Use of Fluorescence Resonance Energy Transfer-based Biosensors for the Quantitative Analysis of Inositol 1,4,5-Trisphosphate Dynamics in Calcium Oscillations*§

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Inositol 1,4,5-trisphosphate (IP$_3$) is an intracellular messenger that elicits a wide range of spatial and temporal Ca$^{2+}$ signals, and this signaling versatility is exploited to regulate diverse cellular responses. In this study, we have developed a series of IP$_3$ biosensors that exhibit strong pH stability and varying affinities for IP$_3$, as well as a method for the quantitative measurement of cytosolic concentrations of IP$_3$ ([IP$_3$]$_i$) in single living cells. We applied this method to elucidate IP$_3$ dynamics during agonist-induced Ca$^{2+}$ oscillations, and we demonstrated cell type-dependent differences in IP$_3$ dynamics, a nonfluctuating rise in [IP$_3$]$_i$, and repetitive IP$_3$ spikes during Ca$^{2+}$ oscillations in COS-7 cells and HSY-EA1 cells, respectively. The size of the IP$_3$ spikes in HSY-EA1 cells varied from 10 to 100 nM, and the [IP$_3$]$_i$ spike peak was preceded by a Ca$^{2+}$ spike peak. These results suggest that repetitive IP$_3$ spikes in HSY-EA1 cells are passive reflections of Ca$^{2+}$ oscillations, and are unlikely to be essential for driving Ca$^{2+}$ oscillations. In addition, the interspike periods of Ca$^{2+}$ oscillations that occurred during the slow rise in [IP$_3$]$_i$ were not shortened by the rise in [IP$_3$]$_i$, indicating that IP$_3$-dependent and -independent mechanisms may regulate the frequency of Ca$^{2+}$ oscillations. The novel method described herein as well as the quantitative information obtained by using this method should provide a valuable and sound basis for future studies on the spatial and temporal regulations of IP$_3$ and Ca$^{2+}$.

Inositol 1,4,5-trisphosphate (IP$_3$)$_2$ is an important intracellular messenger produced by phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$). IP$_3$ releases Ca$^{2+}$ from intracellular stores via IP$_3$ receptors (IP$_3$Rs), and the resulting Ca$^{2+}$ signals often exhibit complex spatial and temporal organizations, such as Ca$^{2+}$ oscillations (1). The mechanism responsible for Ca$^{2+}$ oscillations has been a long-standing question, and a number of experimental approaches and mathematical models have been reported to account for these Ca$^{2+}$ signals, yet the mechanism responsible remains controversial (2). There are two general classes of Ca$^{2+}$ oscillation models (3). In one class, Ca$^{2+}$ oscillations are generated in the presence of constant cytosolic IP$_3$ concentrations ([IP$_3$]$_i$) (4); in the other class, oscillating [IP$_3$]$_i$ are required to drive Ca$^{2+}$ oscillations (5). The physiological relevance of the former class has been supported experimentally by using nonmetabolizable IP$_3$ analogs (6), and by the observation of repetitive Ca$^{2+}$ release in permeabilized cells with clamped IP$_3$ concentrations (7, 8). On the other hand, oscillatory changes in [IP$_3$]$_i$ have been suggested by the observed cyclical translocation of a GFP-tagged pleckstrin homology domain of PLC-δ (GFP-PHD) (9, 10). However, in other experiments using more specific IP$_3$ biosensors, IP$_3$ was shown to accumulate gradually with little or no fluctuation during Ca$^{2+}$ oscillations (11). These discrepant observations may be attributable to differences between various IP$_3$ biosensors and a lack of quantitation.

There are two types of IP$_3$ biosensors, GFP-PHD and IP$_3$R-based FRET sensors. GFP-PHD binds to both PIP$_2$ and IP$_3$; thus it has been thought that changes in [IP$_3$]$_i$ could be monitored indirectly by the release of membrane-bound GFP-PHD (9). IP$_3$R-based FRET biosensors consist of the ligand-binding domain of IP$_3$R and a pair of fluorescent proteins, cyan fluorescent protein and yellow fluorescent protein. Since the successful monitoring of IP$_3$ with LIBRA (12), the first IP$_3$R-based FRET biosensor, several different groups have used similar biosensors for IP$_3$ monitoring (11, 13, 14). In principle, quantitative measurements of [IP$_3$]$_i$ are not possible with GFP-PHD. In addition, it is recognized that GFP-PHD may be released from the plasma membrane by decreases in available PIP$_2$ (15), which could be attributed to PIP$_2$ hydrolysis or the occupation by other molecules. IP$_3$R-based FRET biosensors offer significant benefits for monitoring IP$_3$ based on their high selectivity for IP$_3$ and ratiometric measurement.

