Journal of Biotechnology 167 (2013) 248–254

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

A fluorescence-based method for evaluating inositol 1,4,5-trisphosphate receptor ligands: Determination of subtype selectivity and partial agonist effects

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ARTICLE INFO

Article history:
Received 5 February 2013
Received in revised form 19 May 2013
Accepted 23 June 2013
Available online 3 July 2013

Keywords:
Fluorescence resonance energy transfer (FRET)
Adenophostin A
Inositol 1,4,5-trisphosphate (IP_3)
Fluorescent IP_3 biosensor
Partial agonist
Subtype selectivity

ABSTRACT

Inositol 1,4,5-trisphosphate (IP_3) receptors consist of three subtypes: IP_3R1, IP_3R2, and IP_3R3. Although numerous IP_3 receptor ligands have been synthesized, none of the subtype-selective ligands are known. We have developed a simple fluorescence method to examine the subtype selectivity of IP_3 receptor ligands using FRET-based IP_3 biosensors LIBRAvI, LIBRAvII, and LIBRAvIII. The addition of IP_3 or adenophostin A (ADA) to permeabilized biosensor-expressing cells increased the fluorescence ratios of these biosensors in a concentration-dependent manner, and the potency of ADA relative to that of IP_3 in terms of the changes in the fluorescence ratios of LIBRAvI, LIBRAvII, and LIBRAvIII was 43-, 22-, and 28-fold, respectively. This fluorescence-based method further showed that several ADA analogs had significant differences with respect to subtype selectivity and potency. These results highlight the important role played by the *O*-glycosidic structure of ADA in the selectivity of the ligands for IP_3R1, as evidenced by the modified selectivity following replacement of the 5′-hydroxyl with a phenyl or phenethyl group. We also found that one ADA analog 5′-deoxy-5′-phenyladenophostin A possessed a partial agonistic effect on IP_3R1. Together, the novel fluorescent methods described herein are useful for the evaluation of properties of IP_3R ligands, including potency, efficacy, and subtype selectivity.

1. Introduction

Inositol 1,4,5-trisphosphate (IP_3) is an important intracellular messenger produced by the phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in response to the stimulation of G-protein-coupled receptors or receptor tyrosine kinases. The binding of IP_3 to its receptor (IP_3R) results in the release of Ca^{2+} from intracellular stores (Berridge, 1993). The IP_3R family consists of three subtypes, IP_3R1, IP_3R2, and IP_3R3, which are differentially expressed to varying degrees in mammalian tissues and cell lines (Taylor et al., 1999; Wojcikiewicz, 1995). Despite structural similarities, the three IP_3R subtypes exhibit diversity in terms of their IP_3 sensitivity, distribution, and functional modulations by Ca^{2+}, ATP, and phosphorylation (Foskett et al., 2007). Thus, subtype-selective IP_3R ligands could be highly useful as research tools for investigating the biological roles of the IP_3R subtypes and as a basis for the development of potentially beneficial drugs. Indeed, in light of the biological importance of IP_3Rs, many compounds that activate these receptors, such as analogs of IP_3 and adenophostin A (ADA), have been designed and synthesized (Wilcox et al., 1998). However, the subtype selectivity of these synthesized IP_3 ligands is poorly understood, and none of the subtype-selective ligands are known.

In a previous study, we developed a fluorescence resonance energy transfer (FRET)-based IP_3 biosensor, referred to as LIBRA (Tanimura et al., 2004), which consists of the IP_3-binding domain of rat IP_3R3, the fluorescent proteins ECFP and EYFP (enhanced cyan and yellow fluorescent protein, respectively), and a plasma-membrane-targeting signal. This biosensor was then used in a fluorescence method for the determination of IP_3R ligands (Nezu et al., 2006), which successfully identified several newly synthesized cyclopentane analogs as novel ligands of IP_3Rs (Zhang et al., 2007) as well as ADA analogs (Mochizuki et al., 2010).

Recently, we constructed a series of improved IP_3 biosensors, LIBRAvI, LIBRAvII, and LIBRAvIII, using the IP_3-binding domains of

Abbreviations: ADA, adenophostin A; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; FRET, fluorescence resonance energy transfer; LBD, ligand-binding domain of IP_3R; ICM, intracellular-like medium; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; HBSS-H, Hanks' balanced salt solution buffered with Hepes.

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http://dx.doi.org/10.1016/j.jbiotec.2013.06.012

IP_3R1, IP_3R2, and IP_3R3, respectively (Tanimura et al., 2009). The ligand-binding domain of IP_3R (LBD) is composed of two functional domains, the amino-terminal suppressor domain and the carboxyl-terminal IP_3-binding core domain (Yoshikawa et al., 1996). Unlike other IP_3R-based FRET biosensors (Sato et al., 2005; Matsu-ura et al., 2006), LIBRAvI, LIBRAvII, and LIBRAvIII contain both of these domains of IP_3R1, IP_3R2, and IP_3R3, respectively (Tanimura et al., 2009). The structural differences in the suppressor domains of each IP_3R subtype are known to be responsible for the functional diversity in the ligand sensitivity among these receptors (Iwai et al., 2007). It was therefore expected that the ligand-binding properties of the LIBRAv series would reflect the properties of the IP_3R subtypes.

