Phosphoinositides participate in many signaling cascades via phospholipase C stimulation, which hydrolyzes phosphatidylinositol-4,5-bisphosphate, producing second messengers diacylglycerol and inositol 1,4,5-trisphosphate (InsP3). Destructive chemical approaches required to measure [InsP3] limit spatiotemporal understanding of subcellular InsP3 signaling. We constructed novel fluorescence resonance energy transfer-based InsP3 biosensors called FIRE (fluorescent InsP3-responsive element) by fusing plasmids encoding the InsP3-binding domain of InsP3 receptors (types 1–3) between cyan fluorescent protein and yellow fluorescent protein sequences. FIRE was expressed and characterized in COS-1 cells, cultured neonatal cardiac myocytes, and incorporated into an adenoviral vector for expression in adult cardiac ventricular myocytes. FIRE-1 exhibits an ~11% increase in the fluorescence ratio (F_CFP/F_YFP) at saturating [InsP3] (apparent Kd = 31.3 ± 6.7 nM InsP3). In COS-1 cells, neonatal rat cardiac myocytes and adult cat ventricular myocytes FIRE-1 exhibited comparable dynamic range and a 10% increase in donor (cyan fluorescent protein) fluorescence upon bleach of yellow fluorescent protein, indicative of fluorescence resonance energy transfer. In FIRE-1 expressing ventricular myocytes endothelin-1, phenylephrine, and angiotensin II all produced rapid and spatially resolved increases in [InsP3] using confocal microscopy (with free [InsP3] rising to ~30 nM). Local entry of intracellular InsP3 via membrane rupture by a patch pipette (containing InsP3) in myocytes expressing FIRE-1 allowed detailed spatiotemporal monitoring of intracellular InsP3 diffusion. Both endothelin-1-induced and direct InsP3 application (via pipette rupture) revealed that InsP3 diffusion into the nucleus occurs with a delay and blunted rise of [InsP3] versus cytosolic [InsP3]. These new biosensors allow studying InsP3 dynamics at high temporal and spatial resolution that will be powerful in understanding InsP3 signaling in intact cells.

Cell surface membrane receptor-activation of phospholipase C results in hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (InsP3).3 This signaling step is recognized as a crucial branch point in signal transduction where membrane-delineated diacylglycerol modulates protein kinase C, whereas InsP3 can diffuse into the cytoplasm and mediate Ca release from intracellular stores via the InsP3 receptor (InsP3R).

The InsP3R family of calcium release channels occupies a central role in the initiation and propagation of intracellular calcium-mediated events. This signaling cascade regulates and impinges upon a wide array of cellular processes (1, 2). Despite longstanding recognition of InsP3 as an important intracellular signaling molecule (3, 4), measures of intracellular InsP3 levels have had limitations. These assays mainly either rely on whole cell extracts for mass analysis by competition binding, gas chromatography/mass spectrometry, and ion exchange chromatography or use metabolic measurements of radiolabeled InsP3 precursors and degradation products (5–8). Regrettably, these methods have limited temporal resolution, are tissue destructive, and do not provide any spatial information about subcellular free InsP3 concentration and localization. Because there is increasing appreciation of local spatial compartmentalization and microdomains of intracellular signaling, the ability to measure local [InsP3] in living cells would be highly beneficial in unraveling InsP3-dependent signaling.

Green fluorescent protein (GFP) and its variants with different spectral characteristics have allowed the development of novel biosensors that can be expressed in living cells (9). Moreover, fusion proteins, which include both a cyan and yellow fluorescent protein (CFP and YFP), exhibit fluorescence resonance energy transfer (FRET) from CFP to YFP. By inserting peptide linkers that bind to biological molecules of interest, one can obtain biosensors whose FRET properties change upon binding the molecule of interest (10–12).

Recently, progress has been made to use fluorescent probes to measure [InsP3] dynamically in living cells (11, 13, 14). A key approach is to use the InsP3-binding domain of the InsP3 receptor (11), because this is one of the crucial functional targets in the cell. Here we have developed new InsP3-binding FRET-based sensors using the ligand-binding domains of the type 1, 2, and 3 InsP3R isoforms (FIRE-1, FIRE-2, and FIRE-3). We have characterized FIRE-1 and FIRE-3 in solution and in COS-1 cells exposed to agonists expected to raise InsP3 production.

