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A novel luminescent biosensor for rapid monitoring of IP_3 by split-luciferase complementary assay

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ABSTRACT

Inositol 1,4,5-trisphosphate (IP_3) is a crucial second messenger that regulates complicated signaling processes in various physiological events. Alteration in its content has been observed in many diseases. Hence, development of a high-throughput screening system to monitor temporal changes of IP_3 is essential for screening of new potential therapeutic compounds. Toward a simple, sensitive and rapid method for measuring IP_3, we describe the development and application of a novel biosensor based on luciferase fragment assisted complementation strategy, which converts the ligand-induced conformational changes to light. Designed sensor comprising the IP_3-binding core domain of IP_3-receptor fused between complementary non-functional fragments of firefly luciferase allows direct detection of IP_3 in presence of luciferin substrate both in cell lysate and in living cells. According to the result presented in this manuscript, the screening time was very fast and maximum response was obtained up to 11-fold higher than untreated cells. Moreover, the designed biosensor was able to monitor release of IP_3 upon induction by different inducers like Bradykinin and ATP. The current biosensor not only provides a specific IP_3 detector *in vitro* but also facilitates monitoring of the response of IP_3 in living organisms.

1. Introduction

D-*myo*-inositol 1,4,5-trisphosphate (IP_3) is a key regulatory molecule involved in cell signaling, which has been discovered for the first time since studies on fluid secretion by an insect salivary gland and its controlling pathway (Berridge and Patel, 1968). Taking a broader view, in cellular response to many biological stimuli such as hormones, neurotransmitter and growth factors, IP_3 is generated along with 1,2-diacylglycerol (DAG) following the activation of either G protein-coupled receptors and phospholipase C-β (PLC-β) isoforms or receptor tyrosine kinases and PLC-γ isoforms, that are located on the cell membrane. Whilst DAG stays inside the membrane and activates protein kinase C (PKC), IP_3 is soluble and diffuses through the cell, binds to and activates the intracellular receptors (IP_3 Receptor: IP_3R) located on the surface of intracellular Ca^{2+} stores. Upon activation and opening of Ca^{2+}-channels, Ca^{2+} is released into the cytoplasm. As a ubiquitous second messenger, IP_3 plays a vital role in the control of a large number of cellular processes (Furuichi et al., 1989; Berridge and Dupont, 1994), hence, defect of signaling pathways brings about a broad range of metabolic disorders and diseases (Weber, 2005). Because of great importance of local spatial compartmentalization of intracellular signaling, the ability to measure IP_3 in living cells would be highly beneficial in unraveling IP_3-dependent signaling. Therefore, sensitive, simple and rapid methods for the direct detection of cellular IP_3 are crucial.

Dynamic molecular interaction analysis within the cellular architecture depends on the ability to quantify and image fundamental biological processes at the cellular and subcellular levels (Ozawa, 2006). Development of methods for illuminating key components of the cellular networks requires the application of sophisticated tools. One novel approach for creating such powerful tools relies on structurally engineering reporter proteins to create intracellular sensors. The signal emitted by these sensors is regulated by their interaction with cellular components (Binkowski et al., 2009). One of the important procedures, which has been developed by several groups in the past decade, is the use of split-protein reassembly (also called protein-fragment complementation), wherein initially non-functional fragments of split protein are induced to reassemble through the specific interactions (Luker et al., 2004; Ozawa, 2006; Binkowski et al., 2009; Torkzadeh-Mahani et al., 2012). For the first time, split-protein reassembly was reported on ubiquitin which showed reassembly of fragments upon interaction to potential partners (Johnsson and Varshavsky, 1994). Split protein strategy has subsequently been applied to a variety of monomeric reporter

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proteins including dihydrofolate reductase (Pelletier et al., 1998), β-lactamase (Galarneau et al., 2002), GFP (Hu and Kerppola, 2003), renilla luciferase (Paulmurugan and Gambhir, 2003), gaussia luciferase (Remy and Michnick, 2006), firefly luciferase (Furman et al., 2011), Trp1p (Tafelmeyer et al., 2004), TEV protease (Wehr et al., 2006), thymidine kinase (Massoud et al., 2010), and recently chorismate mutase (Muller et al., 2010). Firefly luciferase is one of the most promising reporter proteins that have been validated for split-protein strategies because of their applicability in imaging of living animals. Moreover, it requires no high-energy excitation source because light emission results from an endogenous chemical reaction, and thus does not suffer from background noise and tissue damage in signal measurements (Contag and Bachmann, 2002).

