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- Note -

Cellular Localization and Translocation of Duplication and Alternative Splicing Variants of Olive Flounder Phospholipase C-δ1

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The purpose of this study was to investigate the cellular characterization of phospholipase C-δ1 in olive flounders (*Paralichthys olivaceus*). In general, phospholipase C signaling pathways are distributed in nuclei at plasma membranes and in cytoplasms, although the pathways' nuclear localization mechanisms are unclear. *P. olivaceus* duplicates type-A PoPLC-δ1 (PoPLC-δ1A), which has a high similarity to the human isoform PLC-δ; type-B PoPLC-δ1 (PoPLC-δ1B [Sf]), which has a low similarity to the human isoform PLC-δ and the alternative splice variant PoPLC-δ1B (Lf), which has a nuclear localization signal (NLS) and a nuclear export signal (NES) for nuclear imports and exports, respectively. This study confirmed the effects of the cellular localization and translocation of GFP-tagged PoPLC-δ 1A, PoPLC-δ1B (Sf) and PoPLC-δ1B (Lf). It administered treatments of Ca2+ ionophore ionomycin and endoplasmic reticulum (ER) - Ca^{2+} pump inhibitor thapsigargin to hirame natural-embryo (HINAE) cells. A laser-scanning confocal microscope was used. GFP-tagged PoPLC-δ1A was distributed to the cellular organelles, rather than to the cytoplasms and cytomembranes, when PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf) were localized at the plasma membranes. The treatments of ionomycin and thapsigargin showed the accumulation of PoPLC-δ1A in the nuclei when PoPLC-δ1B (Lf) nucleocytoplasmic shuttling and PoPLC-δ1B (Sf) nucleocytoplasmic shuttling were not observed. The results were the first evidence that PoPLC-δ1A, which contains functional, intact NES sequences, has a main role in nucleocytoplasmic shuttling and translocation in fish.

Key words : Cell localization, olive flounder, *Paralichthys olivaceus*, phospholipase C (PLC), translocation

Introduction

Among the six phospholipase C (PLC) family enzymes (PLC-β1-4, PLC-γ1-2, PLC-δ1, 3, 4, PLC-ε, PLC-ζ and PLC-η 1, 2), PLC δ types are evolutionarily conserved from lower to higher eukaryotes, and these isozymes are thought to be the primary forms expressed in mammals [6, 13, 17]. There are three PLC δ isozymes, PLC-δ1, -δ3, and -δ4 [5]. It has been suggested that PLC-δ1 is involved in Alzheimer's disease [14] and essential hypertension [7]. Analysis of PLC-δ1 knockout (KO) mice and found that PLC-δ1 has an important role in skin homeostasis [4], and other studies showed that PLC-δ1 and PLC-δ3 play important roles in normal development of the placenta [9, 10]. PLC-δ1 is generally found in the cytoplasm of quiescent cells and it has both nuclear export and import sequences that contribute to its shuttling between the cytoplasm and nucleus [15, 16]. In addition, PLC-δ1 is involved in osmotic response and pathway from hypo-osmotic activation of GAP43 (a membrane-anchored neuronal protein implicated in axonal growth and synaptic plasticity) to Ca^{2+} increase [1].

However, the exact physiological function and activation mechanism of the PLC-δ isoform family has not been completely resolved. Recently, Kim et al. [8] reported that PLC-δ 1 in olive flounder *P. olivaceus* had three variants: two duplicated gene (*PoPLC-δ1A* and *δ1B(Sf)*) and the N-terminal splice variants of *PoPLC-δ1B(Lf)* , which were shared from exon 3 (including PH domain) to exon 16, but differ at the exon 1 (Short form: Sf) and novel exon 2 (Long form: Lf) of the transcripts. Although they have different tissue-specific expression patterns and enzymatic characterization, cellular mechanisms of PLC-δ1 isozymes in fish remain to be studied.

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In this study, we investigated the cellular localization and translocation of duplicated gene variants and N-terminal splice variant phospholipase C-δ1 in fish using laser scanning confocal microscope and GFP-tagged PoPLC-δ1 genes with Ca^{2+} ionophore ionomycin and ER Ca^{2+}-pump inhibitor thapsigargin treatments.

Materials and Methods

Animal cell culture

HINAE cells were maintained in Leibovitz L-15 medium with 10%(v/v) heat-inactivated fetal bovine serum (FBS; Gibco BRL) and 1%(v/v) penicillin-streptomycin (PS; Gibco BRL) at 20°C.