In this study, we developed a series of improved IP$_3$ biosensors that exhibit high pH stability and varying IP$_3$ affinities. They also possess higher selectivity and afford a larger dynamic range than that of original LIBRA. In combination with these

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* This work was supported by Grant-in-aid for Scientific Research 16390532 (to A. T.), by HAITEKU (2007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Japan Science and Technology Agency. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Tables S1 and S2.

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2 The abbreviations used are: IP$_i$, inositol 1,4,5-trisphosphate; EYFP, enhanced yellow fluorescent protein; GFP-PHD, green fluorescent protein-tagged pleckstrin homology domain of phospholipase C-δ; HBSS-H, Hank’s balanced salt solution with Heps; ICM, intracellular-like medium; IP$_3$, inositol 4,5-bisphosphate; IP$_3$R, inositol 1,4,5-trisphosphate receptor; IP$_4$, inositol 1,3,4,5-tetrakisphosphate; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; [IP$_3$]$_i$, cytosolic concentration of IP$_3$; FRET, fluorescence resonance energy transfer.
new biosensors, we developed a method for quantitating IP$_3$ dynamics in single living cells, and we applied this method to measure IP$_3$ dynamics during Ca$^{2+}$ oscillations.

**EXPERIMENTAL PROCEDURES**

*Media*—Hanks’ balanced salt solution with Hepes (HBSS–H) contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl$_2$, 0.41 mM MgSO$_4$, 0.49 mM MgCl$_2$, 0.34 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 5.5 mM glucose, 20 mM Hepes–NaOH (pH 7.4). Intracellular-like medium (ICM) contained 125 mM KCl, 19 mM NaCl, 10 mM Hepes–KOH (pH 7.3), 1 mM EGTA, and 330 mM CaCl$_2$ (for 50 nM free Ca$^{2+}$).

*Plasmid Construction*—Venus, EYFP with F46L, F64L, M153T, V163A, and S175G, was constructed using site-directed mutagenesis of pEYFP-N1 (Clontech) and a QuikChange® II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Primers 1–6 were used for mutagenesis (all primers used in this study are listed in supplemental Table S1). The sequence of the Venus construct (pVenus-N1) was verified by DNA sequencing.

To construct pH-stable LIBRA (LIBRAvIII), the portion of LIBRA containing the membrane-targeting signal, enhanced cyan fluorescent protein, the IP$_3$-binding domain of the rat type 3 IP$_3$R (amino acids 1–604), and Nhel and EcoRl sites at either end was amplified by PCR using primers 7 and 8 and LIBRA plasmid as the template. This PCR product was then ligated into the Nhel and EcoRl sites of pVenus-N1.

To construct LIBRA variants with type 1 IP$_3$R (LIBRAvI), the IP$_3$-binding domain of the rat type 1 IP$_3$R (amino acids 1–604) and Nhel and EcoRl sites incorporated at both ends was amplified by PCR using primers 9 and 10 and a rat brain cDNA library as the template. This PCR product was then ligated into the Nhel and EcoRl sites of pVenus-N1.

To construct LIBRA variants with type 2 IP$_3$R (LIBRAvII), the portion of LIBRA containing the membrane-targeting signal, enhanced cyan fluorescent protein, the IP$_3$-binding domain of the rat type 2 IP$_3$R (amino acids 1–604) using primers 11 and 12 and a rat parotid cDNA library as the template. This PCR product was then ligated into the Nhel and EcoRl sites of pVenus-N1.

We previously constructed an IP$_3$-insensitive mutant (K507A) of LIBRA, which we referred to as LIBRA-N. The IP$_3$-insensitive variant of LIBRAv (LIBRAvN) was constructed by cutting the mutated IP$_3$-binding domain of LIBRA-N-plasmid with Xhol and ligating the fragment into LIBRAvIII-plasmid cut with the same enzyme.

