Short communication

CLONING AND EXPRESSION OF A NEW INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR TYPE 1 SPLICE VARIANT IN ADULT RAT ATRIAL MYOCYTES

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Abstract: Inositol 1,4,5-trisphosphate receptor type 1 (IP_3R1) is already known to be highly expressed in the brain, and is found in many other tissues, including the atrium of the heart. Although the complete primary structure of IP_3R1 in the rat brain has been reported, the complete sequence of an IP_3R1 clone from atrial myocytes has not been reported. We isolated an IP_3R1 complementary DNA (cDNA) clone from isolated adult rat atrial myocytes, and found a new splice variant of IP_3R1 that was different from a previously reported IP_3R1 cDNA clone obtained from a rat brain (NCBI GenBank accession number: NM_001007235). Our clone had 99% similarity with the rat brain IP_3R1 sequence; the exceptions were 39 amino acid deletions at the position of 1693-1731, and the deletion of phenylalanine at position 1372 that lay in the regulatory region. Compared with the rat brain IP_3R1, in our clone proline was replaced with serine at residue 2439, and alanine was substituted for valine at residue 2445. These changes lie adjacent to or within the fifth transmembrane domain (2440-2462). Although such changes in the amino acid sequences were different from the rat brain IP_3R1 clone, they were conserved in human or mouse IP_3R1. We produced a plasmid construct expressing the atrial IP_3R1 together with green fluorescent protein (GFP), and successfully overexpressed the atrial IP_3R1 in the adult atrial cell line HL-1. Further investigation is needed on the physiological significance of the new splice variant in atrial cell function.

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INTRODUCTION

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are ubiquitous intracellular Ca^{2+} release channels that are regulated by Ca^{2+} and IP_3, and mediate the rise in intracellular Ca^{2+} in response to the receptor-activated production of IP_3 [1]. In the heart, IP_3 is generated by many neurohumoral agonists including acetylcholine [2], endothelin, catecholamines, prostaglandins [3], purines and angiotensin II [4, 5] via the activation of phospholipase C, and it activates IP_3Rs. So far, three subtypes of IP_3R (IP_3R1, IP_3R2 and IP_3R3) have been found in mammalian tissues [6, 7]. The most widely studied IP_3R is type 1 IP_3R. IP_3R1 is highly expressed in the brain [8] and also has been detected in vascular smooth muscle, the vas deferens, the heart, and the kidney [9-12].

In the heart, IP_3R1 is expressed in atrial myocytes, and not expressed in ventricular myocytes [12, 13]. IP_3R1 was first purified [14] and cloned [15, 16] from rodent cerebellum. The IP_3R1 isolated from rodents has 2749 amino acids, and can be divided into three functionally distinct regions: the N-terminus, which has an IP_3 binding domain; the channel-forming region localized to the C-terminus; and a large regulatory region connecting the two termini [17]. Different splice variants of IP_3R1 (SI, SII, and SIII) are expressed in specific regions of the brain at different stages of neuronal development, and also in non-neuronal tissues [18]. The expression of each splice variants is regulated in a region-specific and age-specific manner [19]. However, the complete complementary DNA (cDNA) sequence of IP_3R1 from atrial cells has never been reported, and little is known about the heterogeneity of the atrial IP_3R1 in comparison with IP_3R1 expressed in other tissues or other species. The main purpose of our study is to clone complete cDNA, encoding an IP_3Rl from rat atrial myocytes, to analyze its difference from already reported clones of IP_3R1, and to express it in cardiac myocytes for functional study.

MATERIALS AND METHODS

Isolation of cardiac myocytes
Rat atrial myocytes were enzymatically isolated from male Sprague Dawley rats (200-300 g) as described previously [20]. Briefly, rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, *i.p.*), their chest cavities were opened, and the hearts were excised. This surgical procedure was carried out in accordance with the university's ethical guidelines. The excised hearts were retrogradely perfused at 7 ml/min through the aorta, first for 3 min with a Ca^{2+}-free Tyrode solution composed of (in mM) 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, 10 glucose, and pH 7.3, at 36.5°C; and then with a Ca^{2+} free Tyrode solution containing collagenase (1.4 mg/ml, Type 1, Roche) and protease

(0.14 mg/ml, Type XIV, Sigma) for 12 min; and finally with Tyrode solution containing 0.2 mM $CaCl_2$ for 8 min. The atria of the digested heart were then cut into several sections and subjected to gentle agitation to dissociate the cells. The freshly dissociated cells were stored at room temperature in Tyrode solution containing 0.2 mM $CaCl_2$.