In the present study, we utilized these improved biosensors (LIBRAvI, LIBRAvII, and LIBRAvIII) to expand the method for determining the subtype selectivity of IP_3R ligands. In addition, this fluorescence method allowed us to identify an ADA-derived partial agonist of IP_3R1.

2. Materials and methods

2.1. Chemicals

ADA analogs **1–6** were synthesized according to previously reported methods (Mochizuki et al., 2006; Shuto et al., 1998), and the structures of which are shown in Fig. 1. IP_3 was purchased from LC laboratories (Woburn, MA, USA) or Alexis Biochemicals (San Diego, CA, USA). ADA was purchased from Calbiochem (Darmstadt, Germany). All other reagents used were of analytical grade.

2.2. Plasmid construction

The construction of fluorescent IP_3 biosensors was described previously (Tanimura et al., 2009). Briefly, a fusion construct mCY was made by cutting EYFP out of pEYFP-N1 (Clontech) and ligating it into pECFP-mem (Clontech), with subsequent removal of the multiple cloning site originating from pECFP-mem. The IP_3-binding domain of rat IP_3R3 (amino acids 1–604) was PCR-amplified and the resulting fragment was ligated in mCY. This construct is the original version of the IP_3 biosensor LIBRA.

To construct pH-stable LIBRA (LIBRAvIII), the Venus plasmid was constructed using site-directed mutagenesis of pEYFP-N1. The portion of LIBRA containing the membrane-targeting signal, ECFP, and the IP_3-binding domain was then ligated into the Venus plasmid. To construct LIBRAvI and LIBRAvII, the IP_3-binding domains of rat IP_3R1 and IP_3R2 (amino acids 1–604) were PCR-amplified using a rat brain cDNA library and a rat parotid duct cDNA library as the templates, respectively. The PCR products were then ligated into the linker region of the LIBRAvIII-plasmid. The IP_3-insensitive variant of LIBRAv (LIBRAvN) was constructed by site-directed mutagenesis of the IP_3-binding domain (K507A) of LIBRA, with subsequent ligation into the LIBRAvIII-plasmid.

2.3. Cell culture and transfection

HSY-EA1 human parotid duct cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma) supplemented with 10% newborn calf serum (Gibco BRL, Rockville, MD, USA), 2 mM L-glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml (Gibco BRL), as previously described. Cells grown in experimental chambers consisting of 7 mm plastic cylinders and containing fibronectin-coated cover slips were transfected with 10 μg plasmid/ml using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Fluorescence measurements

HSY-EA1 cells expressing the IP_3 sensor were cultured in experimental chambers and then permeabilized by exposing the cells to intracellular-like medium (ICM; 125 mM KCl, 19 mM NaCl, 10 mM Hepes-KOH, pH 7.3, 1 mM EGTA, and 330 μM $CaCl_2$) containing 100 μg (w/v) saponin (ICN, Cleveland, OH, USA)/ml for ~1 min. Permeabilized cells were washed with ICM and then exposed to ICM containing various concentrations of IP_3 and/or other reagents as specified. The fluorescence of the IP_3 biosensors was monitored by excitation at 425 nm and dual emission at 480 and 535 nm.

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 Plan Fluor 60 oil immersion objective (NA 1.25) and a Nikon Plan Fluor 40 oil immersion objective (NA 1.3). Data were analyzed with AQUACOSMOS 2.6 software (Hamamatsu Photonics). All experiments were performed at room temperature.

2.5. Determinations of EC_{50}

The data were analyzed with GOSA-fit software (BIO-LOG, Toulouse, France) to fit the data to the equation:

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

where C is the concentration of IP_3R ligands, $E_{\max}$ is the maximal effect, and n is the Hill coefficient.

Statistical analysis was performed using Bartlett's test for homogeneity of variance followed by Fisher's least significant difference post hoc test for comparison among individual means.

3. Results

3.1. Effects of IP_3 and ADA on the fluorescence of IP_3 biosensors

Fig. 2 shows the IP_3-induced changes in the fluorescence emission ratios of LIBRAvI, LIBRAvII, and LIBRAvIII. An increase in the fluorescence ratio of LIBRAvI was detected in response to 30 nM IP_3, with stepwise increases in IP_3 concentrations inducing concomitant increases in the fluorescence ratio of this biosensor (Fig. 2(A)). The effect of IP_3 on the LIBRAvI fluorescence ratio was nearly saturated at ~3 μM. Similar changes in the fluorescence ratio in response to stepwise increases in IP_3 concentrations were obtained with LIBRAvII and LIBRAvIII at different IP_3 concentration ranges (Fig. 2(B) and (C)). The rank order of the sensitivities of these biosensors for IP_3 was LIBRAvI > LIBRAvII > LIBRAvIII (Fig. 2(D) and (E)), and their EC_{50} values for IP_3 were 176.7, 314.2, and 396.3 nM, respectively.