*Cell Culture and Transfection*—COS-7 cells, obtained from RIKEN Cell Bank (Tokyo, Japan), were cultured in Dulbecco’s modified Eagle’s medium with low glucose (1000 mg/liter), supplemented with 10% fetal calf serum, 584 mg/liter l-glutamine, 110 mg/liter sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen). HSY-EA1 cell was subcloned from HSY human parotid cell line, a generous gift from Dr. Mitsunobu Sato (Tokushima University, Japan), by a dilution plating technique. HSY-EA1 cells were cultured in Dulbecco’s Eagle’s medium nutrient mixture Ham’s F-12 (Sigma) supplemented with 10% newborn calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin, as described previously. These cells were grown in fibronectin-coated experimental chambers consisting of plastic cylinders (7 mm in diameter) glued to round glass coverslips.

*Measurement of Fluorescence*—Cells were washed with HBSS–H and rested for at least 5 min prior to experiments. In some experiments, cells were incubated at room temperature for 1–2 min in HBSS–H containing 2.5 µM fura-2 acetoxyethyl ester (Dojin Chemicals, Kumamoto, Japan). Permeabilization was performed by applying ICM containing 100 µg/ml (w/v) saponin (ICN, Cleveland, OH) for 1–1.5 min. In some experiments, cells were permeabilized with ICM containing 200 µM β-escin (Sigma). Fluorescence images were captured using a dual wavelength ratio imaging system (Hamamatsu Photonics, Shizuoka, Japan) consisting of a C9100-13 EM-CCD camera and W-View optics coupled to a Nikon TE2000 inverted fluorescence microscope equipped with a Nikon S Fluor 60 oil immersion objective (NA 1.3). Fluorescence of IP$_3$ biosensors was monitored with excitation at 425 nm and dual emission at 480 and 535 nm. Fluorescence of fura-2 was monitored with sequential excitation at 345 and 380 nm and emission at 535 nm. AQUACOSMOS 2.6 software (Hamamatsu Photonics) and ImageJ program were used for image analyses. To minimize the effect of photobleaching on the estimation of [IP$_3$], time-dependent decreases in the fluorescence intensities at two emission wavelengths (480 and 535 nm) were monitored for 5–10 min prior to the stimulations, and the photobleaching rates were calculated by fitting the data to linear or exponential equations.

*Determinations of Kd and Hill Coefficient*—Data were analyzed by simulated annealing procedure with GOSA-fit software (BIO-LOG, Toulouse, France) to fit the data to Equation 1,

$$E(% \text{max}) = (E_{\text{max}}C^n)(EC_{50}^n + C^n)^{-1} \quad (\text{Eq. 1})$$

where $C$ is the IP$_3$ concentration; $E_{\text{max}}$ is the maximal effect (100%), and $n$ is the Hill coefficient.

*Estimation of [IP$_3$]$_{i}$*—To estimate resting [IP$_3$]$_{i}$, emission ratios of LIBRAvIII in intact cells before ($R_{\text{rest}}$) and after ($R_{\text{min}}$) treatment with U73122, and emission ratios in the absence ($R_{\text{min}}'$) and presence ($R_{\text{max}}'$) of saturating concentrations of IP$_3$ were monitored after permeabilization. [IP$_3$]$_{i}$ can be calculated by Equation 2,

$$[IP_3] = K_d(\Delta R(R_{\text{max}} - \Delta R)^{-1})^{1/n} \quad (\text{Eq. 2})$$

where $\Delta R$ is $R_{\text{rest}} - R_{\text{min}}$; $R_{\text{max}}$ is $(R_{\text{max}}' - R_{\text{min}}')$ $R_{\text{min}}$ $R_{\text{min}}'; K_d$ is the apparent dissociation constant, and $n$ is the Hill coefficient. Because the variability of resting [IP$_3$]$_{i}$ was reasonably small, we used averaged resting [IP$_3$]$_{i}$ to determine $R_{\text{min}}$ by Equation 3,

$$R_{\text{min}} = R_{\text{rest}} - E_{\text{rest}}\Delta R_{\text{max}} \quad (\text{Eq. 3})$$