Gene amplification, cloning and sequencing
Total RNA was prepared from freshly isolated atrial myocytes using the TRIzol reagent (Invitrogen Co., USA). cDNA was synthesized from total RNA using PrimeScript reverse transcriptase (Takara Bio Inc., Japan), following the manufacturer's instructions, with 100 ng oligo-dT as a primer (19 bp; Bioneer Co., South Korea). The RNA samples were denatured at 65°C for 5 min, reverse transcription (RT) was performed at 42°C for 2 h, and then samples were heated at 70°C for 20 min.
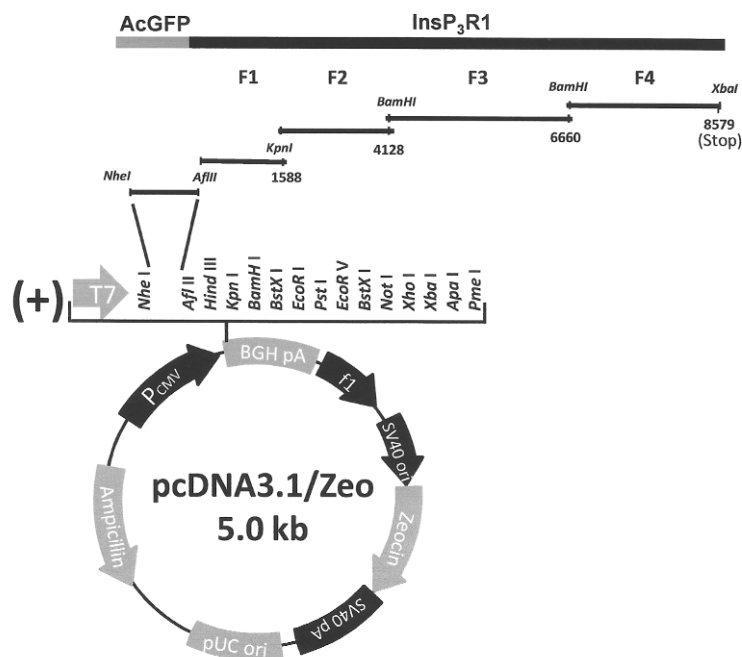

Fig. 1. Insert fragments and cloning strategies for AcGFP-IP$_3$R1 cloning. AcGFP (gray) was inserted in between the *Nhe*I and the *Afl*II sites of pcDNA3.1/Zeo vector as described in the 'Materials and Methods' section. IP$_3$R1 fragments were sequentially (F1, F2, F4 and F3) inserted in between the *Afl*II and the *Xba*I sites. Restriction endonuclease sites used for cloning and corresponding nucleotide numbers in rat IP$_3$R1 are shown.

To construct the expression vector for AcGFP–IP$_3$R1 (Fig. 1), the 5´-untranslated region (containing Kozak's sequence) and the coding sequence of AcGFP (*Aequorea coerulescens* green fluorescent protein) was amplified by PCR with primers 5'-TCC<u>GCTAGC</u>GCTACCGGTC-3' and 5'-GAG<u>CTTAAG</u>CTTG

