Fluorescent Biosensor for Quantitative Real-time Measurements of Inositol 1,4,5-Trisphosphate in Single Living Cells*

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The second messenger inositol 1,4,5-trisphosphate (IP3) plays a central role in the generation of a variety of spatiotemporally complex intracellular Ca2+ signals involved in the regulation of many essential physiological processes. Here we describe the development of “LIBRA”, a novel ratiometric fluorescent IP3 biosensor that allows for the quantitative monitoring of intracellular IP3 concentrations in single living cells in real time. LIBRA consists of the IP3-binding domain of the rat type 3 IP3 receptor fused between the fluorescence resonance energy transfer pair cyan fluorescent protein and yellow fluorescent protein and preceded by a membrane-targeting signal. We show that the LIBRA fluorescent signal is highly selective for IP3 and unaffected by concentrations of Ca2+ and ATP in the physiological range. In addition, LIBRA can be calibrated in situ. We demonstrate the utility of LIBRA by monitoring the temporal relationship between the responses intracellular IP3 and Ca2+ concentrations in SH-SY5Y cells following acetylcholine stimulation.

Many plasma membrane receptors act by stimulating the phospholipase C (PLC)-dependent hydrolysis of phosphatidyl-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ (EBI Data Bank with accession number(s) AB161231.

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The abbreviations used are: PLC, phospholipase C; Ach, acetylcholine; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; EGFP, enhanced GFP; HBSS, Hanks’ balanced salt solution; ICM, intracellular-like medium; IP3, inositol 4,5-bisphosphate; IP4, inositol 4,5,6-trisphosphate; IP6, inositol 4,5,6,7,8-pentakisphosphate; IPi, inositol 1,4,5-trisphosphate receptor; PIP, phosphatidylinositol 4,5-bisphosphate; 1,3,4,5-IP3, inositol 1,3,4,5-tetrakisphosphate; PIP2, pleckstrin homology domain; [Ca2+]i, cytosolic Ca2+ concentration; [IP3], inositol 4,5-bisphosphate (PIP2) to produce the intracellular messenger inositol 1,4,5-trisphosphate (IP3) (1). IP3 in turn releases Ca2+ from intracellular stores via IP3 receptors (IP3Rs) resulting in the activation of a variety of Ca2+-dependent processes. IP3-dependent Ca2+ responses can exhibit complex spatial and temporal patterns, and a number of competing models have been proposed to account for them (1–3). Because of its central role in these responses, the ability to quantitatively monitor temporal changes in [IP3]i in single living cells is of vital importance to understanding these phenomena. Since IP3Rs are the natural physiological target for IP3, we reasoned that their IP3-binding domain (4, 5) might be used to construct an effective and specific intracellular IP3 detector. Here we describe such a ratiometric fluorescent biosensor that in addition allows for in situ calibration. We demonstrate the utility of this biosensor by examining the relationship between the responses of [IP3]i and [Ca2+]i in single living cells.

EXPERIMENTAL PROCEDURES

Media—Hanks’ balanced salt solution with Heps (HBSS-H) contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.4 mM MgSO4, 0.49 mM KH2PO4, 0.34 mM Na2HPO4, 5.4 mM HEPES, 0.04 mM Na2EDTA, 10 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 appropriate concentrations of CaCl2 (330 μM CaCl2 for 50 mM free Ca2+) (6).

Plasmid Construction—A GFP/YFP fusion construct was made by cutting EYFP out of pEYFP-N1 (Clontech) using BamHI and XhoI and ligating into pECFP-C1 (Clontech) cut with the same enzymes. The multiple cloning site of this vector was then removed by cutting with BspEI and BamHI and replaced with a linker generated from two synthetic oligonucleotides. The forward sequence of this linker was GCC GAA ACG CTC GAG GCA GTG GTA AGA AGG CTC GAG GAT GAC GAT GAG GAT AAG GCG GCA TGT GTC GAC GCA GGT GCA TGC, where the reconstituted BspEI and BamHI sites have been included for clarity and the sequence has been parsed into codons. This fusion construct was referred to as pCY-N. The linker plus EYFP sequence of pCY-N was then cut out with BamGI and ligated into pEYFP-mem (Clontech) cut with the same enzyme. Finally the multiple cloning site originating from pEYFP-mem was removed by cutting with Eco47III and Smal and religating. The resultant construct, mCY, codes for ECFP preceded by the N-terminal 20 amino acids of neuraminidin (a membrane-targeting signal) and followed by the above linker and EYFP.