An examination of the effects of ADA on LIBRAvI, LIBRAvII, and LIBRAvIII (Fig. 3) showed an increase in the fluorescence ratios of the biosensors at ~1 nM and their saturation at 30–300 nM. Unlike IP_3, the rank order of the sensitivities of these biosensors for ADA was LIBRAvI ≫ LIBRAvII = LIBRAvIII (Fig. 3(D) and (E)), and the EC_{50} values of ADA were 4.1, 14.2, and 13.5 nM, respectively. Notably, for IP_3 the negative log mol/L (pEC_{50}) was significantly higher using LIBRAvII than LIBRAvIII, whereas for ADA the pEC_{50} values of these two biosensors were comparable (Fig. 4(A)). Fig. 4(B) is a plot of the relative potencies of ADA vs. IP_3 as a function of the increased fluorescence ratios of each IP_3 sensor. It shows the lower ratio of ADA in its relative potency on LIBRAvII than on LIBRAvI or LIBRAvIII.

We also examined the potencies of IP_3 and ADA in the induction of Ca^{2+} release through each IP_3R subtype, using the low-affinity Ca^{2+} indicator mag-fura-2 and three different IP_3R knockout (double-deficient) DT40 cell lines: IP_3R2 and IP_3R3-KO DT40 cells

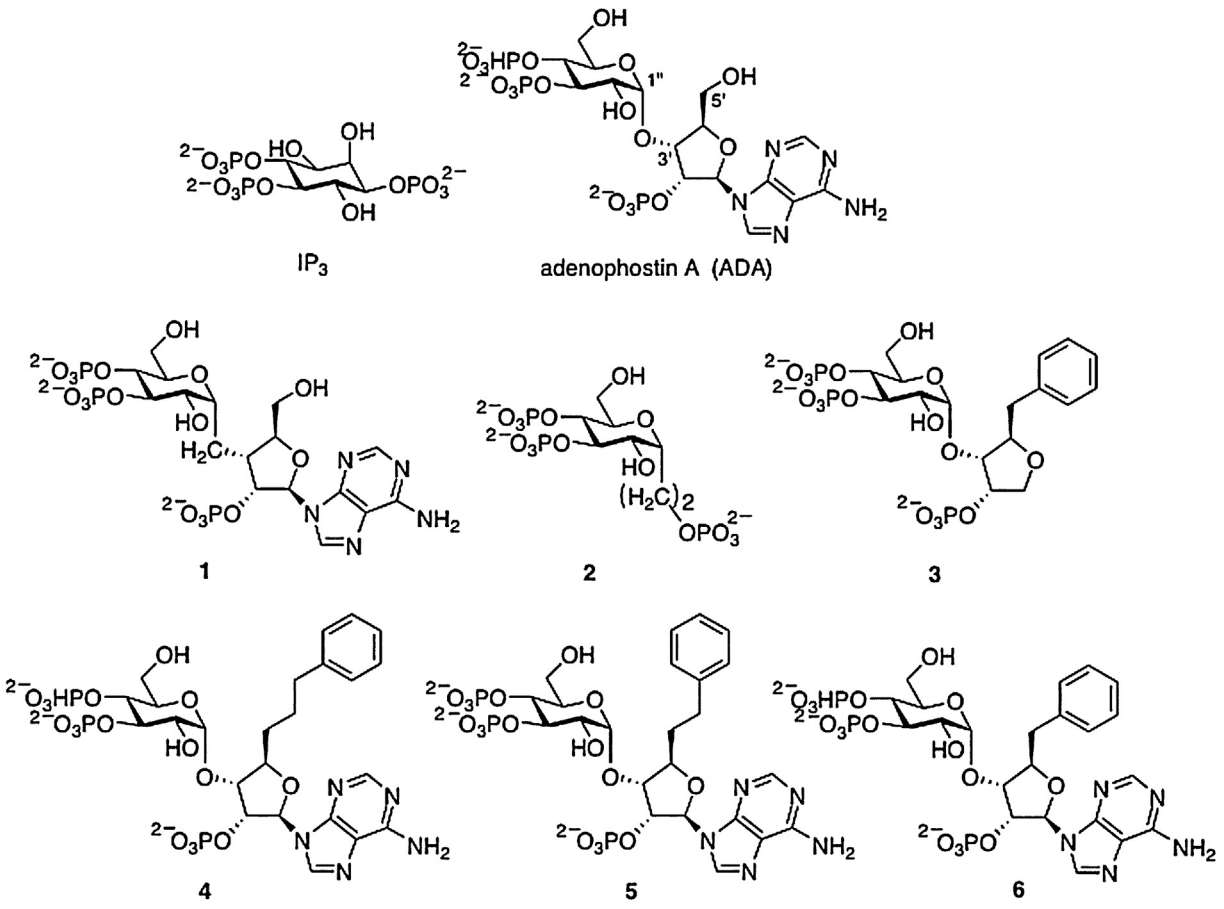

Fig. 1. Structures of IP_3, ADA, and the ADA analogs.

(IP_3R1 cells), IP_3R1 and IP_3R3-KO DT40 cells (IP_3R2 cells), and IP_3R1 and IP_3R2-KO DT40 cells (IP_3R3 cells), each of which expressed a single IP_3R subtype. These Ca^{2+} release experiments demonstrated that both IP_3 and ADA induced Ca^{2+} release in a concentration-dependent manner. The highest potency was achieved with type 2 cells, although in this case the effect of ADA was less profound than that of IP_3 (Fig. S1 and S2). Consistent with the effects on the fluorescence ratios of the IP_3 sensors, the relative potency of ADA vs. IP_3 for Ca^{2+} release was lower for type 2 cells than for type 1 and type 3 cells (Fig. S3).