TACAGCTCATCCATG-3' (the underlined regions indicate *Nhe*I and *Afl*II restriction endonuclease sites respectively), using pAcGFP-C3 (Clontech, Palo Alto, CA, USA) vector as a template. The PCR was performed at 94°C for 30 s, 58°C for 30 s, and 72 °C for 1 min for a total of 30 cycles. The PCR product was digested with *Nhe*I and *Afl*II and cloned into the *Nhe*I and *Afl*II sites of the pcDNA3.1/Zeo+ (Invitrogen, Carlsbad, CA, U.S.A.) vector. The rat atrial IP_3R1 cDNA fragments (F1-F4, Fig. 1) were amplified by PCR with primers that were designed based on the published sequence of rat IP_3R mRNA (NCBI GenBank accession No. NM_001007235). These fragments of IP_3R1 cDNA were digested with appropriate restriction enzymes, purified via agarose gel, and sequentially cloned into the pcDNA3.1/Zeo$^+$/AcGFP vector. The general PCR conditions included 2 µl of the reverse transcription products, each corresponding to 200 ng of the initial total RNA, 0.2 mM of dNTP, PrimeSTAR buffer, PrimeSTAR HS-DNA polymerase (Takara Bio Inc.), and each primer in a total volume of 25 µl. The cDNA samples were initially denatured at 98°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 3 min, and a 72°C final extension for 10 min. After cloning of each fragment, the plasmid was purified and the inserted region was sequenced (Genotech, South Korea). Sequencing primers included CMV-forward and BGH-reverse, as well as synthesized oligonucleotides corresponding to the published sequence of rat IP_3R mRNA. Plasmid DNAs from at least two clones were used for sequencing after cloning of each fragment. The whole IP_3R1 region was re-sequenced after insertion of all the fragments including AcGFP.

To check the homogeneity of the mutation and splice sites, the desired region of IP_3R1 was PCR-amplified through the use of primers flanking the region and atrial cDNA as a template. The reaction products were separated by electrophoresis via 1% agarose gel in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and viewed by Gel Doc 1000. The image density of each PCR product fragment was compared with the density of co-amplified β-actin to determine the ratio of amplification. In addition, the PCR products were subjected to agarose gel purification and sequencing (Genotech, South Korea).

Analysis of DNA and amino acid sequences
Nucleotide and amino acid sequence alignments were done using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2) and ClustalX2 program [21]. The nucleotide sequences of the Type 1 IP_3Rs of various species described in this paper were deposited in the GenBank NCBI Data Bank under the accession numbers NM_001007235 (*Rattus norvegicus*), NM_001099952 (*Homo sapiens*), and NM_010585 (*Mus musculus*).

Cell culture, DNA transfection, and Western blotting
HL-1 cardiomyocytes were obtained from Dr. W. C. Claycomb (Louisiana State University Health Science Center, USA), who first established and characterized the cell line [22]. HL-1 cells were grown onto a matrix of gelatin (0.02%, Difco)

plus fibronectin (12.5 µg/ml, Sigma), in Claycomb medium (JRH Biosciences, USA) supplemented with 10% fetal bovine serum (Sigma), 2 mM L-glutamine (Life Technologies), 0.1 mM norepinephrine (Sigma), and 100 U/ml penicillin-streptomycin (Life Technologies). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For the transfection experiments, cells were grown to a 70-80% confluence (~24 hours) and transfected by using LIPOFECTAMINE^{TM} 2000 reagent (Invitrogen), according to the manufacturer's instructions. The transfected cells were dissolved in a SDS lysis buffer, and 15 µg of total protein was run on 6% SDS-polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane, and the blots were sequentially probed with IP$_3$R1 (Millipore) and α-actinin (loading control, Santa Cruz) antibodies.

Confocal microscopy
We used a confocal microscopy system (A1 Nikon, Japan) to visualize expression of GFP-tagged IP$_3$R1 in HL-1 cells. To measure GFP fluorescence, the cells were exposed to a 488-nm excitation beam (Ar laser). Emission at > 510 nm was imaged using NIS-elements AR (Nikon) software. Images were captured at 512×512 pixel resolution. To localize the nucleus, the cells were incubated with 2.5 µM syto-11 for 30 s after GFP imaging. Syto-11 was also excited by a 488-nm laser with much lower intensity, and its emission was detected by low PMT level, where the GFP signal in the same cell was hardly observed.