To develop a biological sensing system for IP_3, a motif that recognizes the three asymmetrically substituted phosphate groups on the *myo*-inositol backbone is needed. According to a 2.2 Å crystal structure of the IP_3-binding core domain (residues 224–604) of mouse type 1 IP_3R (IP_3R1) in complex with its ligand, a clam-like structure with IP_3 nestle between two domains was observed (Bosanac et al., 2002; Lin et al., 2011). Upon ligand binding, it seems that the protein undergoes a conformational change that brings the two lobes together. However, no functional assay is reported to support this conformational displacement in IP_3R.

Here we report a new IP_3 biosensor based on mouse IP_3R1 crystal structure using split firefly luciferase (FLuc) complementation assay. Toward this goal, based on sequence homology, IP_3-binding core (IBC: residues 224–604) domain of human IP_3R2 that contains all structural determinations reasonable for specific and selective binding of IP_3 ligand with higher affinity than the other isoforms (Chan et al., 2007; Iwai et al., 2007) was used to design an intramolecular sensor which converts the IBC conformational changes to bioluminescence signal: light. This is the first report on an IP_3 detection approach based on bioluminescence and its potential to provide a simple, highly sensitive and extremely rapid assay platform, which enables the study of IP_3 in living cells and a lysate environment that approximates a complex *in vivo* setting.

2. Material and methods

Details on preparation of RNA, reverse transcription (RT)-PCR, PCR, cloning of IP_3R, and luciferase complementary assay in cell lysate and in living cells are provided in Supplementary Data.

2.1. Construction of plasmid

The initial ligand binding domain region of IP_3R was modified by PCR to introduce a unique sequence of linker $(GGGGS)_2$ and a unique enzyme site, *Not*I, to the C-terminus of IBC using reverse primer: 5′ GTAGCGGCCGCGCTACCTCCACCTCCACTTCCGCCACCAC-CTTTTCTGTTGTTGTGC 3′, with the same enzyme site, *BamH*I, in the N-terminus using forward primer: 5′ GCTGGATCCATGAAATA-TAGTTCCTATCG 3′. The cDNA was subcloned into the expression vector pcDNA3.1(+) at the unique enzyme sites *BamH*I and *Not*I. The N-terminal (NLuc: 1–1248 bp) and C-terminal (CLuc: 1194–1650 bp) fragments of FLuc were amplified from pGL3-Control (Promega) with appropriate primers. The NLuc fragment with a Kozak consensus sequence and initiating ATG codon for proper initiation of translation with the *Nhe*I site in the N-terminus was amplified using forward primer: 5′ CTAGCTAGCATGGAAGACGC-CAAAAAC 3′. The linker $(GGGGS)_2$ is connected to the C-terminus of NLuc using reverse primer: 5′ CATGGATCCAGAACCTCCACCTC-CACTTCCGCCACCACCATCCTTGTCAATCAAG 3′. The CLuc fragment was also amplified to add a unique enzyme site, *Not*I, at its N-terminus and *Xho*I at its C-terminus end by forward: 5′ CTA-GCGGCCGCCCTATGATTATGTCCGGTTATG 3′ and reverse: 5′GATC-TCGAGTTACACGGCGATCTTTCC 3′ primers.

The NLuc and CLuc purified fragments were then digested by unique enzymes and ligated to the DNA of IBC in digested/dephosphorylated pcDNA3.1(+) vector harboring gene, respectively (Fig. 1). Thereafter, the ligation product (pcDNA-NLuc-IBC-CLuc) was transformed into *E. coli* DH5α competent cells and screened by antibiotic (100 μg/ml ampicillin) selection. Positive clones were selected by colony PCR and double digestion and then sequenced (Macrogen, Korea) to ensure inserted DNAs.

2.2. Cell culture and transfection

Human embryonic kidney (HEK293T) cells were plated into 24-well dishes and grown in DMEM (Invitrogen) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin solution (Invitrogen) at 37 °C in 5% CO_2. Transient transfection was performed using the calcium phosphate method in 70–80% confluent cultures. Transfection mixture for a 24-well plate was prepared by gently mixing 25 μg of plasmid containing construct, 62.5 μl of sterile $CaCl_2$ and total volume of the above solution was adjusted to 500 μl with sterile water, then 500 μl of sterile 2X HBS (50 mM HEPES (pH 7.05)/10 mM KCl/12 mM Dextrose/280 mM NaCl/1.5 mM Na_2HPO_4) was added drop-wise under gentle agitation. The solution was mixed and applied to each well as mentioned in Supplementary Data.