In cardiac myocytes the role of InsP3 signaling is unresolved, and we also use FIRE-1 to probe InsP3 in ventricular myocytes. The type 2 InsP3R (InsP3R2) isoform predominates in cardiac myocytes (15–17). Although InsP3-dependent calcium release in cardiac tissue has been demonstrated (18–20), the role of InsP3 in cardiac excitation-contraction coupling and other functions is controversial (21–23). In atrial myocytes some InsP3R2 is expressed in sarcoplasmic reticulum, and

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3 The abbreviations used are: InsP3, inositol 1,4,5-trisphosphate; InsP3R, inositol 1,4,5-trisphosphate receptor; FIRE, fluorescent InsP3-responsive element; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; nt, nucleotide(s); ET-1, endothelin-1; ROI, region(s) of interest.
InsP$_3$R-dependent calcium release can modulate excitation-contraction coupling and elicit arrhythmogenesis by facilitating calcium-induced calcium release via ryanodine receptors (24–26). In ventricular myocytes InsP$_3$R are primary localized to the nuclear envelope and complex with calcium/calmodulin-dependent protein kinase II, which can phosphorylate InsP$_3$R, thereby modulating its calcium channel activity (17). Furthermore, agonist-induced InsP$_3$ liberation and calcium release in ventricular myocytes activates calcium/calmodulin-dependent protein kinase II and the translocation of histone deacetylases from the nucleus (27), such that cardiac InsP$_3$R may participate in excitation-transcription coupling.

To pave the way for better understanding of InsP$_3$ signaling in cardiac myocytes, FIRE-1 was incorporated in an adenovirus. This allowed successful tracking of agonist-induced subcellular [InsP$_3$] changes with high spatial and temporal resolution in ventricular myocytes.

**MATERIALS AND METHODS**

**Reagents**—The d-myo-inositol phosphates were purchased from the following vendors: Ins(1,4,5)P$_3$ from Alexis (San Diego, CA); Ins(2,4,5)P$_3$ from Calbiochem (La Jolla, CA); and Ins(1,4)P$_2$, Ins(4,5)P$_2$, and Ins(1,3,4,5)P$_4$ from A.G. Scientific (San Diego, CA). d-myo-Ins(1,4,5)P$_3$, and adeno-photin A were obtained from Calbiochem (La Jolla, CA). Heparin was from Sigma-Aldrich.

**Construction of FIRE Plasmids**—Type 1, 2, and 3 InsP$_3$R biosensors were assembled using the individual ligand-binding domain terminally fused with enhanced CFP and YFP at the amino and carboxyl termini, respectively. The construction of the FIRE plasmids corresponding to the three InsP$_3$R isoforms was as follows. The ligand-binding regions of each receptor isoform (18) encompassing the amino-terminal 604 residues (589 for the type 1 InsP$_3$R SI isoform) were PCR-amplified using the following oligonucleotide primer pairs: GGAGATCTCGAGCTA-TGCTGACAATAATGTC/GCGGATCTTTGCTGGTTGGAAGCATC (type 1), GGAGATCTCGAGCTATGCTGACAAAATGTCC/AGCAG (type 1), GGAGATCTCGAGCTATGAATGAAATGTCCAGC/GCGGATC- GCCACATCTTTCTTTGCGGTTGTTGTAGC (type 2), and GGAGATCTCGAGCTATGCTGACAAAATGTCCAGC/CGCGGATCTTTTGTGGTTGTGAGTAC (type 2). The PCR product from the type 1 receptor ligand-binding region was digested with XhoI, Klenow and inserted into the pEYFP (Clontech) containing the enhanced YFP coding region of pEYFP (Clontech) containing the enhanced YFP coding sequence.

The PCR product from the type 1 receptor ligand-binding region was digested with XhoI and BamHI and ligated into similarly digested pEYFP vector. This vector was constructed by linearizing pEFCF-C1 (Clontech) isolated from a methylation deficient Escherichia coli strain (DM(−)) with XbaI and inserting the XbaI fragment of pEYFP (Clontech) containing the enhanced YFP coding sequence.

The PCR product from the type 1 receptor ligand-binding region was digested with XhoI and BamHI and ligated into similarly digested pEYFP vector to form FIRE-1. The type 2 PCR product was digested with XhoI and BamHI to generate two fragments of 1566 and 255 nt. The 1566-nt fragment was ligated into XhoI/BamHI-digested pEYFP vector. This vector was constructed by linearizing pEFCF-C1 (Clontech) isolated from a methylation deficient Escherichia coli strain (DM(−)) with XbaI and inserting the XbaI fragment of pEYFP (Clontech) containing the enhanced YFP coding sequence.

The PCR product from the type 1 receptor ligand-binding region was digested with XhoI and BamHI and ligated into similarly digested pEYFP vector to form FIRE-1. The type 2 PCR product was digested with XhoI and BamHI to generate two fragments of 1566 and 255 nt. The 1566-nt fragment was ligated into XhoI/BamHI-digested pEYFP vector. This vector was constructed by linearizing pEFCF-C1 (Clontech) isolated from a methylation deficient Escherichia coli strain (DM(−)) with XbaI and inserting the XbaI fragment of pEYFP (Clontech) containing the enhanced YFP coding sequence.