RESULTS AND DISCUSSION

Cloning of IP$_3$R1 cDNA from rat atrial myocytes
The complete IP$_3$R1 cDNA clone from rat atrial myocytes was subcloned into the pcDNA3.1/Zeo vector. Briefly, total RNA was prepared from rat atrial myocytes and RT- PCR was performed by using primers that were designed from the rat IP$_3$R1 mRNA sequence (NCB1 GenBank accession No. NM_001007235) to obtain various IP$_3$R1 fragments (Fig. 1, F1~F4). These fragments (F1~F4) were sequentially inserted in between the *Afl*II and the *Xba*I sites of the pcDNA3.1/Zeo vector as shown in Fig. 1. AcGFP was also inserted in between the *Nhe*I and the *Afl*II sites of the pcDNA3.1/Zeo vector (Fig. 1). The full length of our cloned IP$_3$R1 cDNA sequence had 8130 base pairs. The deduced polypeptide sequence contained 2709 amino acid residues with a calculated molecular mass of ~308 kDa (http://expasy.org/tools/pi_tool.html).

Sequence analysis of IP$_3$R1 cDNA from rat atrial myocytes
After successful cloning of IP$_3$Rl originating from adult rat atrial myocytes, the alignments of nucleotide and amino acid sequences were performed using the "ClustalW" program (http://www.ebi.ac.uk/Tools/msa/clustalw2) and "ClustalX2" software [21]. The amino acid sequence of our cloned IP$_3$R1 has 99% identity with the sequences of both mouse and rat IP$_3$R1s [15, 16]. In fact, the already cloned IP$_3$R1 from rat brain shared approximately 70% and 62% identity with the rat type 2 and type 3 IP$_3$Rs, respectively [23, 24]. Interestingly, our cDNA

clone from rat atrial myocytes encoded a new splice variant of IP$_3$R1 (*i.e.*, SI+, SII−, and SIII−; Fig. 2), which was different from the previously reported IP$_3$R1 cDNA clone from rat brain [16]. Our clone had 99% similarity with the rat brain IP$_3$R1 sequence, with no changes at SI+ and SIII− sites. However, our clone had 39 amino acid deletions at the position of 1693-1731 (SII region) (Fig. 2A), and deletion of phenylalanine at position 1372 that lay in the regulatory region (Fig. 2B). Our sequence data on the lack of a splicing segment SII (SII− subtype) (Fig. 2A, "Cloned IP$_3$R1") was in agreement with the previous observation that the SII+ subtype exists exclusively in neuronal tissues and SII− subtype exists in non-neuronal tissue [25]. In addition to these changes, it had replacement of proline with serine at residue 2439 and substitution of alanine with valine at residue 2445 of rat brain IP$_3$R1 (Fig. 2C). These changes lay adjacent to or within the fifth transmembrane domain, TM5 (2440-2462 of rat brain and mouse IP$_3$R1, 2400-2422 of cloned IP$_3$R1, 2401-2423 of human IP$_3$R1). Interestingly, these specific changes in our aforementioned rat atrial IP$_3$R1 cDNA clone were highly conserved in human and mouse IP$_3$R1.
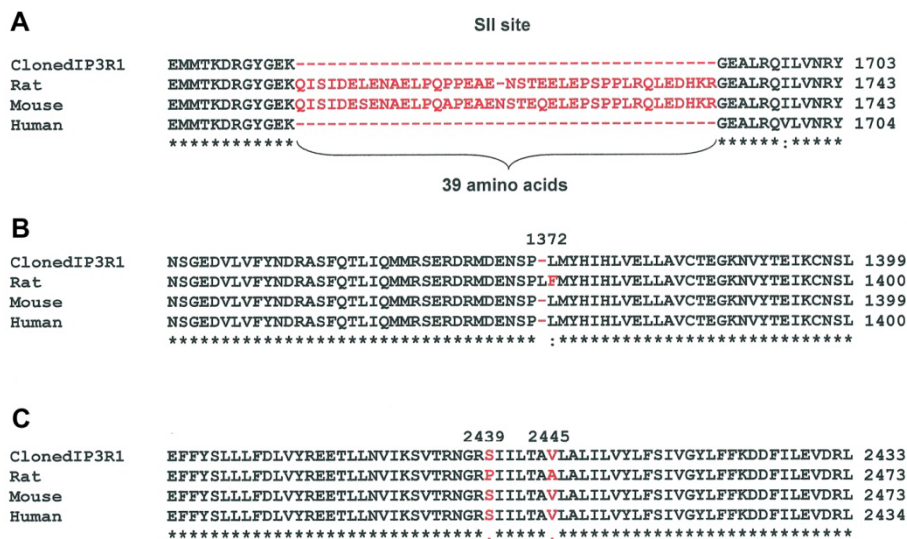