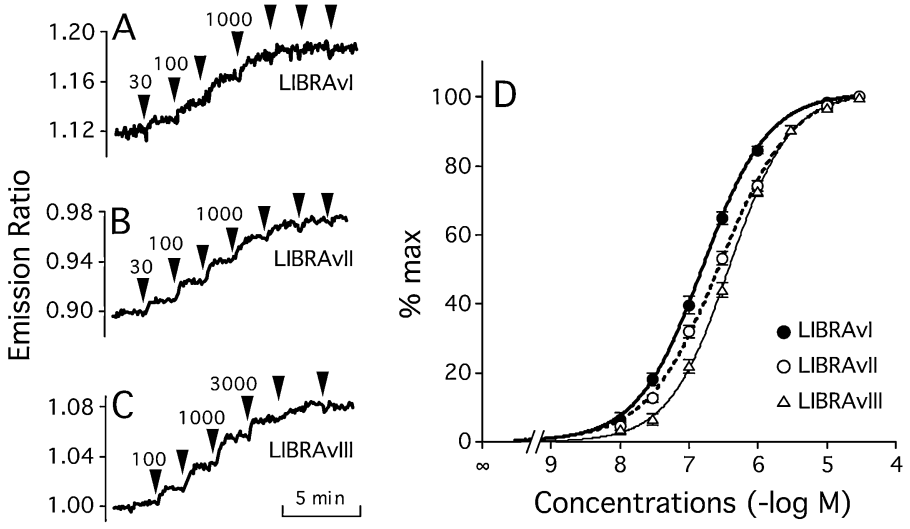

Fig. 2. Effect of IP_3 on the fluorescence ratios of the IP_3 biosensors
(A)–(C) Changes in the fluorescence emission ratios (480/535 nm) of LIBRAvI (A), LIBRAvII (B), and LIBRAvIII (C) following exposure to various concentrations of IP_3. LIBRAvI-, LIBRAvII-, and LIBRAvIII-expressing cells were permeabilized with saponin and consecutively exposed to 30, 100, 300, 1000, 3000, 10,000, and 30,000 nM IP_3. (D) Concentration–response curves (means ± SE, $n \geq 30$) for IP_3 showing changes in the fluorescence ratio of LIBRAvI (closed circles), LIBRAvII (open circles), and LIBRAvIII (open triangles). The changes were normalized to the effects of IP_3 saturation with 10 or 30 μM IP_3.

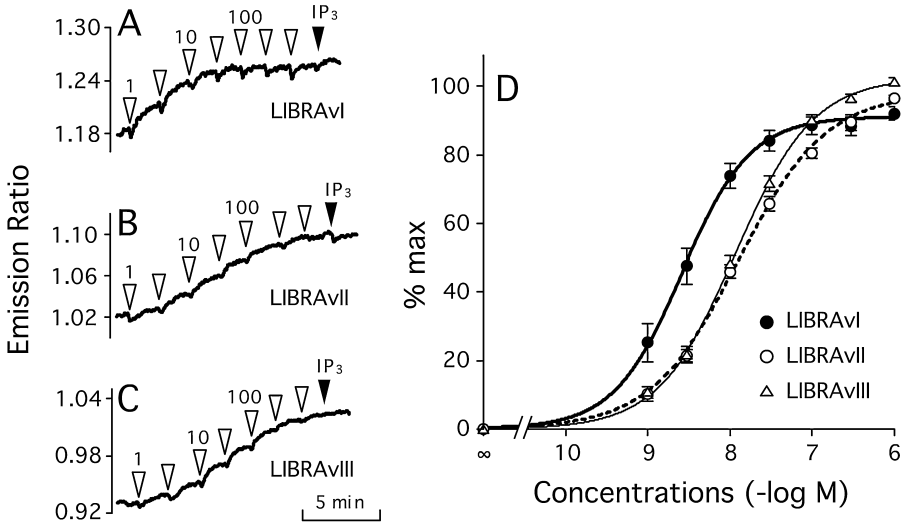

Fig. 3. Effect of ADA on the fluorescence ratios of the IP_3 biosensors
(A)–(C) Changes in the fluorescence emission ratios (480/535 nm) of LIBRAvI (A), LIBRAvII (B), and LIBRAvIII (C) following exposure to various concentrations of ADA and IP_3. LIBRAv variant-expressing cells were permeabilized and consecutively exposed to 1, 3, 10, 30, 100, 300, and 1000 nM ADA and 10 or 30 μM IP_3. (D) Concentration–response curves (means ± SE, $n \geq 10$) for ADA showing the changes in the fluorescence ratios of LIBRAvI (closed circles), LIBRAvII (open circles), and LIBRAvIII (open triangles). The changes were normalized to the effects of IP_3 saturation with 10 or 30 μM IP_3.