Construction of recombinant PoPLC-δ1s/GFP-fused plasmids

Amplification of the ORFs of PoPLC-δ1s was carried out using Ex Taq® DNA polymerase (Takara BIO, Inc., Japan) with each gene specific primers based on their nucleotide sequences. To create GFP-fused PoPLC-δ1s' expression constructs, primers were designed containing *SmaI* and *XhoI* restriction sites at their 5' and 3' ends, respectively. The primer sequences are shown in Table 1. The amplified PoPLC-δ1 cDNA fragments were cloned into pEGFP-N1 vector (Clontech Laboratories, Inc., USA), respectively. The sequences of the constructs were confirmed by sequencing.

Transfection of GFP-tagged PoPLC-δ1s into HINAE cells

HINAE cells were seeded on glass coverslips in 12-well plate and transiently transfected with 1 μg of GFP-tagged PoPLC-δ1A, PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf), or empty vector (GFP only) using FuGENE® 6 transfection reagent (Roche Diagnostics, UK). The day before transfection, confluent HINAE were trypsinized, counted, and plated into 12-well dishes to reach 50-60% confluency on the day of transfection. One microliter (1 ug) of vector and 1.5 ul of FuGENE® 6 transfection reagent were diluted with 47 ul of serum-free DMEM. After 45 min of incubation at room temperature, 50 ul DNA-medium mixtures were added into 2 ml of cell culture medium. The cells were cultured for 2 days until confluency. At 48 hr after transfection, the cells were washed twice with a PBS and fixed with 3.7% formaldehyde for 10 min at room temperature, mounted using VECTASHIELD® with DAPI (VectorLabs, USA), and viewed using an LSM700 laser scanning confocal microscope (CarlZeiss Micro Imaging). The localization of GFP-tagged PoPLC-δ1A, PoPLC-δ1B (Lf), PoPLC-δ1B(Sf) and GFP alone in the cells was demonstrated by direct fluorescence; stacks of optical sections were acquired by sequential acquisition and analyzed using ZEN 2009 software.

Translocation in PoPLC-δ1s

To determine the intracellular distribution of PoPLC-δ1s, GFP-tagged PoPLC-δ1A, PoPLC-δ1B(Lf), PoPLC-δ1B(Sf) and GFP transiently transfected cells were treated with the indicated concentrations of ionomycin (0.1-5 uM) or thapsigargin (0.1-5 uM) for 30 min, after which the culture medium was replaced and the cells grown for 24 hr. Cells were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, and cells were washed twice with PBS, and fixed with 3.7% formaldehyde for 10 min at room temperatures. After fixation, cells mounted using VECTASHIELD® with DAPI (VectorLabs, USA), and we acquired stacks of optical sections using the same methods as for transfected cells.

Results and Discussion

Fish PLC-δ1s have NLS and NES sequences for nuclear import and export

Phosphoinositides (PIs) involved in the PI signaling pathway are localized in the nucleus as well as at the plasma

Table 1. Oligonucleotide primers used for pEGFP cloning of olive flounder phospholipase C isoforms

Primer name	5'-3' sequence	Information
CMV-ProF	AAATGG GCGGTAGGCGTG	Sequencing primers
EGFP-NR	CGTCGCCGTCCAGCTC	
PoPLC DIA-F-GFP	CGGCTCGAGATGGAGGCAAATGGCACAGCTGGA	PoPLC-δ1A
PoPLC DIA-R-GFP	GTGCATCTCATGCTCATGGACGCATCCCGGGGGA	
PoPLC Lf-F-GFP	CGGCTCGAGATGAGCTGCCTGCAGAGACAAGCCAA	PoPLC-δ1B(Lf)
PoPLC Sf-F-GFP	CGGCTCGAGATGGAAATGAATGGGGTCGAAAATACA	PoPLC-δ1B(Sf)
PoPLC DIB-R-GFP	GTACACGTCATGGTCGTCGATGTTAACTCCCGGGGGA	PoPLC-δ1Bs