**Construction of FIRE-1 Adenoviral Vector**—The FIRE-1 plasmid, previously described, was used as the progenitor for the FIRE-1 adenovirus (FIRE-1AdV). The adenoviral vector was constructed using a commercially available kit, the AdEasy TM XL adenoviral vector system (Stratagene, La Jolla, CA.). Briefly, the terminally fluorescent tagged InsP$_3$ ligand-binding domain was excised by digesting with NheI, Klenow repaired followed by digestion with NotI. This fragment was subcloned into the multiple cloning site of the shuttle vector (pShuttle-CMV) by digesting the vector with BglII and following Klenow repair digested with NheI to produce pShuttle-CMV-FIRE-1. The bacterial cell line B5183-1AD-1, pretransformed with the plasmid pAdEasy-1, was used for in vivo homologous recombination with pShuttle-CMV-FIRE-1. The pAdEasy-1-FIRE-1 insert containing plasmid was transformed into DH5α and produced in bulk. Purified, pAdEasy-1-FIRE-1 plasmid was used to transfect/infect AD-293 for virus amplification. FIRE-1AdV virus was plaque-purified, amplified, CsCl gradient-purified, and stored at −80 °C.

**COS-1 Cell Transfection**—COS-1 cells were transiently transfected with expression plasmids for FIRE-1 and FIRE-3 using a diethylaminoethyl-dextran method as previously described (28).

**Neonatal Myocyte Isolation and Transfection**—Ventricular neonatal cardiac myocytes were isolated from 1–2-day-old Sprague-Dawley rat hearts by enzyme digestion as described by Griffin et al. (29). Harvested cells were plated in four-well plates on 1% gelatin-coated 25-mm square cover slips (10⁶ − 7 cells/well) and allowed to recover for 24–48 h in plating medium (4 parts Dulbecco’s modified Eagle’s medium/1 part medium 199, 10% horse serum, 5% fetal bovine serum, 1% antibiotic/antimycotic). After recovery, the medium was changed to serum-free, antibiotic-free medium, and the myocytes were transfected with FIRE-1 plasmid following the method supplied by the manufacturer to transfect a 60-mm culture vessel included in the Lipofectamine 2000 (Invitrogen) eukaryotic transfection kit. The cells were incubated (3% CO$_2$ at 37 °C) for 48 h (medium changed after 24 h) prior to imaging.

**Antibodies**—The InsP$_3$R-specific antibodies directed against the amino termini of the InsP$_3$R1, -2 and -3 isoforms (T1NH, T2NH, and T3NH) used in this study have been described previously (16, 30, 31).

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—SDS-PAGE and Western blotting were performed as described previously (28) using 7.5% SDS-polyacrylamide gels. Visualization was accomplished using ECL reagents (Amersham Biosciences).

**In Vitro Fluorescence Measurement**—FIRE-1 and FIRE-3 fluorescence measurements were performed on a Sim Amino xenon lamp spectrometer (SLM Instruments). Monochromator excitation and emission slit widths were set at 4 nm. Excitation light was 415 nm using an excitation monochromator, and the dual photon counting emission detectors were set at 480 (F$_{530}$) and 530 nm (F$_{480}$ respectively. Fluorescence measurements were recorded (at 22 °C) in polystyrene cuvettes containing 1 ml of 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride containing 250–300 μg of COS-1 cell cytosol fraction expressing FIRE-1 or -3. For inositol phosphate binding experiments, increasing concentrations of inositol phosphates were added directly to the sample and rapidly mixed prior to fluorescence emission recording. Inositol phosphate binding affinity ($K_a$) was calculated from changes of Δ(F$_{530}$/F$_{480}$) or percentage changes of F$_{530}$/F$_{480}$ as a function of [InsP$_3$]$_a$ using nonlinear regression analysis conducted with Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

**Adult Cardiac Myocyte Culture and Adenoviral Infection**—Adult cat ventricular myocytes were isolated as previously described (32) and seeded on laminin-coated glass coverslips, and nonadherent cells were removed after 30–45 min. Culture medium consisted of serum-free medium 199 (M199) supplemented with 25 mM NaHCO$_3$, 5 mM creatine, 5 mM taurine, 2 mM carnitine, and 0.1 mM ascorbic acid. Insulin (100 units/ml), 5′-bromo-2′-deoxyuridine (31 μg/ml), bovine serum albumin (0.2%), and 2% penicillin-streptomycin were also added to the medium. The myocytes were then exposed to recombinant replication-deficient adenovirus expressing the FIRE-1 sensor for 2 h at a multi-
plicity of infection of 1–10. The myocytes were subsequently cultured for 24–36 h, and the medium was changed twice daily.