3. Results

3.1. Design of a split luciferase sensor for detecting IP3-induced intramolecular folding

Based on crystal structure of IBC of mouse IP_3R1 a split-luciferase biosensor was designed. To create a good performance sensor, combination of theoretical data together with empirically driven structure is required. The general and logical scheme for development of an intramolecular sensor for monitoring ligand-induced conformational changes consists of fusing the ligand-binding domain between complementary non-functional fragments of a reporter protein. Several factors need to be evaluated to achieve efficient ligand-induced split protein reassembly. Key factors include: (I) the distance between the complementary fragments before and after the ligand binding, (II) the position of reporter protein fragments in recovering the ligand-binding structure via complementation after binding of the ligand, and (III) the orientation of complementary fragments after the binding of the ligand (Paulmurugan and Gambhir, 2006; Kim et al., 2007a, 2007b). By carefully considering these crucial factors along with comparison between the dissection sites of split firefly luciferase fragments, which was reported (Luker et al., 2004; Paulmurugan and Gambhir, 2005; Misawa et al., 2010), it was found that the

Fig. 1. Diagram of the intramolecular sensor cDNA construct. Schematic structure of the intramolecular sensor construct with the split FLuc fragments (NLuc and CLuc) on either side of the IBC (NLuc–IBC–CLuc) which was used to distinguish IP_3 using ligand-induced intramolecular folding-based FLuc complementation.

best complementary pairs are from 1 to 416 amino acids as N-terminal fragment and from 395 to 550 amino acids as C-terminal fragment. Furthermore, based on crystal structure of IBC, which has been shown to possess a clam-like structure with high affinity for IP_3 (Bosanac et al., 2002; Lin et al., 2011), it would be ideal to produce an intramolecular probe for monitoring ligand interaction.

According to the known information, the construct span of the receptor ligand binding core encompassing the 224–604 residues of human IP_3R2 was therefore utilized in a designed intramolecular split luciferase reassembly system, in which the luciferase NLuc half is attached to the N-terminus of IBC, and the other half of luciferase (CLuc) is attached to the C-terminus of IBC (NLuc–IBC–CLuc). The flexible linkers $(GGGGS)_2$ were used to join the NLuc and CLuc halves to the IBC (Figs. 1 and 2). This linker was chosen due to its flexibility and this length of linkers is empirically observed to be sufficient to allow assembly of reporter protein fragments (Remy and Michnick, 2001).

3.2. Monitoring of IP_3-induced intramolecular probe complementation in vitro

To test ligand-induced intramolecular-folding assisted FLuc complementation activity, the pcDNA–NLuc–IBC–CLuc vector was transiently transfected in the HEK293T cells, incubated for 12–72 h, and thereafter the cells were harvested and lysed to monitor protein expression. As an initial validation of the newly designed sensor, cell extracts were treated with 1 μM IP_3 and the FLuc activity was measured at (0 h), 12, 24, 48 and 72 h after transfection using luciferase assay substrate. As it is obvious in Fig. S-1 (Supplementary Data), no signal was detected from cell extracts before (0 h) and 12 h after transfection. At 24 h and 48 h after transfection, the luminescence signal was significantly increased compared to untransfected cells. No significant increase was observed after 72 h compared to 48 h. Therefore, 48 h post transfection was chosen for further experiments.

Although the results revealed efficient ligand-induced intramolecular folding and FLuc reconstitution with different levels of signal in a time-dependent manner, background luminescence was observed in the absence of ligand. To determine the background luminescence signal, each half of luciferase that binds to IBC alone (NLuc–IBC and IBC–CLuc) was transfected in HEK293T cells independently, and then luciferase activity was assessed. As shown in Table 1, intact luciferase (control plasmid (pGL3)) produced a maximal luminescence of 1.0×10^7 RLU/s/10^5 cells, about 15-fold greater than NLuc–IBC–CLuc (6.7×10^5 RLU/s/10^5 cells), while for NLuc–IBC and IBC–CLuc, it was 3.5×10^2 and 3.0×10^2 RLU/s, respectively, which were much lower than a signal obtained by intact luciferase (pGL3) and also complete construct (NLuc–IBC–CLuc). In fact, NLuc–IBC or IBC–CLuc produced no detectable luminescence relative to untransfected cells under maximal transfection conditions. In addition, in cells expressing complete construct, a detectable background luminescence was observed before IP_3 addition (7.0×10^4 RLU/s) which was approximately 9.5-fold less than emission signal generated after IP_3 addition (Table 1).