3.2. Subtype selectivity of ADA analogs

The fluorescent biosensors were applied to examine the subtype selectivity of ADA analogs **1–6** (Fig. 1), developed in previous studies and described elsewhere (Mochizuki et al., 2006; Shuto et al., 1998). The concentration-dependent effects of **1–6** on the fluorescence ratios of LIBRAvI, LIBRAvII, and LIBRAvIII evidenced the significant differences between these compounds regarding their potencies and subtype selectivity (Fig. 5(A)–(F)). The pEC_{50} values of the ADA analogs with respect to changes in the fluorescence ratios of the IP_3 biosensors are summarized in Fig. 5(G). These plots indicate that the potencies of **2** and **3**, both of which lack an adenine moiety, are reduced on LIBRAvI, LIBRAvII, and LIBRAvIII. The potencies of the 5′-phenethyl derivative **4** and the 5′-benzyl derivative **5** for three of these biosensors were comparable. On the other hand, the subtype selectively of 5′-phenyl derivative **6** was similar to that of ADA, with reduced potencies toward all three subtypes. It is also notable that **1** showed the lowest selectivity for LIBRAvIII.

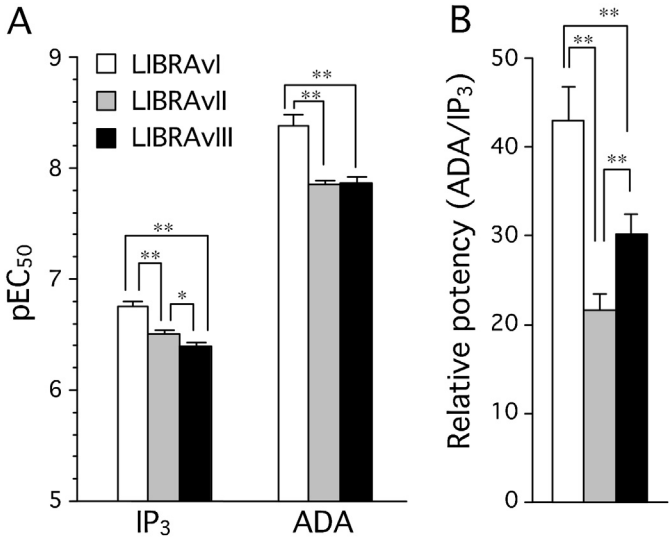

Fig. 4. Potencies of IP_3 and ADA as measured by increases in the fluorescence ratios of the IP_3 biosensors.
(A) The pEC_{50} values (means ± SE) of IP_3 and ADA with respect to changes in the fluorescence ratios of the LIBRAvI (I), LIBRAvII (II), and LIBRAvIII (III) biosensors were calculated based on the results of the experiments shown in Figs. 2 and 3. Asterisks indicate statistical differences (*, $P < 0.05$; **, $P < 0.01$). (B) The relative potency of ADA vs. IP_3. The EC_{50} values of ADA for the fluorescence ratios obtained with LIBRAvI (open column), LIBRAvII (gray column), and LIBRAvIII (black column) were divided by those of IP_3 for each of these sensors. The reciprocals of the calculated values (means ± SE) are plotted as the relative potencies. Asterisks indicate statistical differences ($P < 0.01$).

3.3. Partial agonistic effect of 5′-deoxy-5′-phenyladenophostin A (ADA analog **6**)

Fig. 5(D)–(F) shows that the maximal effects on LIBRAvI of the ADA analogs, **4**, **5**, and **6** were somewhat lower than those of IP_3. In particular, the maximal increase in the LIBRAvI fluorescence ratio obtained with **6** was only 60% of that obtained with IP_3 (Fig. 5(F)). As shown in Fig. 6(A), the effect of **6** was saturated at ~3 μM but the subsequent addition of 10 μM IP_3 induced an additional increase in the LIBRAvI fluorescence ratio. The fluorescence of the negative control constructs LIBRAvN and mCY was not altered by the addition of 10 μM of **6** (data not shown), thus excluding nonspecific effects.

To examine further the partial agonist effects of **6**, its ability to reduce the activity of IP_3 on the fluorescence ratio of LIBRAvI was tested. Fig. 6(B) shows that 1 μM IP_3 increased the fluorescence ratio of LIBRAvI, whereas subsequent additions of **6** decreased the fluorescence ratio in a concentration-dependent manner. A similar inhibitory effect of **6** was observed in the presence of 10 μM IP_3 (Fig. 6(C)), although in that case higher concentrations of **6** were required (Fig. 6(D)).

4. Discussion

The present study demonstrates a method to evaluate the properties of IP_3R ligands using the fluorescent IP_3 biosensors LIBRAvI, LIBRAvII, and LIBRAvIII, which contain the LBD of IP_3R1, IP_3R2, and IP_3R3, respectively. Each LBD (amino acid residues 1–604) contains the IP_3-binding core and suppresser domains, and thus they are expected to reflect the ligand sensitivity of each IP_3R subtype. We