Confocal Microscopy and Patch Clamping—A coverslip with cells expressing the FIRE probe was positioned to the stage of an inverted microscope (Nikon) equipped with a ×40 1.3 NA oil immersion objective lens. The cells were continuously superfused with Tyrode solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM Hepes, pH 7.4 (adjusted with NaOH). Changes in FRET were measured with laser scanning confocal microscopy (Radiance 2000 MP; Bio-Rad, UK). CFP was excited with the 457-nm line of an argon ion laser. CFP and YFP emissions were measured at wavelengths 488 (F₄₈₈) and >530 nm (F₅₃₀), respectively. Changes in InsP₃ activity were defined as the relative change in the ratio F₅₃₀/F₄₈₈ of the background corrected fluorescence intensities measured at the emission wavelengths of CFP and YFP. The experiments were conducted at room temperature.

In experiments where myocytes were dialyzed with InsP₃ (10 μM), the conventional patch-clamp technique was used. Microelectrodes had resistances of 2–4 MΩ when filled with an intracellular solution containing 100 mM potassium glutamate, 40 mM KCl, 1 mM MgCl₂, 4 mM Na₃ATP, 10 mM Hepes, 0.1 mM EGTA, pH 7.2 (adjusted with KOH). The myocytes were voltage-clamped at a holding potential of ~70 mV.

For intracellular calcium measurements ([Ca]ᵢ) cells loaded for 20 min with the membrane permeant fluorescent calcium indicator fluo-4/AM (Molecular Probes/Invitrogen; 20 μM). Fluo-4 was excited with the 488-nm line of an argon ion laser, and emitted calcium-dependent fluorescence was measured at wavelengths >515 nm. [Ca]ᵢ signals are presented as background-subtracted normalized fluorescence (F/F₀), where F₀ is the fluorescence intensity, and F is resting fluorescence recorded under steady-state conditions at the beginning of an experiment.

RESULTS

Construction and Expression of FRET-based Biosensor FIRE—We constructed a set of fluorescent reporter-ligand-binding domain chimeras from the type 1, 2, and 3 InsP₃R isoforms terminally fused with CFP and YFP. The constructs span the receptors ligand-binding core encompassing the amino-terminal 589 residues of the type 1 (SI) spliced form and 604 amino acids for the type 2 and 3 homologues linked amino-terminally with CFP and carboxyl-terminally with YFP. In all cases minimal linker sequences (seven residues CFP-InsP₃R and eight residues for InsP₃R-YFP junctions) were used to join the fluorescent proteins to the InsP₃R-binding core backbone (Fig. 1A). These plasmids are named FIRE with the InsP₃R isoform identified with a numeric suffix (FIRE-1, FIRE-2, and FIRE-3).

COS-1 cells were transiently transfected with the FIRE-1, FIRE-2, and FIRE-3 plasmids, and soluble fractions were examined by western immunoblotting using InsP₃R amino-terminal antibodies specific for the type 1, 2, or 3 InsP₃R (16, 30, 31). In addition, FIRE-1 was also introduced into adult ventricular myocytes using an adenoviral expression system (FIRE-1Adv). As shown in Fig. 1B, all of the constructs express at high levels as soluble proteins with an expected Mr ~118,000.

Calibrations and Selectivity—Fig. 2A shows the [InsP₃]ₜ dependence of the FRET signal (expressed as change in F₅₃₀/F₄₈₈) for both FIRE-1 and FIRE-3 expressed in COS-1 cells. An initial parallel version using the type 2 InsP₃R sequence showed expression as well but was not as good a sensor in initial screening; therefore it was not pursued further here. Cytosolic extracts from FIRE expressing COS-1 cells were suspended in cuvettes and placed in a fluorimeter. Incremental addition of InsP₃ (0–10 μM) resulted in enhanced FRET over a range from 1 nM to 1 μM InsP₃. Both FIRE-1 and FIRE-3 sensors exhibited ~11% change in the fluorescence ratio with apparent Kᵦₜ of 31.3 ± 6.7 nM (n = 7) and 36.4 ± 2.8 nM (n = 4), respectively.

The specificity and selectivity of FIRE-1 to InsP₃ (Ins(1,4,5)P₃) was further examined by characterizing the response to other inositol phosphates, an InsP₃R agonist, and heparin (Fig. 2B). Preincubation of the COS cytosol with heparin (5 mg/ml) followed by the addition of InsP₃ resulted in essentially no change in FRET, confirming that the InsP₃R-induced change in fluorescent ratio is a consequence of InsP₃ binding to the FIRE-1 ligand-binding region. Adenophostin A, a high affinity InsP₃R agonist, showed nearly 11 × higher potency (Kᵦₜ = 2.9 nM, n = 4–8) and slightly less efficacy at increasing FRET (76% of the maximal ΔF₅₃₀/F₄₈₈) measured for Ins(1,4,5)P₃. The inositol phosphates examined (Ins(2,4,5)P₃, which is often used as a more stable, poorly metabolized agonist of the InsP₃R, exhibited ~8-fold lower apparent affinity (250 nM, n = 4) for FIRE-1 than did Ins(1,4,5)P₃. Ins(1,3,4,5)P₄ and Ins(4,5)P₃ induced changes in ΔF₅₃₀/F₄₈₈ with apparent affinities of 563 nM (n = 3) and >14 μM, (n = 4), respectively. The primary cellular degradation product of Ins(1,4,5)P₃ through the activity of InsP₃-5-phosphatase (33), Ins(1,4,5)P₃ did not increase FRET at any concentrations up to 100 μM (n = 4; not shown). L-myo-Inositol (1,4,5)P₃ induced FRET in FIRE-1 with significantly lower potency (n = 4) than the biologically relevant d-myo stereoisomer, consistent with previous reports regarding the stereo specificity of the InsP₃R (34, 35).