Fig. 2. Multiple amino acid sequence alignment of rat atrial IP$_3$R1 clone with published sequences of rat, mouse and human IP$_3$R1s. A − Splice II site. Our clone lacks the SII region (SII−). B − Phenylalanine deletion site in our clone at 1372 amino acid position. C − Replacement of proline with serine at residue 2439, and substitution of alanine with valine at residue 2445.

In order to further confirm the amino acid changes in our clone, we designed forward and reverse primers approximately 250-300 base pairs away from the deletion and substitution sites. RT-PCR was carried out using the cDNA pool obtained from adult rat atrial myocytes. The PCR products were subjected to agarose gel electrophoresis as shown in Fig. 3A. Sequencing for the PCR

products was performed after purification. The individual peaks of each nucleotide were analyzed carefully using "Chromas Lite" software. The sequencing results obtained from the PCR products reconfirmed the amino acid deletion and replacement sites (Fig. 3B and 3C).
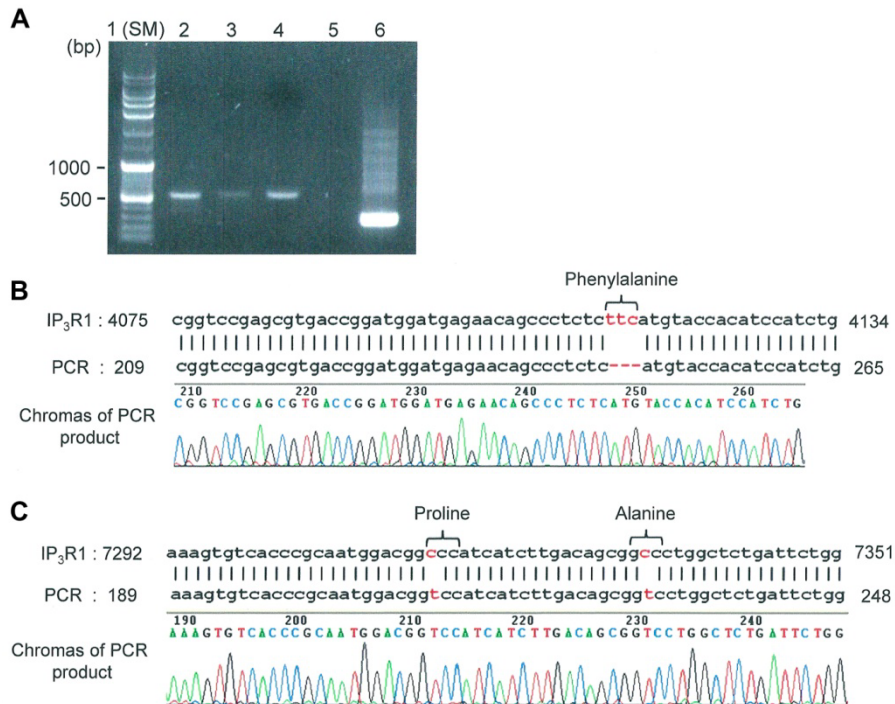

Fig. 3. Homogeneity of the splice and mutation sites in rat atrial myocytes. A – Agarose gel electrophoresis of PCR products of splice and mutation sites from rat atrial IP$_3$R1 cDNA. Lane 1: DNA size marker (base pair). Lane 2: 2439 and 2445 amino acid substitution sites (540 bp). Lane 3: Phenylalanine deletion site at 1372 position. Lane 4: SII− sites (582 bp). (lane 5: none). Lane 6: β-actin (300 bp) as control. B-C – Confirmation of deletion and substitution sites by sequencing PCR products derived from rat atrial cDNA. B – Confirmation of deletion of 3 nucleotides from PCR product of atrial cells' cDNA. An amino acid, phenylalanine, is missing at 1372 position due to deletion of 3 nucleotides which lie in the regulatory region. No additional peaks were seen in the deletion site, indicating the homogeneity of the phenylalanine deletion mutation in rat atrial myocytes. C – Confirmation of replacement of proline with serine at residue 2439 and substitution of alanine with valine at residue 2445. No additional peaks were seen in the replacement/substitution sites, indicating the homogeneity of those mutations in rat atrial myocytes.