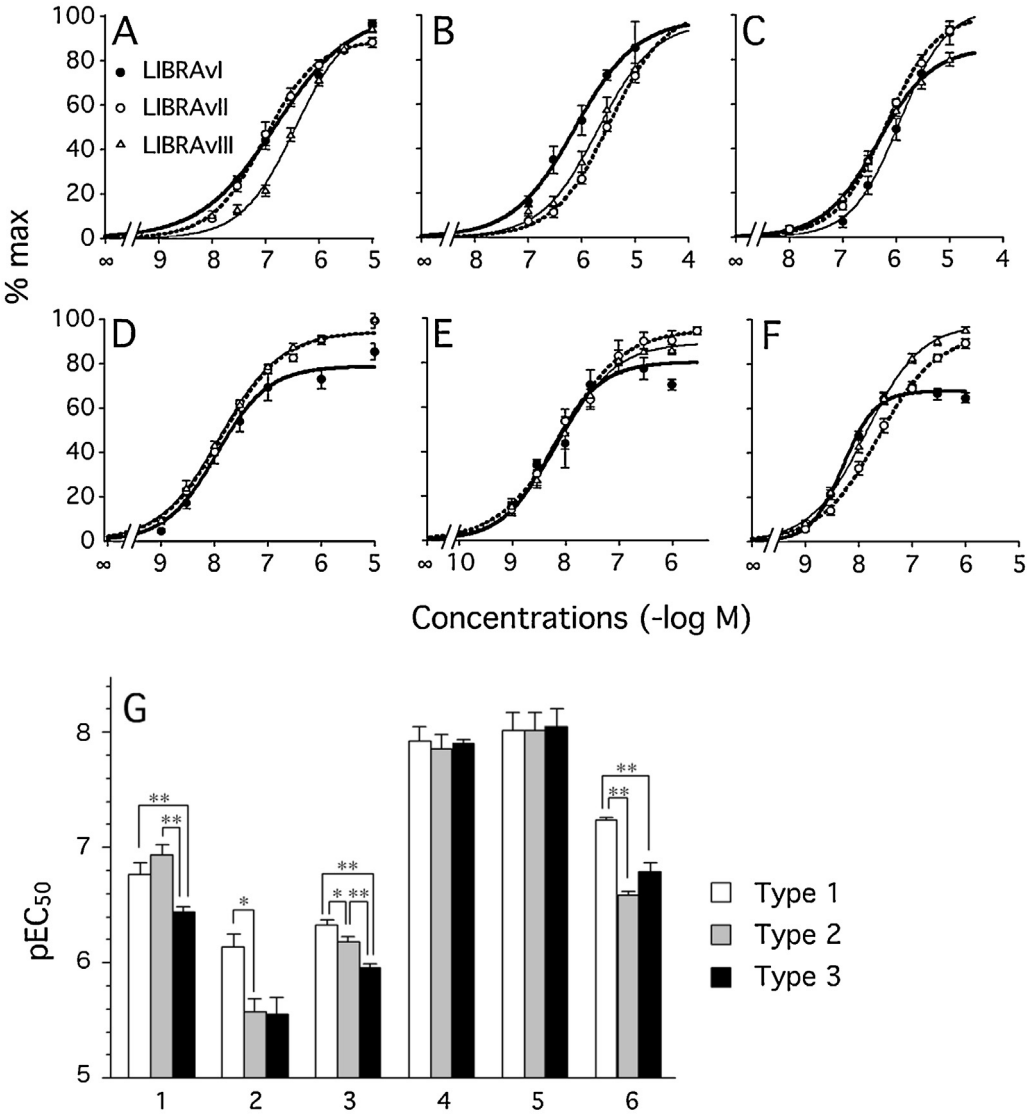

Fig. 5. Effects of ADA analogs on the fluorescence ratios of the IP_3 biosensors.
(A)–(F) Concentration-response curves (means ± SE, $n \geq 3$) for ADA analogs **1** (A), **2** (B), **3** (C), **4** (D), **5** (E), **6** (F) showing the changes in the fluorescence ratios of LIBRAvI (closed circles), LIBRAvII (open circles), and LIBRAvIII (open triangles). Changes in the emission ratios were normalized to the effects of IP_3 saturation with 10 or 30 μM IP_3. (G) pEC_{50} (means ± SE) for ADA analogs with respect to the changes in the fluorescence ratios of LIBRAvI (I), LIBRAvII (II), and LIBRAvIII (III). Asterisks indicate statistical differences (*, $P < 0.05$; **, $P < 0.01$).

took advantage of this to determine the subtype selectivity of ADA and its analogs.

Different IP_3R subtypes have distinct IP_3-binding properties, and these properties are differentially modulated by concentrations of Ca^{2+} and ATP, the presence of interacting proteins, and phosphorylation status (Foskett et al., 2007). In addition, experimental conditions including temperature, pH, and ionic strength strongly affect the ligand-binding properties of IP_3Rs (Ding et al., 2010; Rossi et al., 2010).

The sensitivities of IP_3R subtypes have been studied extensively in ligand-binding analyses using purified IP_3Rs and recombinant LBD. Many published ligand-binding studies have been performed using media with high pH and low ionic strength at 4 °C to maximize specific binding. Although cytosol-like medium is favorable to correlate the results of such assays with IP_3R functions directly, it is impractical to measure [^{3}H]-IP_3 binding reliably in native tissues and mammalian cells because the densities of IP_3Rs are too low (Ding et al., 2010; Rossi et al., 2010). Thus, the results of these IP_3-binding studies may not always precisely reflect the physiological properties of IP_3Rs. The fluorescent method described herein has the advantage that it enables the properties of IP_3R ligands to be determined in physiological conditions.