where $E_{\text{rest}}$ is the E (% max) of resting [IP$_3$]$_{i}$, which is given by Equation 1. [IP$_3$]$_{i}$ was then calculated using Equation 2.
Determinations of Response Rate—LIBRAvIII-expressing COS-7 cells were permeabilized with saponin, and changes in emission ratio were monitored with the first acquisition protocol (20–50 ms/frame). The response rate for the IP3-dependent increase in ratio was examined by application of 10 μM IP3 to the experimental chamber containing saponin-treated LIBRAvIII-expressing cells. To examine the rate of decrease in ratio due to the removal of IP3, permeabilized LIBRAvIII-expressing cells were initially exposed to 1 μM IP3, and subsequently washed out using IP3-free ICM. In this experimental condition, ~70% of medium was replaced by a single wash. Using a nonlinear least squares method, these data were fitted by the equation derived from a solution of the chemical reaction model, Equation 4,

$$R_t = R_0 + A\left\{ (C - 1)^{-1} - (Ce^\gamma - 1)^{-1} \right\}$$

(Eq. 4)
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The ligand-binding domain of IP₃Rs is composed of two functional domains, the amino-terminal suppressor domain and the carboxyl-terminal IP₃-binding core domain (17). Unlike other IP₃R-based FRET sensors (11, 13), LIBRA and LIBRA variants contain both IP₃ suppressor and core domains. Because the molecular properties of IP₃R isoforms have been studied extensively, it is relatively easy to construct biosensors with appropriate levels of affinity. In addition, it has been reported that structural differences in the suppressor domains contribute to functional diversity in ligand sensitivity among IP₃R isoforms (20), assuming that the ligand-binding properties of the LIBRA series reflect this feature of IP₃R isoforms. We previously reported a method using LIBRA for determining IP₃R ligands (21), and we identified novel ligands of IP₃Rs from newly synthesized cyclopentane derivatives (22). This method could be extended easily for identifying subtype-specific ligands of IP₃Rs by using a series of new LIBRA variants.

Imaging of IP₃-dependent Response of LIBRAvIIS—Emission ratios within an individual cell, whether intact or permeabilized, were uneven, whereas the magnitudes of the maximal change in ratio in response to 10 μM IP₃ were reasonably consistent (supplemental Fig. S2). Therefore, we visualized IP₃-dependent changes in the fluorescence emission ratio with normalized ratio images (Fig. 2A), where each ratio image was divided by an image before the application of IP₃. The normalized LIBRAvIIS emission ratio increased homogeneously except for the area including intracellular vesicles in cell 2 (Fig. 2B). It was noticed that abundant accumulation of IP₃ sensors on intracellular vesicles tended to decrease the extent of IP₃-dependent changes in the emission ratio. We then applied this procedure to visualize the ATP-induced increase in LIBRAvIIS emission ratio and subsequent decrease because of the removal of ATP in intact COS-7 cells (Fig. 2, C and D).

Response rates of IP₃ biosensors were examined by high speed monitoring of IP₃-dependent changes in the emission ratio of LIBRAvIIS in permeabilized cells and a least squares curve-fitting technique. The emission ratio increased to the half-maximal level in ~100 ms following the application of 10 μM IP₃. The estimated time for the half-maximal increase (t₁/₂on) and decrease (t₁/₂off) in the emission ratio was 162 and 169 ms, respectively (supplemental Fig. S3). These analyses indicate that the response rate of LIBRAvIIS is sufficiently rapid.

The characteristics of our IP₃ biosensors include the use of Venus, ligand-binding domains of different IP₃Rs, and a membrane-targeting signal. The superior selectivity of IP₃R-based FRET sensors provides a clear advantage over GFP-PHD sensors. In addition, utilizing Venus for a FRET-based biosensor is particularly important to avoid pH-related artifacts (16, 19).
to reflect responses that occur \( \sim 100 \text{ ms} \) or longer. However, the spatial resolution and signal-to-noise ratio necessary to detect subcellular differences in the high-speed monitoring of IP\(_3\) were not achieved in our experimental system.

Inclusion of a membrane-targeting signal provided a rapid means for examining biosensors expressed in permeabilized cells (supplemental Fig. S1) and enabled calibration of FRET signals in single cells (Fig. 3). However, nonuniform distributions of biosensors caused a subcellular variability in the ratio. This variability was overcome by using normalized emission ratio images, although this method is not suitable for measurements in motile cells. Further improvement of IP\(_3\) biosensor dynamic ranges is required for the high-speed monitoring of subcellular IP\(_3\) responses.