3.3. Kinetics of IP3-induced intramolecular FLuc complementation

To determine the kinetics of ligand-induced FLuc reassembly, the lysate from HEK293T cells expressing the fusion protein NLuc–IBC–CLuc was assayed at 0, 5, 30, 60, 120 and 300 s, after exposure to IP_3 (1 μM). As shown in Fig. 3A, the ligand interaction led to FLuc complementation because of extremely stronger signal than that of untransfected cells, also remarkably higher than control (0 s). Total activity measured in 5 s after IP_3 incubation was found to be about 9.5-fold stronger than the background signal in the absence of IP_3 (0 s). Although this fusion probe exhibited a significant signal-to-background ratio, no significant increase in FLuc activity was observed in longer exposure times. Therefore, the time-response data demonstrates that longer incubation time with inducer (IP_3) is not required.

Furthermore, to study the ligand concentration-dependent FLuc folding kinetics of the NLuc–IBC–CLuc fusion based system, equal amounts of the cell lysate were exposed to the different ligand concentrations ranging from 1 nM to 100 μM, and then

Table 1
Comparison of the luciferase activities (RLU/s) of various constructs in HEK293T cells.

	[-IP3]	[+IP3]	Fold induction
pGL3	1.0×10^7 RLU/Sec	1.0×10^7 RLU/Sec	1.0
NLuc–IBC–CLuc	7.0×10^4	6.7×10^5	9.5
NLuc–IBC	2.4×10^2	3.5×10^2	1.45
IBC–CLuc	2.2×10^2	3.0×10^2	1.36
Control	1.5×10^2	1.6×10^2	1.0

HEK293T cells were cultured in 24-well plates and transfected with 1 μg each of plasmid; HEK293T cells with no plasmid transfected was taken as their control. The cells were incubated for 48 h and luminescence was measured. The signal obtained by NLuc–IBC–CLuc is about 9.5-fold greater than background.

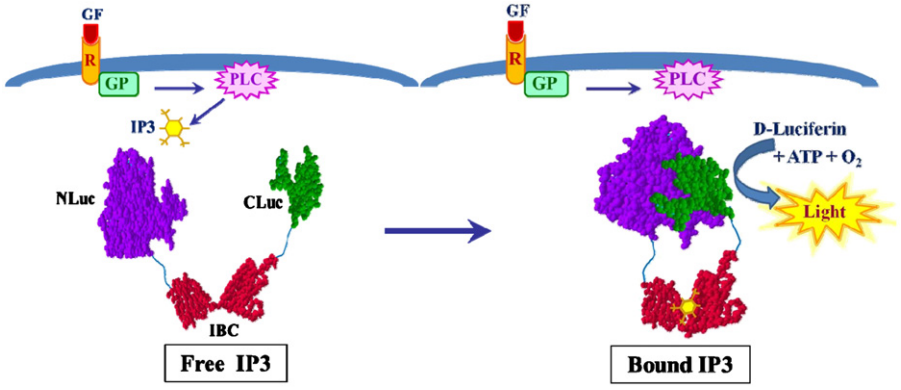

Fig. 2. Schematic strategy of IP_3 interaction via detection of FLuc complementary assay. The NLuc and CLuc fragments of split firefly luciferase were fused to the N- and C-terminals of IBC, respectively. In free IP_3, the intramolecular sensor is present as open conformation. In cellular response to many biological stimuli such as growth factor (GF), IP_3 is generated following the activation of G-protein(GP)-coupled receptors (R) and then phospholipase C (PLC), that are located on the cell membrane. IP_3 is soluble and diffuses through the cell, which binds and induces the IBC conformational changes and brings split luciferase into closer proximity and restores luciferase activity.