The relative potency of these inositol phosphates are very similar to those observed in competition binding assays using NH₄-terminal fragments encompassing the ligand-binding domain of the type 1 and type 2 InsP₃R (36). Additionally the interaction of these inositol phosphates with the ligand-binding region of the FIRE-1 expression product reflects the relative potency of the inositol phosphates in their ability to release calcium from InsP₃-sensitive stores in cultured cells (SH-SY5Y and Swiss-3T3) (37, 38). In those studies Ins(2,4,5)P₃ and Ins(1,3,4,5)P₄ were ~10 and ~18 times less potent than Ins(1,4,5)P₃ in releasing calcium.
Thus, FIRE-1 is an excellent biosensor for signals that are expected to activate the InsP3R.

**Detection of [InsP3] in COS-1 Cells**—The response of FIRE-1- and FIRE-3-transfected COS-1 cells to InsP3 liberating agonists were examined using confocal microscopy. Stimulation of COS-1 cells with ATP (10 μM) reveals oscillatory increases in [Ca2+]i (Fig. 3A) that are mediated by InsP3-dependent calcium release. In parallel experiments, the cells expressing FIRE-1 and FIRE-3 were stimulated with 10 μM ATP (Fig. 3B), resulting in a similar oscillatory increase in FRET assessed by the F530/F488 ratio (excitation at 457 nm). The maximal increase in the F530/F488 ratio induced by ATP or acetylcholine (5–6%; Fig. 3C) would correspond to free [InsP3] in the range of 20–50 nM based on the calibrations in Fig. 2A.

A critical hallmark of FRET is an increase in donor (CFP) fluorescence upon bleaching of the acceptor (YFP). Fig. 4 illustrates that partial photo-bleach of YFP (with the 514-nm laser line; note the 58% decrease in YFP fluorescence) increases CFP donor fluorescence by 12% in a COS-1 cell expressing FIRE-1. Mean values for increased CFP fluorescence after YFP photobleach are 8.9 ± 1.3% (n = 8) and 8.5 ± 1.5% (n = 8) for FIRE-1 and FIRE-3, respectively. These are lower limits for the amount of basal FRET (before [InsP3] increases), because the mean extent of YFP photobleach was only 58.2 ± 3.6% (n = 8), respectively. Correcting for incomplete YFP photobleach, we estimate that the basal extent of FRET is about 22.6 ± 2.1% and 17.2 ± 2.2% for FIRE-1 and -3, respectively.

**Detection of [InsP3] in Cardiac Myocytes**—We next transfected the FIRE-1 plasmid into cultured neonatal rat ventricular myocytes and examined whether the cells exhibited altered FRET upon stimulation with endothelin-1 (ET-1). Although the number of cells transfected was low, the cells expressing FIRE-1 were readily identifiable by their fluorescence, which as in COS-1 cells was cytosolic. Exposure of these neonatal myocytes to 100 nM ET-1 resulted in increased FRET, indicating InsP3 generation (Fig. 5). Compared with untreated controls, 100 nM ET-1 induced an 8% peak increase in the F530/F488 ratio (n = 4; Fig. 5). The InsP3-induced increase in FRET was detected within 20–25 s after addition of ET-1 and reached a maximum within 1–2 min. In the continued presence of ET-1 the F530/F488 ratio leveled off to a sustained
FRET-based InsP₃ Sensor

plateau, suggesting maintained levels of elevated [InsP₃]. Removal of the agonist resulted in a decline of the signal to base-line levels within 1 min.

Similar to our photobleaching experiments in COS-1 cells, FIRE-1 expressed in the neonatal ventricular myocytes also shows comparably increased CFP fluorescence (~11%) after YFP photobleach (data not shown).