Methods for Quantitative Measurement of [IP\(_3\)]\(_i\).—Emission ratios of LIBRA\(_{VIII}\) and LIBRA\(_{VIII}\) in intact cells were applied upon the application of 10 \( \mu \text{M} \) ATP, and returned to basal levels after the addition of 5 \( \mu \text{M} \) U73122 (Fig. 3). These reagents did not change the emission ratio of LIBRA\(_{VIII}\) (data not shown). To obtain the maximal changes in emission ratio \( (\Delta R_{\text{max}}) \) in each individual cell, cells were permeabilized and expressed to IP\(_3\), following the completion of measurements in intact cells (Fig. 3, A and B). Biosensor-expressing cells and LIBRA\(_{VIII}\)-expressing cells showed decreases in their fluorescence ratios upon permeabilization. These changes in fluorescence are thought to be due to effects on the fluorescent proteins rather than to effects on the IP\(_3\)-binding domain, and thus they are unlikely to interfere with IP\(_3\)-dependent changes in emission ratios.

In Fig. 3, C and D, changes in emission ratios are quantitatively shown as \% of maximal change in ratio (\% max). The difference in \% max values of ATP-induced changes in emission ratios between LIBRA\(_{VIII}\) and LIBRA\(_{VIII}\) is thought to reflect the difference in affinities of these IP\(_3\) sensors. We further examined resting [IP\(_3\)]\(_i\) by treating unstimulated cells with U73122. The LIBRA\(_{VIII}\) emission ratio did not change with U73122 treatment (Fig. 3E), whereas the LIBRA\(_{VIII}\) ratio decreased slowly to a new steady state (Fig. 3F). These results indicate that the emission ratio of LIBRA\(_{VIII}\) reflects the resting [IP\(_3\)]\(_i\) and U73122 decreases [IP\(_3\)]\(_i\), to levels below the detectable range of LIBRA\(_{VIII}\). Consistent with this interpretation, Sato et al. (13) reported that U73122-induced decreases in the emission ratio of intact cells were observed using a high-affinity IP\(_3\) biosensor. Based on this idea, we used the U73122-induced decrease in emission ratios, \( K_p \) and Hill coefficient of LIBRA\(_{VIII}\) (shown in Fig. 1D) to estimate the resting [IP\(_3\)]\(_i\) in COS-7 and HSY-EA1 cells. U73122-induced decrease in emission ratio of LIBRA\(_{VIII}\) in COS-7 and HSY-EA1 cells was 13.67 \pm 0.96 \% max (mean \pm S.E., \( n = 60 \)) and 16.03 \pm 1.53 \% max (\( n = 19 \)), respectively. The estimated resting [IP\(_3\)]\(_i\) in COS-7 and HSY-EA1 cells was 15.09 \pm 1.38 and 18.16 \pm 2.49 \text{ nm}, respectively.

Resting [IP\(_3\)]\(_i\) values allowed us to accurately calculate agonist-induced changes in [IP\(_3\)]\(_i\). In calculations for LIBRA\(_{VIII}\), omitting adjustment for resting [IP\(_3\)]\(_i\) caused a noticeable underestimation in concentration (Fig. 4D), whereas the effects of adjusting calculations for resting [IP\(_3\)]\(_i\) was limited for LIBRA\(_{VIII}\) (Fig. 4C). In addition, the impact of basal noise of the LIBRA\(_{VIII}\) fluorescence on the basal level of calculated [IP\(_3\)]\(_i\) was negligible (Fig. 4D, arrowhead), indicating that LIBRA\(_{VIII}\) is suitable for monitoring small increases in [IP\(_3\)]\(_i\), up to 200 \text{ nm}. Changes in the emission ratio of LIBRA\(_{VIII}\) corresponded well over a broad range of [IP\(_3\)]\(_i\), up to \( \sim 400 \text{ nm} \), whereas baseline value fluctuations near the resting [IP\(_3\)]\(_i\) produced a noticeable difference in the estimated [IP\(_3\)]\(_i\) (Fig. 4C, arrowheads). These results indicate that LIBRA\(_{VIII}\) is convenient for monitoring broad ranges of [IP\(_3\)]\(_i\).