membrane and in the cytoplasm, although their nuclear localization mechanisms have not been clarified in detail. In general, PI pathway enzymes that shuttle between the cytoplasm and nucleus contain nuclear localization signal (NLS) and nuclear export signal (NES) sequences for nuclear import and export, respectively [2, 3]. It is important to know when and how these PI pathway enzymes are regulated and localized. Approximately 200 amino acid residues at the N-terminus of PLC-δ1 are necessary for nuclear export and the rest is sufficient for nuclear import. The sequenced of residues 164-177 in PLC-δ1 that functions as an NES [19]. NES sequence is that the first proposed of the Leu-rich NES consensus is 'LLLxLLxxLxLx (LxxxLxxLxL)' where X is any amino acid and some PLC isoforms have canonical leucine-rich NES sequences in their Ca^{2+}-binding helix-loop-helix domain (EF-hand domain) [18]. There is no apparent classical NLS sequence in PLC-δ1. Strategic deletion studies revealed that a basic amino acid-rich region covering the C-terminus X domain and the XY-linker is necessary for the nuclear import of PLC-δ1. Two lysine residues (K_{432} and K_{434}) in the region are important for nuclear import, because a deletion mutant lacking the region or a site-directed mutant of the lysine residues does not accumulate in the nucleus, even in the presence of leptomycin B (LMB), a drug that binds to and inhibits CRM1 (Chromosomal Maintenance 1, also known as Exportin 1) is the major mammalian export protein across the nuclear membrane to the cytoplasm. Thus the NLS-like region was identified in PLC-δ1 [11].

As shown in Fig. 1, PoPLC-δ1A, PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf) have NES sequences and NLS like sequences. Results from amino acid alignment show that only PoPLC-δ1A has the leucine residue (LxxxLxxLxL) like human PLC-δ1 (Fig. 1A). However, three isoforms of olive flounder PLC-δ1 did not exist the leucine residue (LxxxLxxLxL) in EF hand domain although mammalian PLC-δ1s have the leucine residue (Fig. 1A). While most of PLC-δ1 proteins were the lysine residues (K_{432} and K_{434}) in X domain, comparing NLS sequence, both human and mouse were first residue of common lysine residues 'KxKxxKxK' in XY-linker. In olive flounder, only the PoPLC-δ1A has lysine residues (Fig. 1B). Consequently, we expected that PoPLC-δ1A plays a main role in nucleocytoplasmic shuttling in olive flounder.