The rank order of the affinities of the three IP_3R subtypes for IP_3 and the extent of the differences between these affinities vary between studies. There is no simple explanation for these inconsistencies, and the physiological relevance of the different affinities of the IP_3R subtypes for IP_3 has not been clearly established (Foskett et al., 2007). Many ligand-binding studies have indicated that the rank order of the sensitivities of the IP_3R subtypes to IP_3 is $IP_3R2 > IP_3R1 > IP_3R3$, whereas the rank order of the sensitivities of IP_3 biosensors in permeabilized HSY-EA1 cells is LIBRAvI > LIBRAvII > LIBRAvIII.

This difference is due, at least in part, to differences in experimental conditions as discussed above. In addition, differences in the preparation of IP_3Rs are also likely to contribute to this variability; for example, full-length IP_3Rs in microsomes or fragments of recombinant IP_3Rs expressed in mammalian cells, Sf9 insect cells, or bacterial cells. Therefore, it is impractical to compare the

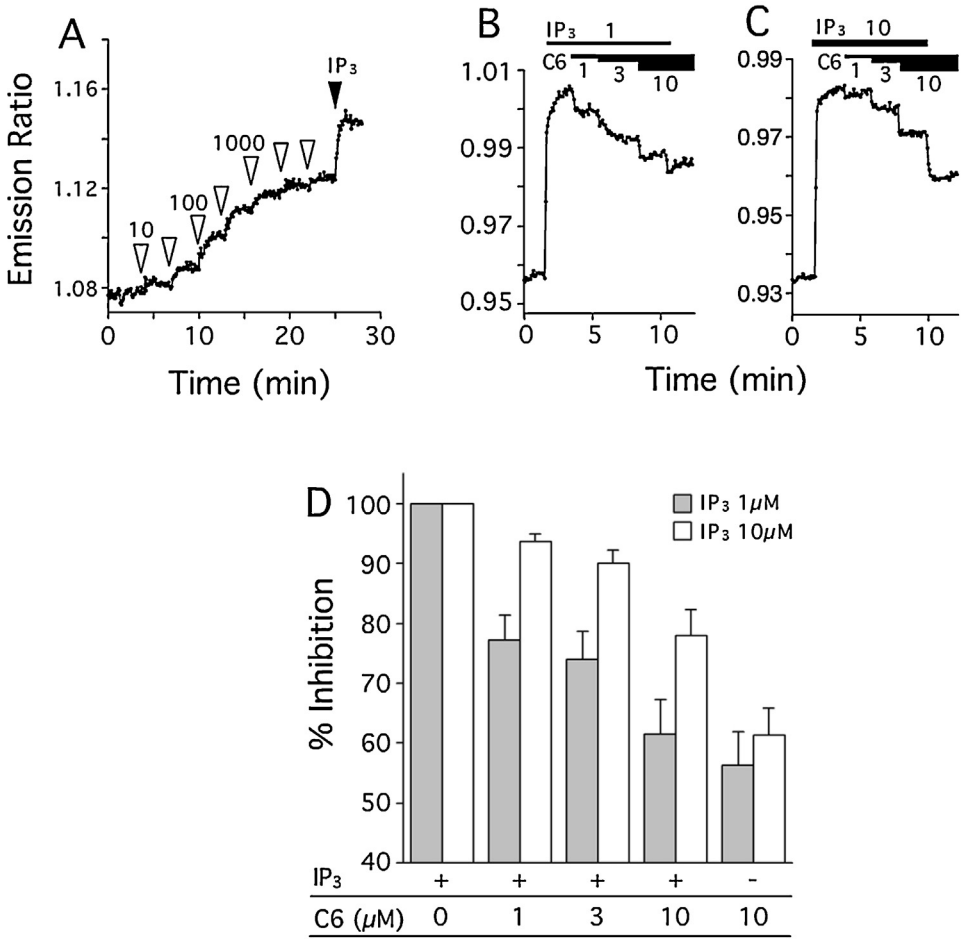

Fig. 6. Partial agonistic effect of ADA analog **6** on the fluorescence ratio of LIBRAvI.
(A) Changes in the fluorescence emission ratio (480/535 nm) of LIBRAvI following exposure to various concentrations of **6** and 10 μM IP_3. LIBRAvI-expressing cells were permeabilized and consecutively exposed to 10, 30, 100, 300, 1000, 3000, and 10,000 nM of **6** or 10 μM IP_3. (B, C) Changes in the fluorescence emission ratio (480/535 nm) of LIBRAvI following exposure to IP_3 and the subsequent addition of **6**. LIBRAvI-expressing cells were permeabilized and initially exposed to 1 μM IP_3 (B) or 10 μM IP_3 (C), and then to 1, 3, or 10 μM of **6** in the presence of IP_3 and 10 μM of **6** without IP_3. (D) Effect of **6** on the reduction of IP_3-mediated changes in the fluorescence ratio of LIBRAvI (means ± SE, $n \geq 4$). Changes in the emission ratio (480/535 nm) of LIBRAvI obtained with **6** and IP_3 were normalized to the effects of IP_3 without **6**. The presence (+) or absence (−) of IP_3 and the concentrations (μM) of **6** are shown at the bottom of the figure.

absolute affinities of IP_3 and other IP_3R ligands for IP_3Rs and LBD in different experimental conditions. In addition, additional components of biosensors, such as fluorescent proteins and linkers, might also have an effect. Thus, it is reasonable to use these IP_3 biosensors to determine the potency of target compounds (ADA and its derivatives) relative to that of IP_3 for IP_3R subtypes rather than to estimate their absolute affinities.