Expression of cloned IP$_3$R1 in atrial cells
Since introduction of the plasmid into intact atrial cells is technically difficult, we used an HL-1 atrial cell line to express the cloned rat atrial IP$_3$R1. HL-1 cells are autorhythmic cells that beat spontaneously, continuously divide, and retain an adult atrial phenotype in culture [22]. It has been thought that this cell line is

a suitable adult cardiac culture system for the study of cardiac cell function using genetic manipulations [22]. It has been previously reported that HL-1 cells endogenously express type 1 and type 2 IP$_3$Rs as well as ryanodine receptors [26]. We transiently transfected our cloned IP$_3$R1 expression plasmid in HL-1 cardiomyocytes and successfully overexpressed our clone in HL-1 cells (Fig. 4). Since HL-1 cells expressed IP$_3$R1 endogenously, protein bands for IP$_3$R1 were also observed in control lanes (lane 1 and lane 2, Fig. 4). Additionally, a prominent increase of IP$_3$R1 protein took place in IP$_3$R1 plasmid transfected cells (lane 3 and lane 4, Fig. 4). No such changes in band intensity were observed in the case of α-actinin.
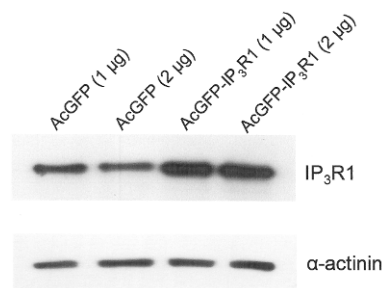

Fig. 4. Overexpression of IP$_3$R1 in HL-1 atrial myocytes. Representative immunoblots showing expression level of IP$_3$R1 in HL-1 cells transfected with IP$_3$R1 expression plasmids for 24 hours. α-actinin was used as a loading control. The cells were dissolved in an SDS containing buffer, and 15 μg of total protein was run on 6% SDS-polyacrylamide gel. Blots were sequentially probed with IP$_3$R1 (*upper* panel) and α-actinin (*lower* panel) antibodies. Since HL-1 cells expressed IP$_3$R1 endogenously, protein bands for IP$_3$R1 were also observed in the control lane, but there was an increase of band intensity in InsP$_3$R1 plasmid transfected lanes. No such changes in band intensity were observed in the case of α-actinin. "AcGFP": HL-1 cells transfected with pcDNA3.1-AcGFP plasmid DNA. "AcGFP-IP$_3$R1": HL-1 cells transfected with pcDNA3.1-AcGFP-IP$_3$R1 plasmid DNA.

We next confirmed the overexpression of IP$_3$R1 in HL-1 cells by visualizing GFP that is co-expressed with IP$_3$R1 using confocal microscopy (Fig. 5). The GFP fluorescence in IP$_3$R1-AcGFP transfected cells was highly localized to the peri-nuclear region, while that in AcGFP transfected (wild-type) cells was evenly observed throughout the cytoplasm (Fig. 5). The localization of GFP fluorescence in the peri-nuclear region is consistent with peri-nuclear localization of endogenous IP$_3$R1 in HL-1 atrial cells [12].