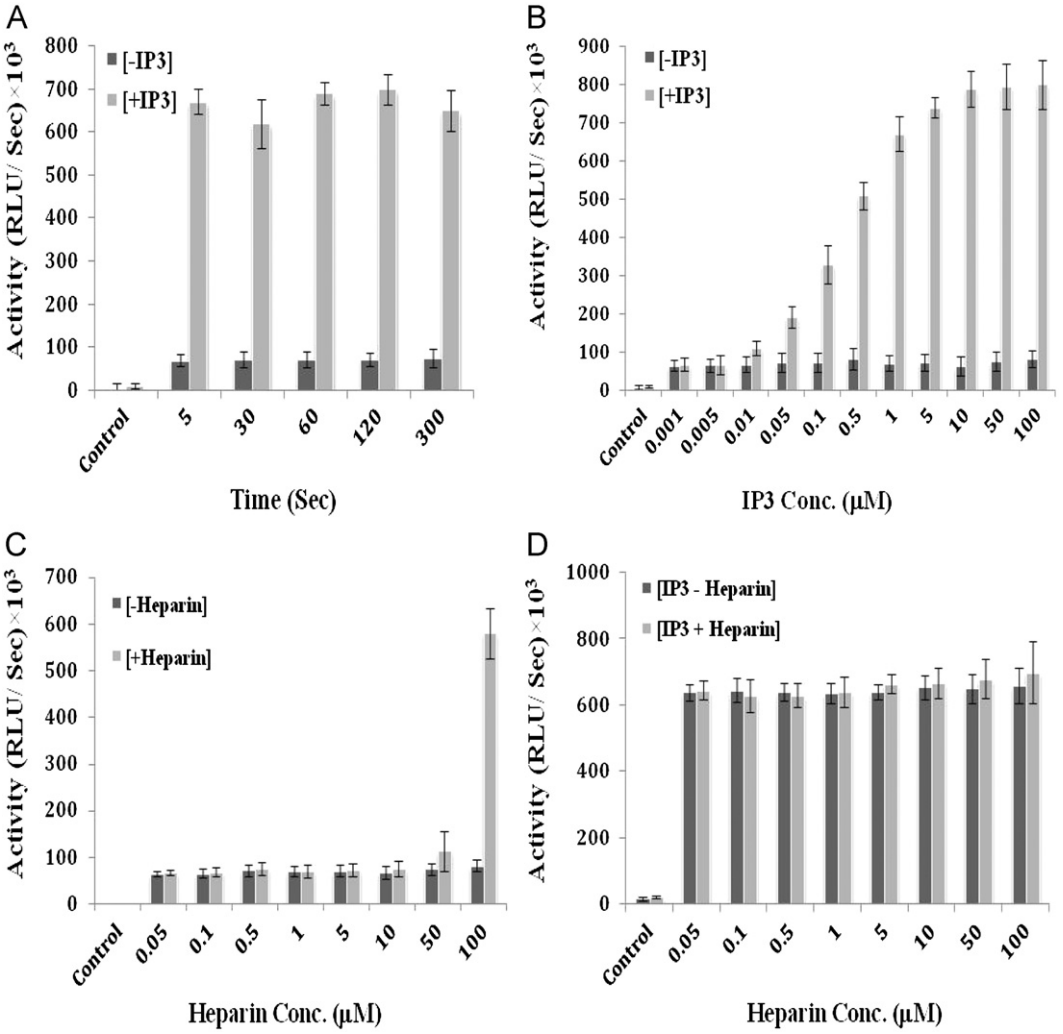

Fig. 3. Determination of the relative luciferase activity of the intramolecular FLuc system in response to ligand. (A) Time course and (B) dose–response curves for IP_3 effect on the fusion protein folding based on the luminescence intensity in HEK293T cell lysate harboring reconstituted protein. (C) Effect of heparin on the luminescence intensity of the intramolecular system in the lysate from transfected cells in the absence, and (D) in the presence of 10 μM IP_3. (Control: nontransfected cells), (mean ± SD from three independent experiments).

FLuc activity was determined. A dose-dependent increase in FLuc activity was observed, over a range from 10 nM to 10 μM of IP_3. The maximum level of ligand-induced FLuc complementation at 10 μM was obtained which is 11.0-fold higher than the background signal. No detectable changes in the luciferase activity were observed at concentrations lower than 10 nM and no further increase was also observed upon treatment to higher concentrations of 10 μM IP_3 (Fig. 3B).

3.4. *Specificity of intramolecular FLuc probe for ligand recognition*

The IP_3Rs are highly selective for the 1,4,5-isomer of inositol phosphate, displaying very little cross-reactivity with the other inositol phosphates or related compounds (Worley et al., 1987; Remus et al., 2006). Using the developed intramolecular FLuc probe, it was examined to confirm that the intensity of photons emitted by the probe protein precisely reflected the level of D-myo-inositol 1,4,5-trisphosphate (IP_3) in the cell extract. In this manner, various concentrations of the different inositol phosphates and ATP (as analyte with three phosphates) were added to the cell extract expressing the FLuc fusion protein and the luciferase activity was measured. As predicted, no significant cross-reactivity was observed (data not shown), confirming the specificity of the intramolecular FLuc reporter system for 1,4,5-isomer. In fact, these results indicate that a direct specific ligand interaction would be necessary to promote FLuc complementation assay.

3.5. *Effect of heparin, an IP_3R antagonist, on induction of intramolecular FLuc complementation*

To further establish the functionality of the reporter construct, the transfected cell lysate was subjected to a test to determine whether it could be effectively utilized for reporting of inhibitors against IP_3Rs. Heparin is a well-known potent competitive inhibitor of the IP_3Rs that inhibits IP_3 binding to receptor as well as release of Ca^{2+} from intracellular stores (Kobayashi et al., 1988). Toward this goal, and under the conditions optimized for IP_3-mediated FLuc complemented reassembly, the small molecule inhibitor was present at a concentration from 50 nM to 100 μM in the cell lysate to allow the identification of its effects.