In the present study, the potency of ADA relative to that of IP_3 in terms of the changes in the fluorescence ratio of LIBRAvI, LIBRAvII, and LIBRAvIII was 43-, 22-, and 28-fold, respectively. These results are consistent with the notion that the affinity of ADA is 10–100-fold higher than that of IP_3 (Rossi et al., 2010; Shuto et al., 1998; Takahashi et al., 1994). In addition, our data show differences between the IP_3R subtype selectivities of IP_3 and ADA, and suggest that ADA is slightly less selective for IP_3R2. Although more modest differences in the relative affinities of IP_3Rs for IP_3 and ADA (~3-fold) have been reported (Morris et al., 2002; Vanlingen et al., 2000), this seems to be observed only for shorter fragments of LBD in non-physiological (high pH and low ionic strength) experimental conditions (Ding et al., 2010).

The Ca^{2+} release experiments using IP_3R-KO DT40 cell lines do not allow the absolute sensitivities of the IP_3R subtypes to IP_3 or ADA to be compared between cell lines, because it is impossible to obtain cells expressing identical levels of IP_3Rs. However, determining the potency of ADA relative to that of IP_3 allowed us to compare the effects of ADA between cell lines, and this functional analysis confirmed that the relative potency of ADA was lowest in the DT40 cell line expressing IP_3R2. Based on these results, we conclude that the fluorescence method using LIBRAvI, LIBRAvII, and LIBRAvIII is useful to determine the relative potencies and the IP_3R subtype selectivities of IP_3R ligands in physiological experimental conditions. We used this novel fluorescence method to examine the IP_3R subtype selectivities of ADA analogs **1–6**.

The fluorescence analysis of ADA analogs **1–6** confirmed the importance of the adenine moiety for the high affinity of ADA analogs for IP_3R. The data also suggested that modification of the 5′-moiety of ADA changes its relative potency for IP_3R subtypes. IP_3Rs are ubiquitously expressed and regulate many physiological processes. Differences between IP_3R subtypes in terms of their localizations, their affinities for IP_3, and the differential effects of Ca^{2+} and phosphorylation, play important physiological roles. Therefore, ligands that selectively bind specific IP_3R subtypes offer important experimental tools in functional studies of these receptors and in the eventual development of therapeutic agents. The method described herein provides a simple approach to evaluate such IP_3R ligands.

Interestingly, ADA analog **6** (5′-deoxy-5′-phenyladenophostin A) did not induce the maximal change in the fluorescence ratio

of LIBRAvI. Furthermore, the effects of IP_3 on the increase in the fluorescence ratio of LIBRAvI were decreased by **6** in a concentration-dependent manner. These results clearly indicated that **6** competes with IP_3 for binding to LIBRAvI and acts as a partial agonist in increasing the fluorescence emission ratio of LIBRAvI.

Although many ADA analogs have been synthesized, those examined to date are full IP_3R agonists, with the exception of acyclophostin, which is a partial agonist at high pH (Beecroft et al., 1999). Thus, **6** is the first ADA-derived partial IP_3R agonist that is active under physiological conditions. This new ADA-derived partial agonist has a higher apparent affinity than IP_3 for IP_3Rs. The high affinity of ADA for IP_3Rs makes this family of analogs attractive to develop novel subtype-selective ligands for IP_3Rs and high affinity partial agonists and antagonists of IP_3Rs. From this viewpoint, it is important that ADA analog **6** acts as a partial agonist. While IP_3R antagonists will be useful in studies of IP_3-related signal transduction pathways, these compounds have yet to be developed. Since partial agonists can be effective prototype compounds in the development of corresponding antagonists, the 5′-aromatic modification approach may provide entry to the exploration of IP_3R antagonists. Thus, **6** is a potential lead compound in the synthesis of IP_3R antagonists.

5. Conclusions

The present study established a new method for evaluating the subtype selectivity of IP_3Rs ligands using fluorescent IP_3 biosensors. This method allowed us to resolve the subtype selectivity of ADA and its analogs, with the identification of partial agonist effects. The compound 5′-deoxy-5′-phenyladenophostin A (**6**) was identified as a potent partial agonist and thus as a potentially effective lead compound for developing IP_3 receptor antagonists. These results highlight the efficiency of fluorescent IP_3 biosensors and the methods presented herein in evaluating the properties of IP_3R ligands. The subtype-selective ligands of IP_3Rs, including specific agonists and antagonists, offer important experimental tools in functional studies of these receptors and in the eventual development of therapeutic agents. Although further improvements of the fluorescent indicators are required, this direction of research will be accelerated by applying this florescent method to a high-throughput screening system aimed at examining the properties of large numbers of IP_3R ligands.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was supported in part by a Grant-in Aid for Scientific Research 16390532 (A. Tanimura) and by the “High-Tech Research Center” Project for Private Universities 2007–2012, with a matching fund from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.06.012.

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