</td>
<td>1.1 µM</td>
<td>2 µM</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>0.97</td>
<td>1</td>
</tr>
<tr>
<td>$R$</td>
<td>(0)</td>
<td>0.4</td>
</tr>
<tr>
<td>$I_r$</td>
<td>1 s$^{-1}$</td>
<td>0.03 s$^{-1}$</td>
</tr>
</tbody>
</table>
Figure S3. Simulation of IP$_3$ and Ca$^{2+}$ dynamics. IP$_3$ and Ca$^{2+}$ dynamics were calculated using the following model:

\[
\frac{d[Ca^{2+}]}{dt} = J_1 - J_2 \tag{14}
\]

\[
J_1 = c_i \left( v_1 x_{110} + v_2 \right) \left( [Ca^{2+}]_{\text{ER}} - [Ca^{2+}]_i \right) \tag{15}
\]

\[
J_2 = \frac{v_3 [Ca^{2+}]^2}{[Ca^{2+}]_i^2 + k_2^2} \tag{16}
\]

\[
\frac{d[IP_3]}{dt} = v_4 \left( 1 - \frac{\alpha k_4}{[Ca^{2+}]_i + k_4} \right) \left( 1 + R \right) - I_r [IP_3] \tag{17}
\]

where $[Ca^{2+}]_i$ is the cytosolic free Ca$^{2+}$ concentration, $[Ca^{2+}]_{\text{ER}}$ is the ER luminal free Ca$^{2+}$ concentration, $J_1$ is the outward flux of Ca$^{2+}$, $J_2$ is the inward flux, and $x_{110}$ is the fraction of IP$_3R$ subunits activated by both IP$_3$ and Ca$^{2+}$ but not yet inactivated by Ca$^{2+}$ (De Young and Keizer, 1992). The denomination of all parameters is shown in Table S2. (A and B) Solutions using the parameters originally described in De Young and Keizer (1992). (C and D) Solutions using the parameters that produce slow IP$_3$ metabolism. Stimulus-induced IP$_3$ synthesis was turned on during the period shown by the horizontal bars (A–D). (E) The rates of IP$_3$ production used in A and B (broken line) and in C and D (solid line) are shown. (F) The rates of IP$_3$ degradation used in A and B (broken line) and in C and D (solid line) are shown.
where [IRIS*] is the concentration of IP₃ unbound IRIS-1 with a low FRET efficiency, $k'_f$ is the rate constant of forward conformational change, $k'_r$ is the reverse conformational change, $k'_{on}$ is the association rate constant, and $k'_{off}$ is the dissociation rate constant. In this model, there are two conformations of IRIS-1 with different FRET efficiencies (IRIS and IRIS*), and only IRIS* is able to bind to IP₃. IP₃ binding itself does not induce FRET efficiency change of IRIS-1. In this model, the relationship between the fraction of the low FRET efficiency forms (IRIS* and IP₃·IRIS*) and [IP₃] at equilibrium is

$$\frac{[\text{IRIS}^*] + [\text{IP}_3 \cdot \text{IRIS}^*]}{[\text{IRIS}]_{\text{total}}} = \frac{1}{(K'_2 + 1)K'_1/[\text{IP}_3] + 1} + \frac{1}{K'_2 + [\text{IP}_3]/K'_1 + 1}$$

(9),

where $K'_1 = k'_{off}/k'_{on}$ and $K'_2 = k'_{r}/k'_f$. The changes of the concentration of each form are described in the following equations:

$$\frac{d[\text{IRIS}]}{dt} = k'_f [\text{IRIS}^*] - k'_r [\text{IRIS}]$$

(10),

$$\frac{d[\text{IRIS}^*]}{dt} = -k'_f [\text{IRIS}^*] + k'_r [\text{IRIS}] - k'_{on} [\text{IP}_3] [\text{IRIS}^*] + k'_{off} [\text{IP}_3 \cdot \text{IRIS}^*]$$

(11),

$$\frac{d[\text{IP}_3]}{dt} = -k'_{on} [\text{IP}_3] [\text{IRIS}^*] + k'_{off} [\text{IP}_3 \cdot \text{IRIS}^*]$$

(12), and

$$\frac{d[\text{IP}_3 \cdot \text{IRIS}^*]}{dt} = k'_{on} [\text{IP}_3] [\text{IRIS}^*] - k'_{off} [\text{IP}_3 \cdot \text{IRIS}^*]$$

(13).

We found that when $[\text{IRIS}]_{\text{total}} = 2.5$ nM, equations 9–13 provide good fits with the parameters $k'_f = 4.8$ (s⁻¹), $k'_r = 96$ (s⁻¹), $k'_{on} = 2.1 \times 10^8$ (M⁻¹ s⁻¹), and $k'_{off} = 2.1$ (s⁻¹) and both the kinetic data (Fig. S2 B, broken line) and the equilibrium data (Fig. S2 C, broken line). The equilibrium constants, $K'_1$ and $K'_2$, are $1 \times 10^{-8}$ (M) and 20, respectively. We calculated the change of the fraction of the low FRET efficiency forms (IRIS* and IP₃·IRIS*) by the equations 10, 11, and 13 with the above parameters in response to the addition of a 1-s IP₃ pulse and found that IRIS-1 signals return to their basal level within ~3 s after the termination of IP₃ pulses (Fig. S2 D).

**References**