Heparin unlike IP_3 increased the luminescence signal in an eventual high concentration (50 μM). No significant luciferase activity was observed upon addition of lower concentrations of inhibitor to the cell lysate. However, a large increase in luciferase activity at 100 μM heparin was obtained which is almost equal to maximal effect of IP_3 (Fig. 3C). On the contrary, addition of various

concentrations of heparin to cell lysate pre-incubated with effective IP_3 concentration (10 μM) did not bring about any change in complementary assay signal (Fig. 3D).

3.6. Monitoring of IP_3-induced FLuc complementation in living cells

Bradykinin, a vasoactive peptide (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg), is a biologically active hormone of the kinin family, which stimulates many cellular functions such as phosphatidyl-inositol turnover, through a mechanism requiring the influx of extracellular calcium and activation of phospholipases followed by hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) and formation of IP_3 (Lambert et al., 1986; Bascands et al., 1991).

To check out the ability of this intramolecular sensor to monitor the endogenously released IP_3 on specific drug treatment, the selected cell line transfected with NLuc–IBC–CLuc was monitored for IP_3 levels during Bradykinin stimulation by the complementary FLuc activity with *in vitro* cell-based assay. The response time after stimulation is important for high-throughput drug screening. Hence, the intact transfected cells were investigated after treatment with 1 μM Bradykinin over time (for 0, 15, 30, 60, 120 and 300 s); then cells were harvested, and their lysate assayed to monitor the recovery of the luminescence. As it is shown in Fig. 4A, a significant level of photon emission was observed as a result of the ligand interaction and subsequent intramolecular FLuc reassembly that confirmed release of IP_3 into target cells. Compared with untreated transfected cells, Bradykinin induced approximately 8-fold increase in the luciferase activity within 30 s after its addition, and thereafter the signal declined back to base-line level gradually. Although the background signal was observed in the absence of Bradykinin, the maximum signal in the presence of Bradykinin was found to be efficiently stronger (8-fold) than background.

In order to obtain response curve to Bradykinin, its various concentrations were tested on IP_3 release and monitored by luciferase recovery assay. The transfected cells were harvested after 48 h and stimulated with increasing Bradykinin concentrations from 1 nM to 10 μM for 30 s; next the cell lysate was prepared and luciferase activity was measured. As indicated in Fig. 4B, no change in signal was observed at concentrations lower than 10 nM. The maximum response in the presence of 1 μM Bradykinin was 8-fold stronger than that in the absence of Bradykinin. No further increase in FLuc activity was observed upon addition of higher concentrations of Bradykinin. Another interesting result related to this study was the effect of Bradykinin on inducing of IP_3 release within whole living cells which was confirmed by recovery of luciferase activity in intact cells (without cell disruption) by incubation with luciferin.

3.7. Intramolecular FLuc complementation in response to ATP treatment in living cells

It has been reported that binding of extracellular signals such as hormones increases intracellular IP_3 level within 20–30 s after induction which then declines as part of the IP_3 pathway within the cell (Tanimura et al., 2009). To confirm the time course of IP_3 induction levels and in agreement with Badykinin data, the response of NLuc–IBC–CLuc transfected HEK293T cells to an IP_3 liberating agonist (ATP) was also examined that is explained under Supplementary Data. Interestingly, the FLuc activity increased rapidly within 30 s upon the application of 10 μM ATP on whole cells, whereas it returned to basal level 5 min after treatment (Fig. 4C). To validate the effects of ATP on IP3 production in whole cells, the cell lysate harboring NLuc–IBC–CLuc proteins that was not treated with ATP was analyzed separately;