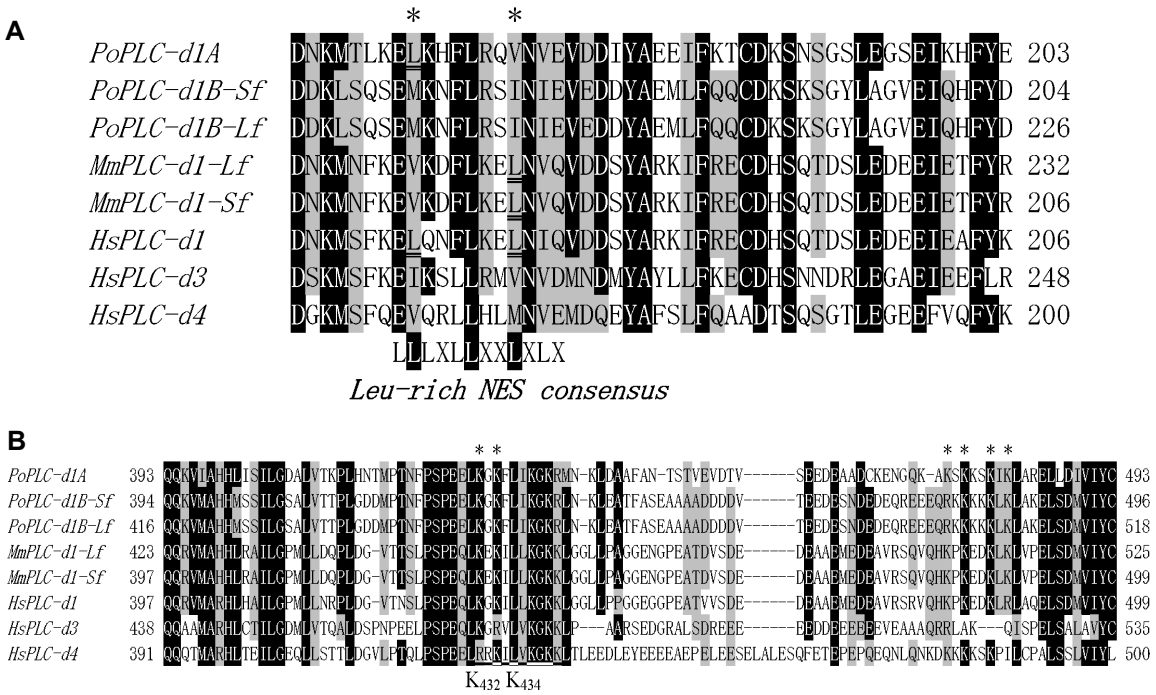

Fig. 1. The putative NES and NLS sequences of PoPLC-δ1s. (A) Corresponding sequences in the EF-hand domains of olive flounder PLC-δ1A, -δ1B (Lf), -δ1B (Sf), mouse PLC-δ1A, -δ1B (Lf), -δ1B (Sf) and human PLC-δ1A, -δ3, -δ4 are aligned for comparison with typical leucine-rich NES sequences in EF hand domain. A consensus sequence 'LLLxLLxxLxLx (LxxxLxxLxL)', in which a white "x" denotes any amino acid, is shown at the bottom. (B) Sequence alignment of the C-terminus of the X domain and XY-linker in various PI-PLC-δs. Both the lysine residues (K_{432} and K_{434}) in X domain, which are important for the nuclear import of PLC, was is shown at the bottom. Conserved lysine residues of the X domain and XY-linker in in various PLC-δ1s are indicated by asterisks (*).

PoPLC-δ1A is main role of cell localization and translocation in fish

To understand the cellular localization and translocation of fish PLC-δ1 isozymes, GFP fused PoPLC-δ1s expression vectors were constructed to study the cellular localization. As shown in Fig. 2, PoPLC-δ1A was observed as scattered patches and localized in cell organelles when PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf) were localized at the plasma membrane.

To study changes in the cellular localization and translocation of fish PLC-δ1s in the occurrence of calcium signals, we used treatment with ionomycin and thapsigargin into GFP fused PoPLC-δ1s, which transfected into HINAE cell lines (Fig. 3). After ionomycin treatment, we were able to see the accumulation of expressed PoPLC-δ1A after treatment of serum-starved cells with drug (1 uM-5 uM). However, PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf) were not accumulated in the nucleus. Treatment of thapsigargin, which is useful in examining the effects of increasing cytosolic calcium concentrations, also facilitates the nuclear import of PoPLC-δ1A. These results suggest that raising the extracellular Ca^{2+} concentration would facilitate the nuclear import PLC-δ1 in fish. In fact, a portion (10%-20%) of serum-starved MDCK cells accumulated PLC-δ1 within 2 min after the treatment of cells with ATP (100 mM), suggesting that the activation of purinergic receptors causes the nuclear import of PLC-δ1. Recent studies have indicated that the Ca^{2+} increase activates PLC isoforms and causes a decrease in