In this study, [IP\(_3\)]\(_i\) was estimated under the following assumptions: 1) that the properties of IP\(_3\) biosensors in permeabilized cells are comparable with those in intact cells, and 2) that U73122 decreases [IP\(_3\)]\(_i\) below the detectable level of the biosensors. To examine possible effects of cytosolic proteins on biosensor response, we compared IP\(_3\)-induced changes in the ratios of LIBRA\(_{VIII}\) in \( \beta \)-escin-permeabilized cells and in saponin-permeabilized cells and found comparable IP\(_3\)-induced responses (supplemental Fig. S4). \( \beta \)-Escin-permeabilized cells have been used to examine the functions of cytosolic proteins.\(^3\)

\(^3\) A. Tanimura, unpublished observations.
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FIGURE 4. Estimation of [Ins(1,4,5)P_3]_i with LIBRAvIII and LIBRAvIIS. ATP-induced changes in emission ratios of LIBRAvIII (A) and LIBRAvIIS (B) in intact COS-7 cells are shown. These % max values were converted to [IP_3]_i in C and D, with (solid lines) or without (dashed lines) adjustment for the resting [IP_3]_i.

whereas cytosolic proteins are lost in saponin-permeabilized cells (7, 23). Thus, the effects of endogenous cytosolic proteins on the IP_3-induced response of biosensors could be ruled out. In addition, our previous study indicates that IP_3-dependent changes in the ratio of LIBRA are not altered by Ca^{2+} or ATP, and that the effect of pH on fluorescent proteins does not alter the IP_3-dependent changes in the fluorescence of LIBRA (12). Although these experiments do not exclude possible effects of other small endogenous molecules, it is reasonable to assume at this stage that IP_3-dependent changes in the ratios of these types of biosensors in permeabilized cells are comparable with those in intact cells.

We also examined the effects of U73122 on ATP-induced Ca^{2+} responses, and we found that the pretreatment of COS-7 cells with 5 μM U73122 completely blocked the rise in [Ca^{2+}]_i, obtained by 3 μM ATP, and strongly decreased responses obtained with 10 μM ATP (data not shown). It is therefore thought that U73122 pretreatment is sufficient to block the low level of PLC activity in unstimulated cells. Indeed, we have used this method to estimate the [IP_3]_i required to elicit Ca^{2+} responses (see below), and these values are reasonably close to the threshold concentration of photoreleased [IP_3]_i (60 nM) that triggers Ca^{2+} spikes in Xenopus oocytes (24).

Changes in [IP_3]_i during Agonist-induced Ca^{2+} Oscillations—The mechanism responsible for Ca^{2+} oscillations and the associated dynamics of IP_3 have been of long-standing interest (2). Experiments based on the translocation of GFP-PHD have suggested that [IP_3]_i oscillate (9, 10), whereas an IP_3R-based FRET biosensor has shown that IP_3 accumulates gradually in the cytosol with little or no fluctuation during Ca^{2+} oscillations (11). Because of conflicting data and a lack of quantitation of IP_3 dynamics, it remains controversial whether [IP_3]_i truly fluctuates and whether proposed [IP_3]_i fluctuations drive Ca^{2+} oscillations. To clarify these important questions, we quantitatively monitored IP_3 dynamics during Ca^{2+} oscillations using LIBRAvIIS.

The upper panels of Fig. 5, A–D, show changes in emission ratios of LIBRAvIIS and fura-2, and the lower panels indicate calculated [IP_3]_i during ATP-induced Ca^{2+} oscillations. In COS-7 cells, stimulation with either 1 or 3 μM ATP increased [IP_3]_i slowly to a sustained level without detectable fluctuations, and Ca^{2+} oscillations were observed during this increase and sustained elevation of [IP_3]_i (Fig. 5, A and B). Unlike ATP-induced increases in [Ca^{2+}]_i, the large increase in [Ca^{2+}]_i, caused by ionomycin (2 μM) treatment had no effect on the emission ratio of LIBRAvIIS, indicating that Ca^{2+} itself does not activate IP_3 generation in COS-7 cells.