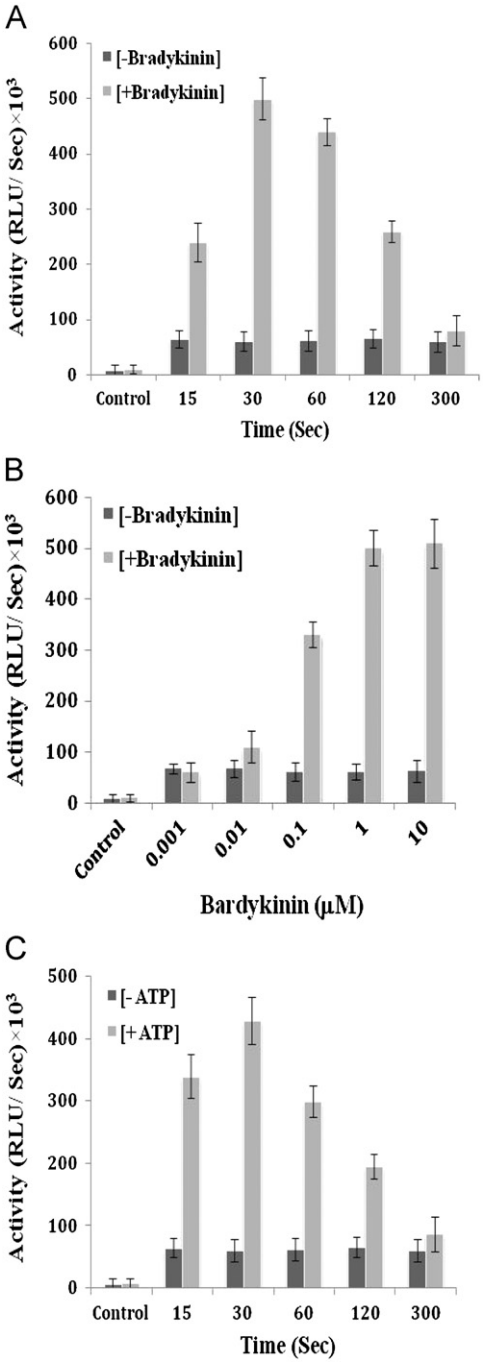

Fig. 4. Validation of the intramolecular FLuc sensor for detection of endogenously stimulated production of IP_3. The intact HEK293T cells carrying the fusion protein (A) stimulated with a fixed concentration of Bradykinin (1 μM) for various times, (B) treated with various concentrations of Bradykinin for an exact time (30 s), and (C) incubated with 10 μM ATP for different time periods as indicated; the resulting luminescence intensities were determined, (Control: nontransfected cells), (mean ± SD from three independent experiments).

the luciferase activity showed no difference between the presence and absence of ATP.

4. Discussion

Knowledge of the dynamic molecular processes within living cells has brought recently increased efforts toward structurally engineered reporter proteins to serve as intramolecular biosensor

for cellular and molecular imaging. In order to create a good performance sensor for IP_3 monitoring a rational design was employed. IBC of the human IP_3R2 that consists of all specific binding sites of IP_3 was selected as a ligand binding domain known to undergo conformational changes. The NLuc of firefly luciferase is fused to the N-terminus of IBC and its CLuc is attached to the C-terminus of IBC (NLuc–IBC–CLuc). A flexible peptide linker $(GGGGS)_2$ was inserted between the fragmented luciferase and the IBC to assist in proper protein folding and FLuc complementation (Figs. 1 and 2). By this design, it seems that light output is modulated upon binding of IP_3, presumably due to conformational changes in the IBC that influence subsequently on the efficiency of folding of the single-chain fusion protein. In this way, the functional relevance of the ligand binding/intramolecular probe folding was evaluated in terms of its modulator role on luciferase activity through *in vitro* incremental addition of IP_3 and ensuing effects on IP_3 production by living cells stimulation.

In order to test our designed biosensor, HEK293T cells were transiently transfected with either NLuc–IBC–CLuc or NLuc–IBC and IBC–CLuc separately as specificity controls to detect changes in the fusion protein structural folding in response to ligand binding. As a primary test, cell lysate was collected to measure luciferase activity as a function of ligand interaction. As demonstrated in Table 1, transfection of control constructs did not result in detectable luminescence recovery compared to untransfected cells, indicating that the luciferase fragments alone have no remarkable activity, while transfection with both luciferase fragments in the same molecule (NLuc–IBC–CLuc) brought about a high level of signal to background ratio. The overall signal obtained was sufficiently greater than background luminescence obtained in the absence of IP_3, for which we speculate that the noise may possibly arise from an increase in assembling split-luciferase halves in the expression system, suggesting that composition and length of the linkers joining the protein fragments may be effective in the efficiency of reassembly. Moreover, the background signal might be also originated from overlapping the luciferase fragments of amino acids 395–415 that has been previously reported (Luker et al., 2004; Misawa et al., 2010). Alternatively, this result might indicate that other processes (e.g. oligomerization of the fusion protein, thereby reconstituting luciferase activity) may occur rather than just a simple conformational change. Moreover, as IP_3R is a calcium-dependent protein, its proper refolding and function may be dependent on calcium presence. Reconstituted split luciferase activity in the presence of Ca^{2+} was brought about without effect on activity upon induction with IP_3 while its background level was increased (data not shown).