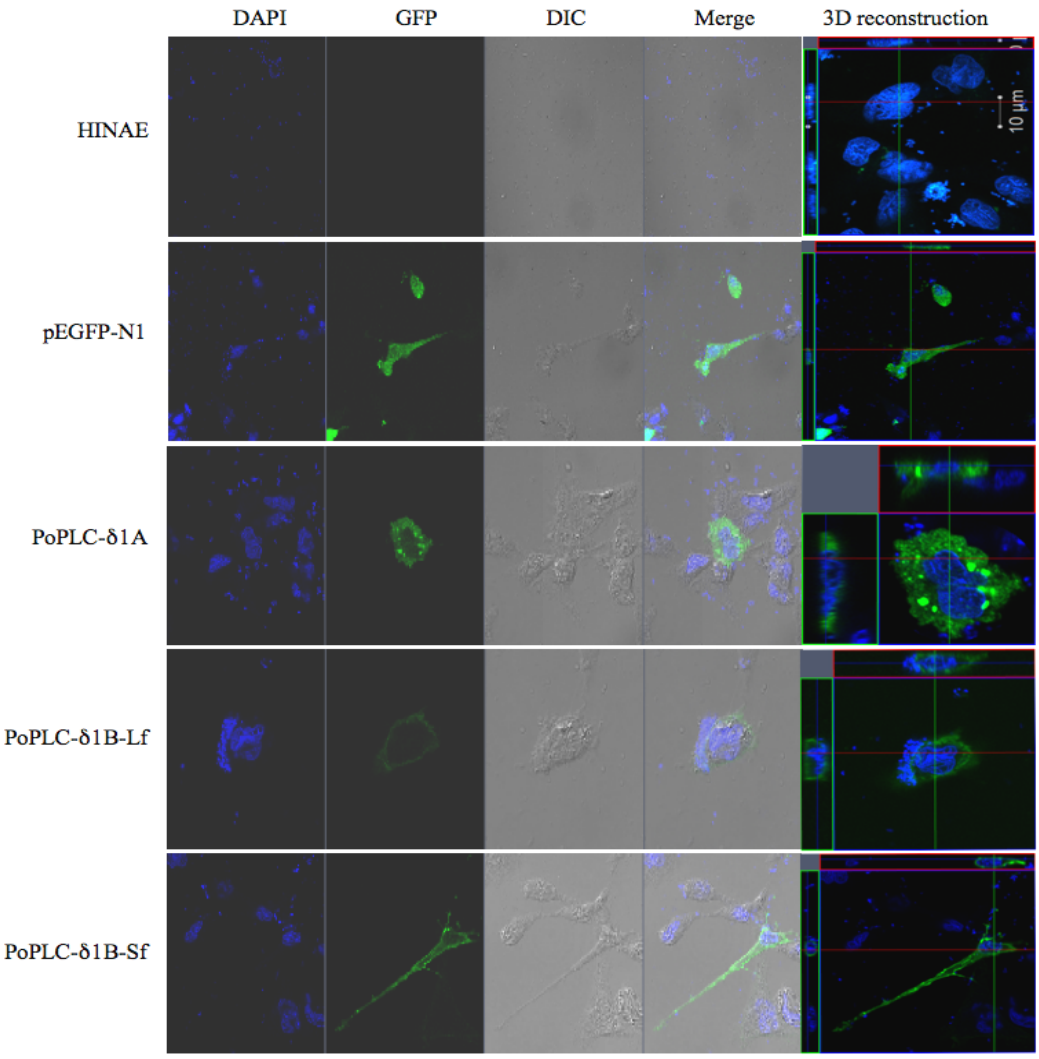

Fig. 2. Cellular characterization of PoPLC-δ1A, PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf). Transfection with GFP-tagged PoPLC-δ1A, PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf) isoforms in HIANE cell and viewed using an LSM700 laser scanning confocal microscope (Carl Zeiss Micro Imaging).