To construct LIBRA (luminous inositol trisphosphate-binding domain for ratiometric analysis) the IP3-binding domain of the rat type 3 IP3 receptor (amino acids 1–604) was amplified by PCR using pCB-EGFP: IP3R (7) as the template and incorporating XhoI sites at either end. This sequence was then ligated into the XhoI site in the linker region of mCY using standard methods. LIBRAAN was constructed in the same way using amino acids 227–604 of the rat type 3 IP3R (8). The forward PCR primers used were ACG CAT ACT CGA GAT GAG TGA AAT GTC CAG C for LIBRA and AAG CAT ACT CGA GGT TCC C for LIBRAAN. The same reverse primer, AGC GTA TCG CTA GCC GCT GTC GTC GCA G, was used for both PCR reactions. The correctness of all constructs was confirmed by restriction digestion and sequencing.

Cell Culture and Transfection—SH-SY5Y cells purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with low glucose (100 mg/ml), supplemented with 10% newborn calf serum, 584 mg/ml l-glutamine, 110 mg/ml sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were transfected with plasmids using LipofectAMINE 2000 (Invitro-
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Measurement of Fluorescence—SH-SY5Y cells were grown on fibronectin-coated glass cover slips incorporated into an experimental chamber (6). Cells were washed with HBSS-H and rested for at least 15 min prior to experiments. In some experiments, cells were loaded with fura-2 by incubation for 5 min at room temperature in HBSS-H containing 0.25–0.5 µM fura-2-AM (Dojin Chemicals, Kumamoto, Japan). Cells in HBSS-H or Ca²⁺-free HBSS-H (in which 1.3 mM CaCl₂ was replaced with 0.1 mM EGTA) were stimulated by exposure to various concentrations of acetylcholine (Ach) or 1 µM ionomycin as indicated.

Permeabilization was performed by exposing cells to ICM containing 100 µg/ml (w/v) saponin (ICN, Cleveland, OH) for ~1 min. Permeabilized cells were washed with HBSS and then exposed to HBSS containing various concentrations of IP₃ and other reagents.

Fluorescence images were captured using a dual-wavelength FRET-based imaging system (Hamamatsu Photonics, Shizuoka, Japan) consisting of a cooled CCD camera (HiSCA) and W-View optics coupled to a Nikon Diaphot 300 inverted fluorescence microscope equipped with a Nikon Fluor 40 oil immersion objective (NA 1.3). Fluorescence of LIBRA, LIBRA/N, and mCY were monitored with excitation at 425 nm and dual-emission at 480 and 535 nm. Simultaneous monitoring of LIBRA and fura-2 was performed with sequential excitation at 380 nm (for fura-2) and 425 nm (for LIBRA) and dual-emission at 480 nm (for LIBRA) and 535 nm (for LIBRA and fura-2). Data were analyzed with AQUACOSMOS software (Hamamatsu Photonics). [Ca²⁺] was calculated from the fura-2 fluorescence intensity, F, using the formula

\[ F_\text{ratio} = \frac{F_{\text{calc}}}{F_{\text{max}}} \]

where \( F_\text{calc} \) and \( F_{\text{max}} \) are the values of F at zero and limiting high [Ca²⁺], respectively (9). \( F_{\text{max}} \) was determined by assuming that the resting [Ca²⁺] was 50 nM; \( F_{\text{max}} \) was measured following the application of 1 µM ionomycin.

RESULTS AND DISCUSSION

We designed two potential IP₃ biosensors (Fig. 1a and see “Experimental Procedures”) consisting of the IP₃-binding domain of the rat type 3 IP₃R (8) fused between the well-established FRET pair CFP and YFP (10, 11) and preceded by the N-terminal 20 amino acids of neuromodulin (12), a plasma membrane-targeting signal. The first of these constructs, which we refer to as LIBRA contains residues 1–604 of the rat type 3 IP₃R, while LIBRA/N contains residues 227–604. The control molecule mCY lacks the IP₃-binding domain. Using confocal microscopy we found that these recombinant proteins localized to the plasma membrane and Golgi area when expressed in SH-SY5Y human neuroblastoma cells (data not shown); also ~90% of their fluorescence was retained by the cells after permeabilization with saponin (Fig. 1b).

We looked for FRET between CFP and YFP by exciting CFP at 425 nm and recording emitted fluorescence at 480 and 535 nm. When permeabilized SH-SY5Y cells transfected with LIBRA were exposed to 10 µM IP₃, an increase in the 480 nm signal (~4.5%) and a parallel decrease in the 535 nm signal (~3.6%) were observed within ~1 s (Fig. 1c). By contrast neither LIBRA/N nor mCY showed any detectable fluorescence changes in response to 10 µM IP₃ (Fig. 1d). The CFP donor quenching of LIBRA that was relieved by YFP acceptor photo-bleaching (13) was ~3.9% in the presence of 10 µM IP₃ and ~8.6% in its absence, confirming that the FRET efficiency of LIBRA is decreased in its IP₃-bound form. It has been demonstrated that residues 226–576 of the mouse type 1 IP₃R constitute the essential “core” region required for high affinity IP₃ binding and that the N-terminal 225 residues act to suppress the IP₃ binding affinity of the core (14). Interestingly, LIBRA/N, which lacks this N-terminal suppressor region, shows no IP₃-dependent changes in fluorescence. Accordingly we speculate that the conformational change underlying the IP₃-dependent LIBRA FRET signal could be related to an effect of IP₃ on the interaction between the core and suppressor regions.

Changes in the LIBRA 480/535 nm emission ratio (ΔRatio) were highly sensitive to [IP₃] and selective for 1,4,5-IP₃ versus other inositol phosphates (Fig. 2). ΔRatio had a monophasic dependence on [IP₃] with apparent dissociation constant (Kₐ) of 404 nM and Hill coefficient (n) of 1.118 (Fig. 2b). The Kₐ for adenophostin A, a more potent agonist for the IP₃R than IP₃ (15), was 65 nM and we estimate that inositol 1,3,4,5-tetrakisphosphate (IP₄), inositol 4,5-bisphosphate (IP₂), and inositol 1,3,4-trisphosphate (1,3,4-IP₃) bind to LIBRA with Kₐ values of ~15, ~40, and >10 µM, respectively (Fig. 2b). It is known that the activity of the IP₃R is modulated by Ca²⁺ (16, 17) and ATP (18), although their effects are not mediated by the IP₃-binding domain (5, 19). In control experiments we have confirmed that both Ca²⁺ (0–1 µM) and ATP (0–3 mM) have no effect on LIBRA fluorescence or on the ΔRatio seen in response to IP₃ (Fig. 2c). We also found that, while changes in pH affected the basal emission ratio as a result of the well known effects of pH on YFP fluorescence (13), the ΔRatio elicited by IP₃ was unaffected over the pH range 7.0–7.6 (Fig. 2d). Taken together the above results show that ΔRatio (at constant pH) is a sensitive and specific measure of [IP₃]ₐ.

We next used the ΔRatio of LIBRA to follow the dynamics of [IP₃]ₐ after muscarinic stimulation of intact SH-SY5Y cells (Fig. 3a). In the presence of extracellular Ca²⁺⁻, application of 10 or 100 µM Ach increased ΔRatio in >80% of LIBRA-expressing cells, and 1 µM Ach elicited a response in ~60% of the cells tested. Increasing [Ca²⁺], with ionomycin had no effect on ΔRatio (Fig. 3, a and c) and the application of 100 µM Ach caused no changes in fluorescence in either mCY-expressing...
cells or in cells loaded with the intracellular pH indicator BCECF (data not shown), confirming the specificity of the LIBRA response. The PLC inhibitor U73122 (20) completely blocked the Ach-induced increase in ΔRatio but had no effect on the resting emission ratio (Fig. 3b) suggesting that the resting [IP$_3$]$_i$ in these cells is below the detectable range of LIBRA.

The magnitude of the LIBRA ΔRatio response increased with [Ach] reaching a maximum 2–5 min after stimulation (Fig. 3, a and c). As discussed in more detail below the time scale of these responses is much longer than the onset of Ach-induced Ca$^{2+}$ spikes in this cell type (5–20 s). Peak ΔRatio values were similar in the presence and absence of extracellular Ca$^{2+}$ (Fig. 3c); however, significant differences were seen in the time courses of these responses (Fig. 3a). In the presence of Ca$^{2+}$, in 20 out of 32 responding cells, ΔRatio rose monotonically to a sustained maximum level, while in the remaining 12 cells ΔRatio rose to a peak then fell to a plateau level that was >50% of the maximal response. In contrast, in the absence of Ca$^{2+}$, only 9 out of 43 responding cells showed a sustained rise in ΔRatio in response to Ach; in the remaining 34 cells ΔRatio rose to a peak then fell to a value close to resting levels. In both experimental conditions the application of Ach typically elicited a rapid spike in [Ca$^{2+}$]$_i$ (monitored using fura-2), which then fell to a lower sustained level above base line in the presence of extracellular Ca$^{2+}$ and to base-line levels in its absence (data not shown). The sustained rise in ΔRatio in the presence of extracellular Ca$^{2+}$ and the transient rise in its absence (Fig. 3a) suggest a role for a sustained rise in [Ca$^{2+}$]$_i$ in maintaining increased [IP$_3$]$_i$. To look for effects of LIBRA expression on Ach-induced Ca$^{2+}$ release, we examined the latency for the onset of Ca$^{2+}$ spikes after Ach stimulation. Following the application of 1 μM Ach in the absence of extracellular Ca$^{2+}$ this latency was 16.7 ± 4.2 s (n = 11) in LIBRA-expressing cells and 18.9 ± 2.0 s (n = 24) in non-expressing cells. Since any buffering of intracellular [IP$_3$]$_i$ by LIBRA would have been expected to increase this latency (i.e. to delay the Ca$^{2+}$ spike), these results are consistent with the hypothesis that LIBRA expression has little if any buffering effect.
on intracellular [IP$_3$] levels under our experimental conditions.

To directly explore the relationship between the Ach-induced [IP$_3$], response and Ca$^{2+}$ release from intracellular stores, we monitored the fluorescence of both LIBRA and the [Ca$^{2+}$], indicator fura-2 simultaneously in the same cells in Ca$^{2+}$-free medium. In experiments where cells were exposed to two successive applications of a low [Ach], the second application of Ach consistently elicited a significant but smaller increase in [IP$_3$], than the first and was accompanied by little or no increase in [Ca$^{2+}$]. (Fig. 3d), consistent with previous observations that submaximal [IP$_3$] leads to a partial depletion of stored Ca$^{2+}$ (21). In contrast, in similar experiments in Ca$^{2+}$ containing medium both applications of Ach resulted in comparable increases in both [IP$_3$], and [Ca$^{2+}$], (data not shown). Interestingly, at Ach concentrations ≥3 μM (and in some instances at 1 μM) the peak of the [IP$_3$], response consistently occurred after the peak in [Ca$^{2+}$], so that [IP$_3$], was still rising as [Ca$^{2+}$], fell (Fig. 3, d and e). Similar results were observed in the presence of extracellular Ca$^{2+}$ (data not shown). At low [Ach] a subsequent application of a higher [Ach] resulted in additional Ca$^{2+}$ release (Fig. 3d) indicating that this temporal dissociation of the [IP$_3$], and [Ca$^{2+}$], responses is not due to the emptying of intracellular Ca$^{2+}$ stores. Additional experimentation will be required to clarify this interesting relationship between [IP$_3$], and [Ca$^{2+}$].

By assuming that the response of the LIBRA ΔRatio to IP$_3$ is the same in intact and permeabilized cells, one can convert the above LIBRA signals to [IP$_3$], using the concentration-response curve shown in Fig. 2b. Consistent with this assumption we have confirmed that the increase in ΔRatio due to microinjection of a supramaximal concentration of IP$_3$ into intact LIBRA-expressing cells is not significantly different from the maximal increase in ΔRatio due to IP$_3$ in permeabilized cells (data not shown). Thus we estimate that the threshold [IP$_3$], required to elic a Ca$^{2+}$ spike in SH-SYSY cells (ΔRatio − 0.008) is ~50 nM. This value is very close to the threshold concentration of photoreleased IP$_3$ (~60 nM) previously found to be required for triggering [Ca$^{2+}$], spikes in Xenopus oocytes (22). We also estimate that the peak [IP$_3$], achieved by 1, 10, and 100 μM Ach in SH-SYSY cells are ~100, 210, and 360 nM, respectively.

Our results demonstrate the utility of LIBRA to directly follow physiologically relevant changes in [IP$_3$], in intact mammalian cells in real time. LIBRA has several important advantages over a previous method for monitoring [IP$_3$], that employs the intracellular redistribution of a chimeric protein consisting of the pleckstrin homology domain (PHD) of PLC81 fused to GFP (GFP-PHD) (23). This probe was originally designed to detect changes in membrane PIP$_2$ levels (24, 25) but subsequently was also shown to be sensitive to [IP$_3$], (2, 23). More recent experiments have confirmed that GFP-PHD redistribution can reflect physiologically relevant changes in both IP$_3$ and PIP$_2$ and that its selectivity varies with GFP-PHD expression level (26, 27). In addition to these complexities, for technical reasons (26, 27) it has not been possible to quantitate the GFP-PH signal.

The temporal dynamics of G protein coupled receptor-mediated Ca$^{2+}$ responses have been shown to depend on the effects of cytosolic and luminal [Ca$^{2+}$] on IP$_3$Rs (1, 6, 17, 28) as well as on positive feedback loops to enhance IP$_3$ production (29). In addition, recent studies suggest the involvement of PKC and scaffolding proteins on IP$_3$ synthesis (30). We anticipate that the ability to quantitatively monitor [IP$_3$], with LIBRA and related ratiometric biosensors will aid in the clarification of the roles of these and other effects on the generation of spatiotemporally complex Ca$^{2+}$ signals.

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