Adult ventricular myocytes are a main focus of cardiac research, although work on cultured neonatal rat ventricular myocytes has been used extensively for studying signaling pathways. Adult ventricular myocytes cannot be transfected by plasmids. Therefore, the FIRE-1 coding region was excised from the plasmid vector and introduced into the AdEasy XL adenoviral vector system to produce FIRE-1AdV. Infection of adult cat ventricular myocytes resulted in the expression of a protein with a molecular mass ~118 that immuno-reacts with our type 1-specific amino-terminal antibody (T1NH) (Fig. 1 right panel). This expression product is soluble and essentially indistinguishable from the plasmid-based FIRE-1 expression products. Unlike COS-1 or neonatal myocytes, expression of FIRE-1 in adult cells revealed some sequestration of the sensor to the nuclei (Fig. 6A). Whether this apparent nuclear enrich-

ment results from fluorescent protein overexpression or another feature unique to expression of this chimera in this cell type is unknown.

In FIRE-1 expressing adult myocytes, we evaluated increased CFP fluorescence upon YFP photobleach, with or without saturating amounts of InsP₃ (Fig. 6). As observed in COS-1 cells, YFP photobleach (by 47% in the example shown in Fig. 6A) resulted in an increased CFP fluorescence by 8%. To elevate [InsP₃] and saturate FIRE-1, adult myocytes were patch-clamped with pipettes containing 10 µM InsP₃, such that upon rupture of the patch InsP₃ diffused into the cell (Fig. 6B; see below). In this cell dialyzed with InsP₃, a 41% bleach of YFP resulted in increase of CFP fluorescence by 13%. Mean data from these experiments (Fig. 6C) show that for a comparable extent of YFP photobleach (51.9 ± 2.3%; n = 7 and 57.0 ± 1.6%; n = 5, respectively), there was considerably greater increase of CFP fluorescence at high [InsP₃] (11.8 ± 1.2%) versus 5.4 ± 1.0%). This confirms that increased [InsP₃] augments FRET in FIRE-1 in vivo in adult cardiac myocytes.

Fig. 7A shows the influence of three G-protein coupled receptor agonists (ET-1, phenylephrine, and angiotensin II) to induce increases in [InsP₃], in adult cat ventricular myocytes expressing FIRE-1. All three agonists induced rapid and readily detectable increases in free [InsP₃] in adult cardiomyocytes. ET-1 tended to produce the fastest increases (note time bars), but phenylephrine at 10 µM produced the largest increase in [InsP₃]. To assess the dynamic range of FIRE-1 in these adult cardiac myocytes, we dialyzed the cell (via patch pipette) with either InsP₃-free solution or 10 µM InsP₃ (Fig. 7B). Having no InsP₃ in the pipette did not significantly affect the F₅₃₀/F₄₈₈ ratio, suggesting that resting [InsP₃] may be low. In contrast, internal perfusion with 10 µM InsP₃ increased the ratio in cells by 13.2 ± 0.9% (n = 6) (comparable with our in vitro calibrations in Fig. 2). Assuming the Kᵣ from Fig. 2, this would correspond to increases of [InsP₃], in the range of 10–30 nM with these three agonists in adult ventricular myocytes. Fig. 7C summarizes the average changes in InsP₃ (F₅₃₀/F₄₈₈) elicited with G-protein coupled receptor agonists and InsP₃ perfusion, respectively, in adult ventricular myocytes. These results demonstrate the ability of FIRE-based FRET sensors to detect temporal changes in [InsP₃] in isolated cells and underscore the utility of this indicator to measure InsP₃ levels in real time.

Spatially Resolved [InsP₃] Signals—FIRE-1 can also provide spatially resolved information concerning InsP₃ signaling. The top panel of Fig. 8A shows the spatial distribution of FIRE-1 in an adult cat ventricular myocyte (excitation, 457 nm; emission, >530 nm). In this experiment the FIRE-1-expressing myocyte was internally dialyzed with InsP₃ via a patch pipette containing 10 µM InsP₃. The pseudocolor image represents spatially resolved local F₅₃₀/F₄₈₈ ratio (reflecting [InsP₃]), recorded 5 min after the membrane patch was ruptured, allowing InsP₃ dialysis. The line trace in Fig. 8A shows the longitudinal profile of F₅₃₀/F₄₈₈ 5 min after patch rupture. As expected, [InsP₃] was highest directly beneath the pipette and declined as a function of distance from the InsP₃ source. Fig. 8B shows the time course of changes of [InsP₃] in four regions of interest (ROI) (1) under the pipette (black), (2) in a nearby nucleus (blue), (3) cytosol just beyond the nucleus (red), and (4) distant cytosol (green) as a function of time. Clearly the rise in [InsP₃] is slower in more distant regions, and notably the rise in the nearby nucleus (2) is no faster than a more distant cytosolic ROI (3). This indicates that the nuclear envelope slightly retards InsP₃ diffusion. The inset in Fig. 8B plots the half-time (t₁/₂) of rise of local [InsP₃], as a function of distance from the pipette (two experiments). The t₁/₂ increases roughly linearly as a function of distance in the cytosol (open symbols), whereas InsP₃ diffusion into the nucleus was substantially delayed (filled symbols) compared with the cytosol. This further indicates slower InsP₃ diffusion into the nucleus than along the cytosol.
We also used a more physiological stimulus to explore spatiotemporal differences in [InsP$_3$]$_i$ between the nucleus and cytosol. Fig. 9 shows an intact adult ventricular myocyte expressing FIRE-1 upon exposure to ET-1 (100 nM). Changes in [InsP$_3$]$_i$ [$F_{530}/F_{488}$ ratio] were followed in the cytoplasm (white oval in Fig. 9A) and the nucleus (black oval). ET-1 caused [InsP$_3$]$_i$ to rise more rapidly and to higher levels in the cytoplasm as compared with the nucleus (Fig. 9B). Average data indicate that the amplitude of rise in nuclear [InsP$_3$]$_i$ was 70 ± 9% ($n = 6$) of that in the cytosol, and the $t_{1/2}$ was about twice as long (Fig. 9C). The data from Figs. 8 and 9 suggest that InsP$_3$ can diffuse over long distances in the cytosol, that the nuclear pores slows down this diffusion into nuclei, and that neurohumoral activation of InsP$_3$ in intact ventricular myocytes causes [InsP$_3$]$_i$ to rise rapidly in both cytosol and nucleus.