We also examined IP_3 dynamics in HSY-EA1 cells. This cell line is characterized by long lasting Ca^{2+} oscillations in response to wide ranges of agonist concentrations (23, 25). In fact, 65% of HSY-EA1 cells (55 of 84 cells) exhibited Ca^{2+} oscillations following treatment with 3–100 μM ATP or 10–100 μM carbaccol. Interestingly, 56% of Ca^{2+}-oscillating HSY-EA1 cells (31 cells) showed associated fluctuations in LIBRAvIIS emission ratios (Fig. 5, C and D). Similar fluctuations in the LIBRAvIIS emission ratio were observed when IP_3 dynamics were examined in the absence of fura-2 loading (data not shown). In contrast, no change in emission ratio was observed during Ca^{2+} oscillations in LIBRAvN-expressing HSY-EA1 cells (Fig. 5E). These experiments exclude the possibility of resulting artifacts derived from IP_3-independent changes in LIBRAvIIS fluorescence and possible interference by fura-2 fluorescence. These results demonstrate cell type-specific differences in IP_3 dynamics, nonfluctuating rises in [IP_3]_i, and repetitive IP_3 spikes in COS-7 cells and HSY-EA1 cells, respectively. Quantitative examinations revealed that repetitive IP_3 spikes in HSY-EA1 cells occurred concomitantly with a slow basal accumulation of [IP_3]_i. The size of IP_3 spikes varied from 10 to 100 nM, and the second and later spikes were initiated before the decline of [IP_3]_i to resting levels, resulting in a slow increase in the [IP_3]_i interspike.

It is generally thought that IP_3 diffuses rapidly within the cell, and thus we think that the fluorescence of membrane-bound biosensors reflects overall cytosolic [IP_3]_i. In agreement with this idea, we observed nonfluctuating IP_3 responses in COS-7 cells and repetitive IP_3 spikes in HSY-EA1 cells in both cytosolic and nuclear areas using a biosensor lacking the membrane targeting sequence.³

Although repetitive IP_3 spikes were observed in HSY-EA1 cells, our observations do not support the requirement of IP_3 fluctuations in Ca^{2+} oscillations. Although [IP_3]_i showed clear fluctuations at the beginning of Ca^{2+} oscillations, repetitive spikes of [IP_3]_i were gradually obscured during Ca^{2+} oscillations in 64% of HSY-EA1 cells. In addition, the [IP_3]_i spike peak was preceded by a Ca^{2+} spike peak (Fig. 5, C and D). These results suggest that repetitive IP_3 spikes in HSY-EA1 cells are passive reflections of the Ca^{2+} oscillations, and are unlikely to be essential for driving Ca^{2+} oscillations.

[IP_3]_i fluctuations could be induced by the effects of Ca^{2+} on IP_3 synthesis and/or IP_3 degradation. Applications of 2 μM ionomycin had little or no effect on the emission ratio of LIBRAvIIS in HSY-EA1 cells (Fig. 5C), suggesting that the direct effect of Ca^{2+} on IP_3 production is very small or absent in this cell line. Thus, [IP_3]_i fluctuations are likely to be caused by Ca^{2+}-induced potentiation of agonist-dependent IP_3 generation. The IP_3 spikes described here resemble the pattern pre-
dicted by the oscillator model, in which positive feedback via Ca\(^{2+}\)/H\(_{11001}\)-dependent activation of PLC is added to the dual positive and negative feedback effect of Ca\(^{2+}\)/H\(_{11001}\) on IP\(_3\)R, rather than the model including negative feedback via Ca\(^{2+}\)/H\(_{11001}\)-dependent IP\(_3\) degradations by IP\(_3\) 3-kinases (26).

Ca\(^{2+}\) oscillations were observed primarily when [IP\(_3\)]\(_i\) was less than 300 nM (Fig. 6, A and B). More than 50% of COS-7 cells exhibited Ca\(^{2+}\) oscillations when [IP\(_3\)]\(_i\) was 50–100 nM, and the percentage of oscillating COS-7 cells decreased as [IP\(_3\)]\(_i\) increased (Fig. 6C). In HSY-EA1 cells, Ca\(^{2+}\) oscillations were observed in 50–70% of cells, when interspike [IP\(_3\)]\(_i\) was less than 250 nM, and the percentage of oscillating cells decreased abruptly (11.1%) when interspike [IP\(_3\)]\(_i\) was greater than 250 nM (Fig. 6D). These results indicate that low concentrations of IP\(_3\) (≤100 nM) induce Ca\(^{2+}\) oscillations in both cell types, whereas HSY-EA1 cells are more likely to exhibit Ca\(^{2+}\) oscillations at higher concentrations of [IP\(_3\)]\(_i\) (100–250 nM) than are COS-7 cells. Large increases in [IP\(_3\)]\(_i\) (>250 nM) induced the peak plateau-type Ca\(^{2+}\) response in both cell types.