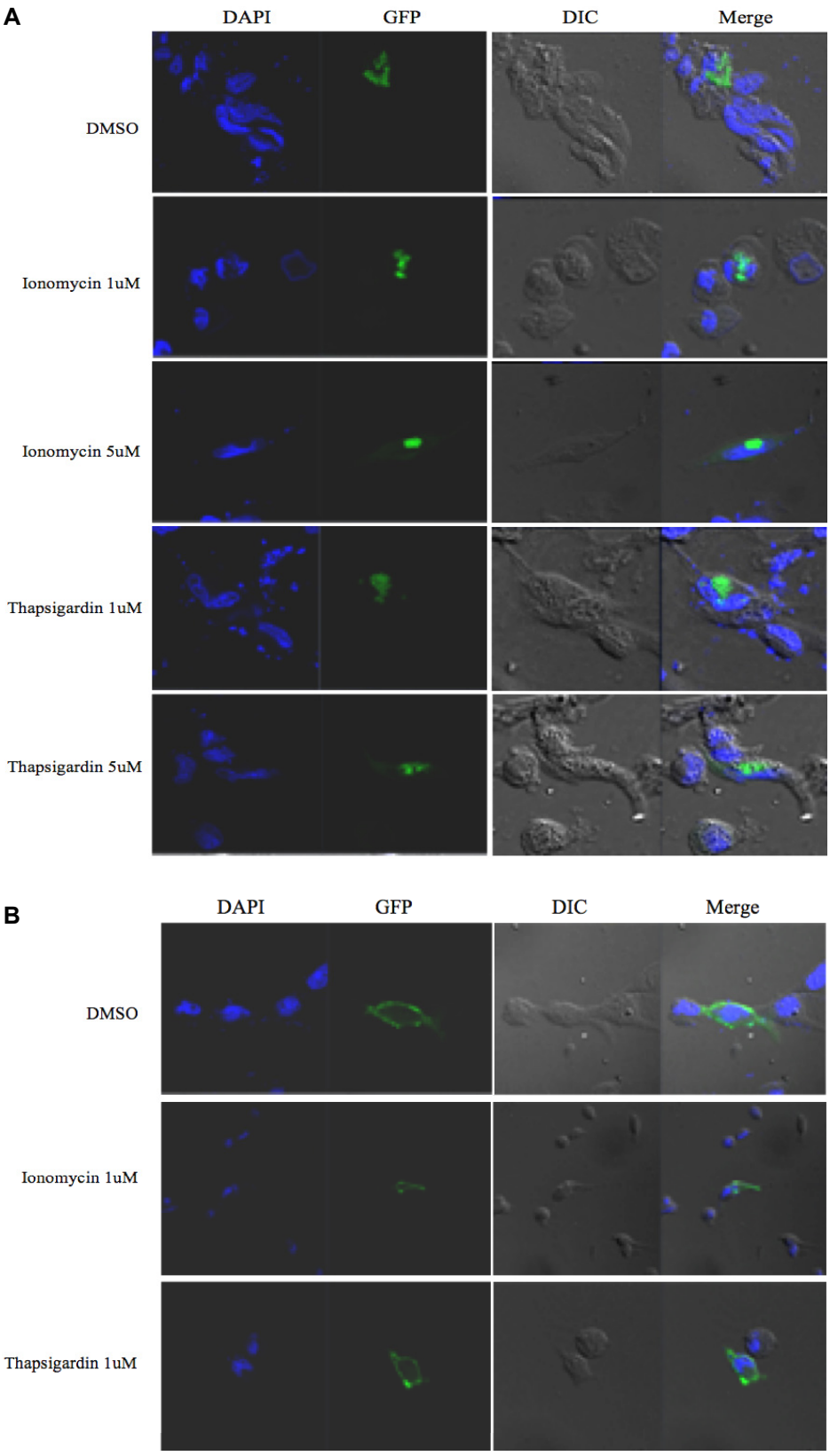

Fig. 3. Effect of ionomycin and thapsigargin after transfection with GFP-fused PoPLC-δ1A, PoPLC-δ1B (Lf) and PoPLC-δ1B (Sf) isoforms in HIANE cell. (A) PoPLC-δ1A transfected in HINAE cell and treatment two drugs for 20 min. (B) PoPLC-δ1B (Lf). (C) PoPLC-δ1B (Sf).

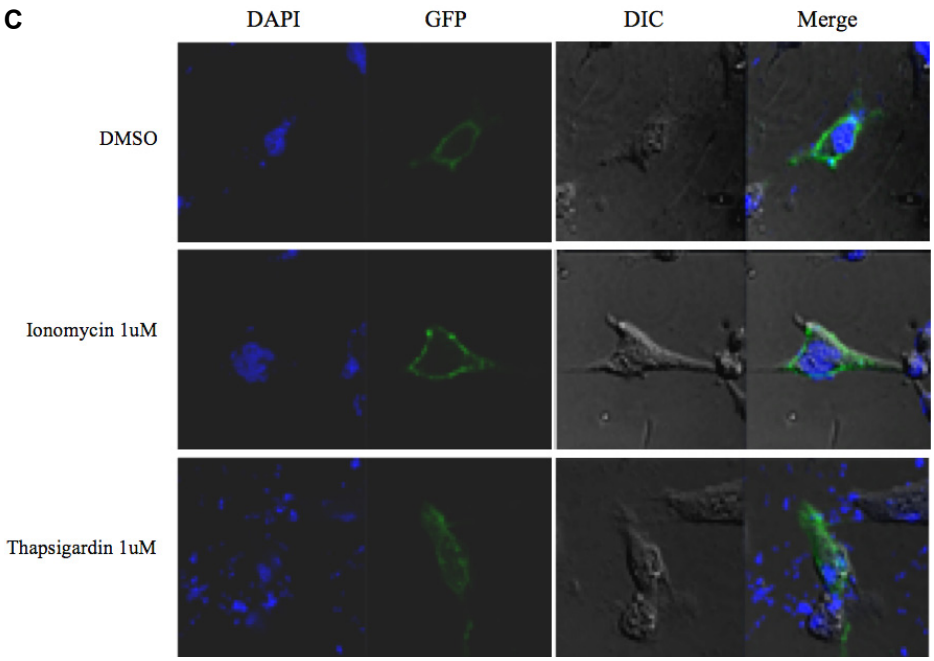

Fig. 3. