PROCEEDING

Monitoring of IP₃ dynamics during Ca²⁺ oscillations in HSY human parotid cell line with FRET-based IP₃ biosensors

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Abstract: Inositol 1,4,5-trisphosphate (IP₃) is an intracellular messenger that elicits a wide range of spatial and temporal Ca²⁺ signals, and this signaling versatility is exploited to regulate diverse cellular responses. In the present study, we have developed a series of IP₃ biosensors that exhibit strong pH stability and varying affinities for IP₃, as well as a method for the quantitative measurement of cytosolic concentrations of IP₃ ([IP₃]ᵢ) in single living cells. We applied this method to elucidate IP₃ dynamics during agonist-induced Ca²⁺ oscillations, and demonstrated cell type-dependent differences in IP₃ dynamics; a non-fluctuating rise in [IP₃]ᵢ and repetitive IP₃ spikes during Ca²⁺ oscillations in COS-7 cells and HSY-EA1 cells, respectively. The size of the IP₃ spikes in HSY-EA1 cells varied from 10 to 100 nM, and the [IP₃]ᵢ spike peak was preceded by a Ca²⁺ spike peak. These results suggest that repetitive IP₃ spikes in HSY-EA1 cells are passive reflections of Ca²⁺ oscillations, and are unlikely to be essential for driving Ca²⁺ 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 IP₃ and Ca²⁺. J. Med. Invest. 56 Suppl.: 357-361, December, 2009

Keywords: inositol 1,4,5-trisphosphate, calcium, FRET, biosensor

INTRODUCTION

Inositol 1,4,5-trisphosphate (IP₃) is an important intracellular messenger produced by phospholipase C (PLC)-dependent hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PIP₂). IP₃ releases Ca²⁺ from intracellular stores via IP₃ receptors (IP₃Rs), and the resulting Ca²⁺ signals often exhibit complex spatial and temporal organizations, such as Ca²⁺ oscillations (1). The mechanism responsible for Ca²⁺ oscillations has been a long-standing question, and a number of experimental approaches and mathematical models have been reported to account for these Ca²⁺ signals, yet the mechanism responsible remains controversial (2). There are two general classes of Ca²⁺ oscillation. In one class, Ca²⁺ oscillations are generated in the presence of constant cytosolic IP₃ concentrations ([IP₃]ᵢ) (3); in the other class, oscillating [IP₃]ᵢ are required to drive Ca²⁺ oscillations (4).

In the present study, we developed a series of improved IP₃ biosensors which exhibit high pH stability and varying IP₃ affinities. They also possess higher selectivity and afford a larger dynamic range than that of the original biosensor LIBRA. In combination with these new biosensors, we developed a method for quantitating IP₃ dynamics in single living cells.
cells, and applied this method to measure IP$_3$ dynamics during Ca$^{2+}$ oscillations.

RESULTS AND DISCUSSIONS
Characterization of improved IP$_3$ biosensors

Fig. 1A shows schematic diagrams of IP$_3$-biosensor domain structures. To improve the pH-stability of the LIBRA emission ratio (480 nm/535 nm), EYFP (enhanced YFP) was replaced with Venus, a pH-stable YFP mutant (5). The resulting construct (LIBRAvIII) showed strong pH stability in the range of pH 6 to pH 8 (Fig. 1B). Next the LIBRAvIII ligand-binding domain was replaced with that of type 1 or type 2 IP$_3$R, to create high-affinity IP$_3$ biosensors. These constructs were designated LIBRAvI and LIBRAvII, respectively. It is known that changing Arg to Gln at position 441 (R441Q) increases the affinity of type 1 IP$_3$R (6). To further increase the affinity, comparable amino acid substitutions in type 2 and type 3 IP$_3$Rs (R440Q) were made in LIBRAvII and LIBRAvIII. We also generated IP$_3$-insensitive LIBRAv (LIBRAvN) by substituting Ala for Lys at position 508 (K508A) in the IP$_3$-binding domain of LIBRAvIII. These biosensors were distributed on the plasma membrane and in vesicular structures (Fig. 1C), and most of the associated fluorescence (>90%) was retained after permeabilization.

Concentration-response curves of the new IP$_3$ biosensors for IP$_3$, inositol 4,5-bisphosphate (IP$_2$) and inositol 1,3,4,5-tetrakisphosphate (IP$_4$) are shown in Fig. 1D. The apparent $K_d$ (nM) of LIBRAvIII,
LIBRAvI, LIBRAvII, and LIBRAvIIS for IP$_3$ were 491.5, 268.7, 233.6, and 117.2, respectively. A high concentration (10 μM) of IP$_2$ or IP$_1$ induced 20–40% of the maximal response of these biosensors. LIBRAvII and LIBRAvIIS exhibited greater selectivity than LIBRA, and the selectivity of LIBRAvIIS for IP$_3$ was 300-fold higher than for IP$_2$ and 96-fold higher than for IP$_4$.

The characteristics of our IP$_3$ biosensors include the use of Venus, ligand-binding domains of different IP$_3$R isoforms, and a membrane-targeting signal. The superior selectivity of IP$_3$R-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.

**METHODS FOR QUANTITATIVE MEASUREMENT OF [IP$_3$]$_i$**

Emission ratios of LIBRAvIII and LIBRAvIIS in intact cells increased upon the application of 10 μM ATP, and returned to basal levels after the addition of 5 μM U73122. To obtain the maximal changes in emission ratio in each individual cell, cells were permeabilized and exposed to IP$_3$ following the completion of measurements in intact cells.

The LIBRAvIII emission ratio did not change with U73122 treatment, whereas the LIBRAvIIS ratio decreased slowly to a new steady-state. These results indicate that the emission ratio of LIBRAvIIS reflects the resting [IP$_3$]$_i$, and U73122 decreases [IP$_3$]$_i$ to levels below the detectable range of LIBRAvIIS. Based on this idea, we used the U73122-induced decrease in emission ratios, $K_d$, and Hill coefficient of LIBRAvIIS (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 LIBRAvIIS in COS-7 and HSY-EA1 cells was 13.67 ± 0.96% (Mean ± S.E., $n = 60$) and 16.03 ± 1.53% ($n = 19$), respectively. The estimated resting [IP$_3$]$_i$ in COS-7 and HSY-EA1 cells was 15.09 ± 1.38 nM and 18.16 ± 2.49 nM, respectively (5).

**CHANGES IN [IP$_3$]$_i$ DURING AGONIST-INDUCED Ca$^{2+}$ OSCILLATIONS**

Resting [IP$_3$]$_i$ values allowed us to accurately calculate agonist-induced changes in [IP$_3$]$_i$. Figs. 2 shows changes in [IP$_3$]$_i$ during ATP-induced Ca$^{2+}$

![Figure 2](image-url)

**Figure 2.** IP$_3$ dynamics during Ca$^{2+}$ oscillations in COS-7 and HSY-EA1 cells. LIBRAvIIS-expressing cells were loaded with fura-2, and sequentially stimulated with various concentrations of ATP. (A) COS-7 cells showed no detectable fluctuation in [IP$_3$]$_i$ during ATP-induced Ca$^{2+}$ oscillations. Traces are representative of 27 oscillating cells. (B) HSY-EA1 cells showed repetitive IP$_3$ spikes during ATP-induced Ca$^{2+}$ oscillations. Traces are representative of 31 out of 55 oscillating cells. Traces show estimated [IP$_3$]$_i$ (black lines), excitation ratio of fura-2 (red lines). Black arrowhead, peak of IP$_3$ spike; red arrowhead, peak of Ca$^{2+}$ spike. Reproduced from ref. 5 with permission.
oscillations. In COS-7 cells, stimulation with either 1 μM or 3 μM ATP increased [IP₃]ᵢ slowly to a sustained level without detectable fluctuations, and Ca²⁺ oscillations were observed during this increase and sustained elevation of [IP₃]. (Fig. 2A). Unlike ATP-induced increases in [Ca²⁺]ᵢ, the large increase in [Ca²⁺]ᵢ caused by ionomycin (2 μM) treatment had no effect on the emission ratio of LIBRAvIIS, indicating that Ca²⁺ itself does not activate IP₃ generation in COS-7 cells (5).

We also examined IP₃ dynamics in HSY-EA1 cells (5). This cell line is characterized by long lasting Ca²⁺ oscillations in response to wide ranges of agonist concentrations (7). In fact, 65% of HSY-EA1 cells (55 out of 84 cells) exhibited Ca²⁺ oscillations following treatment with 3-100 μM ATP or 10-100 μM carbachol (CCh). Interestingly, 56% of Ca²⁺-oscillating HSY-EA1 cells (31 cells) showed associated fluctuations in [IP₃]ᵢ (Fig. 2B). Similar fluctuations in the LIBRAvIIS emission ratio were observed when IP₃ dynamics were examined in the absence of fura-2 loading (data not shown). In contrast, no change in emission ratio was observed during Ca²⁺ oscillations in LIBRAvN-expressing HSY-EA1 cells. These experiments exclude the possibility of result artifacts derived from IP₃-independent changes in LIBRAvIIS fluorescence and possible interference by fura-2 fluorescence. These results demonstrate cell type-specific differences in IP₃ dynamics; non-fluctuating rises in [IP₃]ᵢ and repetitive IP₃ spikes in COS-7 cells and HSY-EA1 cells, respectively. Quantitative examinations revealed that repetitive IP₃ spikes in HSY-EA1 cells occurred concomitantly with a slow basal accumulation of [IP₃]ᵢ. The size of IP₃ spikes varied from 10 to 100 nM, and the second and later spikes were initiated before the decline of [IP₃]ᵢ to resting levels, resulting in a slow increase in the [IP₃]ᵢ interspike.

Although repetitive IP₃ spikes were observed in HSY-EA1 cells, our observations do not support the requirement of IP₃ fluctuations in Ca²⁺ oscillations. While [IP₃]ᵢ showed clear fluctuations at the beginning of Ca²⁺ oscillations, repetitive spikes of [IP₃]ᵢ gradually obscured during Ca²⁺ oscillations in 64% of HSY-EA1 cells. In addition, the [IP₃]ᵢ spike peak was preceded by a Ca²⁺ spike peak (Figs. 2B). These results suggest that repetitive IP₃ spikes in HSY-EA1 cells are passive reflections of the Ca²⁺ oscillations, and are unlikely to be essential for driving Ca²⁺ oscillations.

[IP₃]ᵢ fluctuations could be induced by the effects of Ca²⁺ on IP₃ synthesis and/or IP₃ degradation. Applications of 2 μM ionomycin had little or no effect on the emission ratio of LIBRAvIIS in HSY-EA1 cells, suggesting that the direct effect of Ca²⁺ on IP₃ production is very small or absent in this cell line. Thus, [IP₃]ᵢ fluctuations are likely to be caused by Ca²⁺-induced potentiation of agonist-dependent IP₃ generation. The IP₃ spikes described here resemble the pattern predicted by the oscillator model, in which positive feedback via Ca²⁺-dependent activation of PLC is added to the dual positive and negative feedback effect of Ca²⁺ on IP₃-R, rather than the model including negative feedback via Ca²⁺-dependent IP₃-degradations by IP₃-3-kinases (8).

Ca²⁺ oscillations were observed primarily when [IP₃]ᵢ was less than 300 nM. More than 50% of COS-7 cells exhibited Ca²⁺ oscillations when [IP₃]ᵢ was 50-100 nM, and the percentage of oscillating COS-7 cells decreased as [IP₃]ᵢ increased. In HSY-EA1 cells, Ca²⁺ oscillations were observed in 50-70% of cells, when interspike [IP₃]ᵢ was less than 250 nM, and the percentage of oscillating cells decreased abruptly (11.1%) when interspike [IP₃]ᵢ was greater than 250 nM. These results indicate that low concentrations of IP₃ (< 100 nM) induce Ca²⁺ oscillations in both cell types, while HSY-EA1 cells are more likely to exhibit Ca²⁺ oscillations at higher concentrations of [IP₃]ᵢ (100-250 nM) than are COS-7 cells. Large increases in [IP₃]ᵢ (> 250 nM) induced the peak-plateau type Ca²⁺ response in both cell types.

The present study showed the time delay of IP₃ spike peaks from Ca²⁺ spike peaks in HSY-EA1 cells, and Ca²⁺ oscillations with non-fluctuations of [IP₃]ᵢ in COS-7 cells. Together these results suggest it is likely that IP₃ spikes are not essential to drive Ca²⁺ oscillations. Regarding the mechanism of Ca²⁺ oscillations, the importance of dual feedback effects of Ca²⁺ on IP₃-Rs has been demonstrated experimentally (9-11). However, it has been pointed out that this dual feedback effect explains relatively short period Ca²⁺ oscillations but it cannot reproduce long interspike intervals. Thus, additional mechanisms responsible for establishing these oscillations remain unclear. One model study reported that frequency properties of oscillation are modulated by the incorporation of Ca²⁺ activation of PLC into the Ca²⁺ oscillation model based on dual feedback regulations of IP₃-R properties, which enhances the range of frequency encodings of agonist stimulations (8). Interestingly, HSY-EA1 cells exhibited Ca²⁺ oscillations for a wider range of agonist and [IP₃]ᵢ concentrations than observed in COS-7 cells. Thus, it may be possible that repetitive IP₃ spikes or fluctuations play
some role in supporting and/or regulating Ca\textsuperscript{2+} oscillations. The quantitative information provided here would be useful for future studies of the mechanisms and roles of IP\textsubscript{3} oscillations.

REFERENCES

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