**DISCUSSION**

The second messenger InsP$_3$ occupies a central position in the initiation and propagation of intracellular calcium release through InsP$_3$Rs that regulate a myriad of cellular events. Until very recently the real time analysis of InsP$_3$ liberation, concentration dynamics, and spatial distribution in a living cell have not been possible, and measurements have relied upon destructive methodologies (5–8). These methods unfortunately are of limited utility in deciphering the spatiotemporal organization of this second messenger system at the cellular and subcellular level. Recently, novel fluorescent probes have been developed to study intracellular InsP$_3$ dynamics. For example, a type 3 InsP$_3$R-derived biosensor called LIBRA was employed to measure InsP$_3$ concentrations in SH-SY5Y cultured cells, which represents an important first step in the generation of physiologically relevant reagents to evaluate InsP$_3$ in living cells (11). Additionally, a pleckstrin homology domain from phospholipase C81-GFP fusion was developed (14) to evaluate plasma membrane phosphatidylinositol 4,5-bisphosphate concentration dynamics using a cytoplasmic translocation assay, and it was found that this construct bound InsP$_3$ at high affinity and could be used to estimate [InsP$_3$]$_i$. With a similar approach agonist-induced oscillatory changes of [InsP$_3$]$_i$ could be measured in Chinese hamster ovary cells (13). Although these approaches represent remarkable progress for the study of the subcellular dynamics of InsP$_3$ signaling, they either lack specificity and sensitivity, were only assessed in generic cultured cells, or are restricted to the measurement of [InsP$_3$]$_i$ in specific subcellular (e.g., membrane-bound) domains of the cell.

In this study we report the construction and characterization of prototypic fluorescent biosensors that allow quantitative measurement of cellular InsP$_3$ levels in a living cell with temporal and spatial resolution. These sensors utilize the InsP$_3$R type 1 and type 3 ligand-binding domain s expressed as chimeras terminally linked to CFP and YFP fluorescent proteins. These biosensors have been coined FIRE. They are expressed as soluble proteins and are uniformly distributed throughout the cytoplasm of all cells tested. Our in vitro fluorimetric characterization of FIRE-1 and FIRE-3 revealed that they respond by exhibiting increased FRET upon incremental additions of InsP$_3$ with an enhanced dynamic range and with a superior sensitivity (~12 times higher apparent affinity) than the previously described LIBRA sensor (11). At variance with FIRE, the LIBRA sensor exhibits a decrease in FRET in response to InsP$_3$ binding, whereas our sensor demonstrates an increase in FRET. Perhaps this is a consequence of the plasma membrane targeting sequence present in LIBRA, and membrane insertion results in a conformation that positions the two fluorophors in proximity, which is reduced upon ligand binding. Both FIRE-1 and
**FRET-based InsP₃ Sensor**

**FIGURE 7. Agonist-induced changes of [InsP₃] in adult ventricular myocytes.** A, adult ventricular myocytes were stimulated with ET-1 (100 nM; top panel), phenylephrine (Phe, 10 μM; middle panel), and angiotensin II (Ang II, 10 μM; bottom panel). B, internal perfusion of ventricular myocytes with an internal solution containing a saturating concentration of InsP₃ (10 μM) and internal solution containing no InsP₃. C, average percentage changes in [InsP₃] ([F₁/F₄₀₈/F₄₈₈] ratio image) elicited by agonist stimulation or internal perfusion with or without InsP₃. The numbers in parentheses indicate number of individual cells tested under each experimental condition.