Time–response data (Fig. 3A) demonstrates that longer incubation time with IP_3 did not affect luciferase activity. Therefore, these results suggest that the clam-like IBC undergoes immediate conformational changes toward FLuc complementation and signal production upon IP_3 binding. In addition, cell lysate assay showed that the luminescence signal increased concomitantly with the increased concentration of the ligand; the maximum response was obtained at 10 μM IP_3. However, no further increase in luminescence was observed at higher concentration of IP_3 (Fig. 3B). In fact, the detection range of this luminescent biosensor was observed over a linear range from 10 nM to 10 mM of IP_3. It may be concluded that the lack of higher signal is probably due to the saturation of split-reporter protein with the IP_3 ligand. Therefore, if it had not been for this concentration limitation, higher luminescence intensities would have been obtained in the presence of the designed reporter. Similar dose-dependent inhibition has been reported for a FRET-based assay of IP_3 (Tanimura et al., 2009). Based on the time and dose kinetics data, it is worth commenting briefly on availability of the intramolecular FLuc probe for possessing unique properties of rapid response of bioluminescence to ligand interaction within the physiological concentration range and strong signal to discriminate from the background signal.

The effect of a competitive inhibitor of IP_3Rs, Heparin, on binding to IBC revealed its ability in induction of conformational changes at its saturating concentration which is much higher than that reported for IP_3. No concentration dependent increase of activity of split luciferase was observed upon addition of heparin to cell lysate (Fig. 3C). The role of Heparin in the presence of IP_3 was also examined which did not show binding at high concentration of IP_3 (Fig. 3D). These results have disproved the idea that heparin act primarily on the specific IP_3-binding site; nonspecific effects appear at high concentrations (Nezu et al., 2006).

To validate the selectivity of this sensor for its ligand (1,4,5-isomer), application of various inositol phosphate derivatives showed high specificity and selectivity (data not shown). This is in accordance with those reported evidences that indicate specificity and selectivity of intracellular IP_3Rs for IP_3 (Remus et al., 2006).

To assess chemical and pharmacological effects of IP_3 modulation in living cells, the designed intramolecular sensor was used to investigate cellular process dynamics for liberating of this key player in signaling cascades. As illustrated in Fig. 4, kinetics data demonstrate that the intramolecular FLuc complementary assay is time and dose dependent. Stimulation of intact cells released a rapid increase in intensity of luminescence signals upon incremental addition of Bradykinin (Fig. 4B). The time to obtain maximal luminescence was approximately 30 s after stimulation with chemical compounds (Bradykinin and ATP), thereafter the intensity of the signals gradually decreased to the background level (Fig. 4A and C). No difference between time of IP_3 release and concentration of inducers was observed with those reported earlier (Bascands et al., 1991; Lambert et al., 1986). Therefore, it may be suggested, 30 s is the most suitable time for high throughput screening of the chemical compounds and drug candidates in accordance with the intended use. Taken together, results obtained of the quantitative quality from living cells demonstrate the applicability of this sensor to resolve the temporal nature of agonist-induced IP_3 generation. In fact, living cell experimental results support the view that this intramolecular IP_3 probe can be potentially used for monitoring endogenous IP_3 upon release by specific induction.

Another interesting finding of the current study was the ability of the designed split luciferase system to monitor IP_3 from living cells without cell disruption. Induction of IP_3 release from living cells with Bradykinin brought about an eventual rise in luciferase activity upon cell treatment with IP_3-releasing effector. Therefore, it may be concluded that this ability will make the designed reporter suitable for high-throughput screening of drug candidates and bioluminescence imaging of living organism.

Although the present study demonstrates that the intramolecular luminescent probe can be useful in drug screening, high-throughput screening will need to be improved further, especially in the background signal. Even though the background signal is a common drawback of the complementation reporter systems, recent attempts to solve this problem have met with considerable success, as significant increase in absolute signal and lower background have been achieved (Luker et al., 2004; Remus et al., 2006). It sought to overcome limitations of split reporter system to develop further improvement systems that can also be used for high-throughput screening of interactions in cells and expand its range of applications in the imaging of IP_3 signaling and drug screening.

5. Conclusion

In the current study, we developed and assessed a specific intramolecular sensor based on split firefly luciferase complementation assay for quantitative detection of IP_3 in living cells and *in vitro*

conditions. Moreover, according to the result presented in this manuscript, for the first time, the ability of human IP_3-binding core in interaction and conformational changes was examined experimentally. The response of this fusion system upon binding of IP_3 is very fast, which enables high-throughput screening and characterization of therapeutic drugs and inhibitors that regulate activity of PLC and kinase cascade with subsequent production of IP_3. Finally, the successful development of our IP_3 probe based on luminescence assay has firmly established the split-luciferase system as a powerful approach to study the other intracellular interactions, as well as to investigate the important intramolecular interactions.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.09.037.

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