The expression levels of different IP$_3$R subtypes vary in different regions of the heart (e.g., atrium, ventricle, and conduction tissue cells) and cardiac patients. Type 2 IP$_3$R is the major subtype expressed in adult and neonatal ventricular myocytes and in atrial myocytes [27-29]. The type 1 IP$_3$R was detected exclusively in atrial tissue [30], rat atrial myocytes [26], and rat Purkinje cells [31]. The expression of IP$_3$R1 is higher in the human atrium in cases of chronic atrial fibrillation [30] and in the left ventricle of patients with cardiomyopathy (when compared to patients who do not have cardiomyopathy) [32].
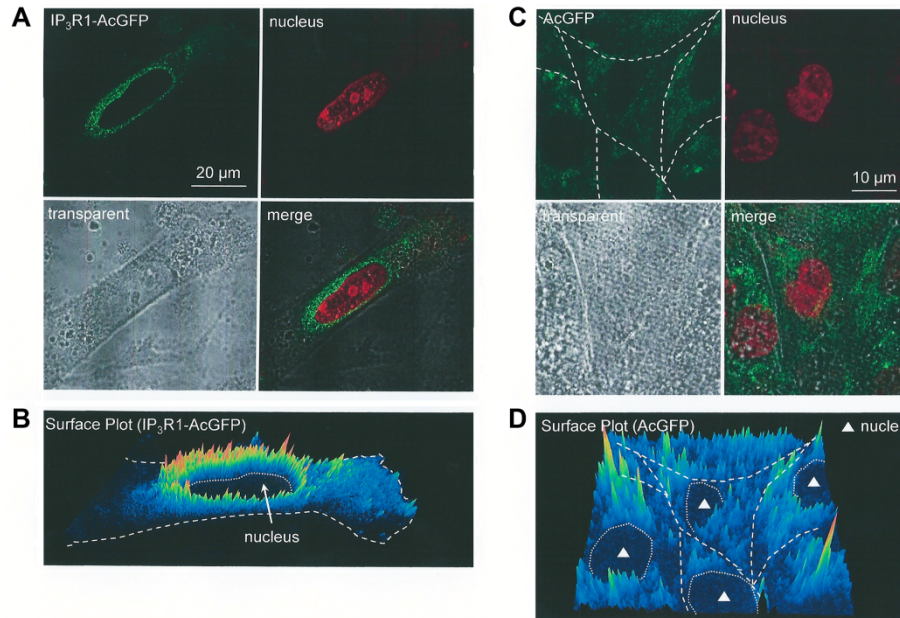
Fig. 5. Peri-nuclear localization of GFP-linked IP$_3$R1s in IP$_3$R1-AcGFP transfected HL-1 cells. After green fluorescence was imaged (A and C, *upper left*), the nucleus specific fluorescence dye syto-11 was added to the same cells to localize the nucleus (A and C, *upper right*; red). Merged images show a clear difference in the subcellular distribution of the GFP signal between wild-type (WT) and overexpressed cells. Panels B and D show the surface plot of the GFP fluorescence images captured from WT ("IP$_3$R1-AcGFP") and overexpressed ("AcGFP") HL-1 cells.

Function of IP$_3$R1 and its splice variant

The specific function of IP$_3$R1 is largely unknown in atrial myocytes and in cardiac cells under pathologic conditions. Regarding this, the spontaneous Ca^{2+} sparks in the peri-nuclear regions of atrial cells have been found to be suppressed in HL-1 atrial cells, in which IP$_3$R1 was knocked down [26]. The approach of overexpressing IP$_3$R1 in HL-1 cells may be used to further characterize and understand pathophysiological functions of the IP$_3$R1 in atrial myocytes.

Although we found some unique deletions or substitutions of a single amino acid in our rat atrial IP$_3$Rl clone compared with rat brain IP$_3$R1 (Fig. 2B and 2C), the changes in amino acids were not observed in crucial regions, such as the N-terminal IP$_3$ binding domain, the regulatory domain, and more importantly in the channel-forming domain. However, a possible role of those changes in single amino acids in the regulation of IP$_3$R1 may not be completely excluded. Interestingly, however, the SII site of alternative splicing is located in the coupling domain of IP$_3$R1 [6]. The excision of the SII insert is known to change the protein kinase A phosphorylation pattern of IP$_3$R1 [25, 33] and create additional ATP and calmodulin binding sites in the IP$_3$R1 sequence.

A functional difference between IP$_3$R1-SII(−) and IP$_3$R1-SII(+) has also been found by Tu et al. (2002) [34]. They have demonstrated that IP$_3$R1-SII(−) has a higher conductance and different regulation by ATP compared with IP$_3$R1-SII(+) using lipid bilayer experiments [34].

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