FIRE-3 have very similar concentration-dependent FRET responses, even though the reported apparent affinities for InsP₃ to InsP₃R1 and InsP₃R3 differ considerably (39). However, those affinity measurements were made in the context of either larger fragments or whole receptor protein and not chimeric assemblies terminally linked to fluorescent proteins.

Analysis of the response of FIRE-1 to other inositol phosphates reveals that they react very similarly to the intact InsP₃R. The two primary products of cellular InsP₃ metabolism, Ins(1,3,4,5)P₄ and Ins(1,4)P₂, as well as the other inositol phosphates examined (Ins(2,4,5)P₃ and Ins(4,5)P₂) interacted with FIRE-1 consistent with previous competition binding and calcium release studies (35–37, 40). As expected, Ins(1,4)P₂, which has no activity in calcium signaling (41), did not induce FRET in cytosolic extracts expressing FIRE-1. The other major metabolite of InsP₃ via the 3-kinase, Ins(1,3,4,5)P₄, induced an increase in FRET with FIRE-1 yet at a significantly lower apparent affinity (563 nM) compared with InsP₃ (~31 nM). The likelihood that [InsP₃]ₜ is overestimated as a consequence of Ins(1,3,4,5)P₄ accumulation, for example in the agonist-induced FRET whole cell experiments, is low because the predominant pathway for InsP₃ degradation is through the 5-phosphatase. Although the 3-kinase has a relatively high affinity for InsP₃ (submicromolar to low micromolar range), its Vₘₐₓ is significantly less than the 5-phosphatase (33). Furthermore, the rapid decline of the FRET signal after removal of the agonist ET-1 (e.g. Fig. 5) strongly suggests that FIRE-1 reports changes in [InsP₃] rather than accumulating InsP₃ degradation products. Accordingly, the specificity of the FIRE sensor is high and effectively mimics the same specificity and selectivity for InsP₃ that the intracellular receptor (InsP₃R) exhibits. Thus, the FIRE-1 sensor cannot only be used to evaluate cellular [InsP₃] but may be of significance as an indicator of InsP₃R activation independent of calcium release because of altered channel gating.

We have demonstrated the effectiveness of FIRE in measuring the liberation of InsP₃ in culture cell lines as well as in acutely isolated cells. In addition to the quantitative qualities of this indicator, our results show that these sensors are readily able to resolve the temporal nature of agonist-induced InsP₃ generation. In the cellular context these sensors have a very similar dynamic range to that observed in vitro.

Our InsP₃ perfusion and agonist stimulation experiments in adult cardiac myocytes infected with FIRE-1 AdV demonstrate that we cannot...
only deduce [InsP$_3$]$_0$, but we can measure the spatial properties of the second messenger signal as a function of the ubiquitous distribution of FIRE in the cell. This is considered advantageous over the membrane-associated LIBRA expression product (11) or the pleckstrin homology domain–GFP construct (13) that relies on translocation of the indicator from the plasma membrane to the cytosol bound to InsP$_3$, which may impinge upon the second messengers diffusion. Our results demonstrating the diffusional properties of InsP$_3$ in myocytes (Figs. 8 and 9) also reveal that InsP$_3$ diffuses into the nucleus with a delay and reaches lower levels than in the cytosol. These observations suggest that the spatiotemporal pattern of nuclear InsP$_3$ signaling differs from the cytosol. This observation is an important finding with regard to the specific role of InsP$_3$R type 2 localized to the nuclear envelope. For example, on the nuclear envelope InsP$_3$R2 associate with calcium/calmodulin-dependent protein kinase II$_{b}$, and this protein complex has been implicated in specific functions in cardiac myocytes (27). In cardiac muscle, for example, FIRE sensors will be important tools to study the kinetics and localization of [InsP$_3$] during events that induce translocation of the transcription factor histone deacetylase as well to characterize spatiotemporal patterns of InsP$_3$ production involved in neurohumoral stimulation of cardiac myocytes during excitation-contraction coupling and nuclear factor of activated T cell (NFAT) translocation in hypertrophy and heart failure. In other cells where InsP$_3$ directs oscillatory calcium transients (e.g. Fig. 3), this sensor will allow determination of whether the calcium oscillations are driven by InsP$_3$ oscillations (42–44). The current FIRE constructs are capable of being subcellularly targeted to discreet localizations for measurement of InsP$_3$ generation in specific subcellular microdomains or as a complementary spatiotemporal indicator relative to other indicators (e.g. calcium indicators to characterize the interplay between calcium and InsP$_3$ signaling pathways).

In summary, we have produced an intracellular FIRE-based sensor capable of measuring InsP$_3$ concentration in living cells with high sensitivity and spatiotemporal resolution. These biosensors are invaluable novel tools to study the microdomains of the phosphoinositide second messenger signaling cascade.

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