This study showed the time delay of IP\(_3\) spike peaks from Ca\(^{2+}\) spike peaks in HSY-EA1 cells, and Ca\(^{2+}\) oscillations with nonfluctuations of [IP\(_3\)]\(_i\) in COS-7 cells. Together these results suggest it is likely that IP\(_3\) spikes are not essential to drive Ca\(^{2+}\) oscillations. Regarding the mechanism of Ca\(^{2+}\) oscillations, the importance of dual feedback effects of Ca\(^{2+}\)/H\(_{11001}\) on IP\(_3\)Rs has been demonstrated experimentally (6–8). However, it has been pointed out that this dual feedback effect explains relatively short period Ca\(^{2+}\) oscillations, but it cannot reproduce long interspike intervals. Thus, additional mechanisms responsible for establishing these oscillations remain unclear (3, 26, 27).

One model study reported that frequency properties of oscillation are modulated by the incorporation of Ca\(^{2+}\)/H\(_{11001}\) activation of PLC into the Ca\(^{2+}\)/H\(_{11001}\) oscillation model based on dual feedback regulations of IP\(_3\)R properties, which enhances the range of frequency encodings of agonist stimulations (26). Interestingly, HSY-EA1 cells exhibited Ca\(^{2+}\) oscillations for a wider range of agonist and [IP\(_3\)]\(_i\) concentrations than observed in COS-7 cells. Thus, it may be possible that repetitive IP\(_3\) spikes or fluctuations play some role in supporting and/or regulating Ca\(^{2+}\) oscillations. The quantitative information provided here would be useful for future studies of the mechanisms and roles of IP\(_3\) oscillations.
Effect of [IP$_3_i$] on the Refractory Period of Ca$^{2+}$ Oscillations—We analyzed the effects of [IP$_3_i$] on the interspike period of Ca$^{2+}$ oscillations. In COS-7 cells, increases in agonist concentrations resulted in either shortening of interspike periods of Ca$^{2+}$ oscillations (Fig. 5A) or shifting of oscillations to the peak plateau-type Ca$^{2+}$ response (open square), or no response (blue triangle). C and D show [IP$_3_i$] histograms for the % of oscillating COS-7 (C) and HSY-EA1 cells (D).

However, interspike periods of Ca$^{2+}$ oscillations occurring during a slow rise in [IP$_3_i$] were not shortened by the rise in [IP$_3_i$] (Fig. 5A and C). Small increases or no change in interspike periods during an increase in [IP$_3_i$] was observed in 18 of 55 HSY-EA1 cells and in 12 of 25 COS-7 cells. These data suggest that IP$_3$ is an important, but not exclusive, regulator of Ca$^{2+}$ oscillation frequency.

Although the mechanism underlying the dissociation of the interspike period from the rise in [IP$_3_i$] is unknown, it might be associated with decreases in IP$_3$R sensitivity. Consistent with this idea, Matsuura et al. (11) described progressive decreases in apparent IP$_3$ sensitivity for generation of Ca$^{2+}$ spikes during Ca$^{2+}$ oscillations. It is known that Ca$^{2+}$ within the lumen of the endoplasmic reticulum regulates IP$_3$R sensitivity. Thus, a slow rise in [IP$_3_i$] may be balanced by a decrease in IP$_3$R sensitivity via a slow decrease in stored Ca$^{2+}$ (28). In addition, the involvement of stored Ca$^{2+}$ and sarco/endoplasmic reticulum Ca$^{2+}$-ATPase activity in determining the frequency of Ca$^{2+}$ oscillations has been proposed recently (27). Additional work is required to explore the possible involvement of stored Ca$^{2+}$ and other cytosolic factors in controlling the frequency of Ca$^{2+}$ oscillations.

In summary, we developed improved IP$_3$ biosensors and a relatively simple method for the quantitative measurement of [IP$_3_i$], and we applied this method for monitoring IP$_3$ dynamics during Ca$^{2+}$ oscillations. This method revealed cell type-specific differences in IP$_3$ dynamics as follows: nonfluctuating rises in [IP$_3_i$] and repetitive IP$_3$ spikes. Our results provide the first quantitative information for repetitive IP$_3$ spikes and present new aspects concerning the regulation of Ca$^{2+}$ oscillation frequency. In addition, the method demonstrated here offers a powerful means for studying the IP$_3$ dynamics of many cellular processes.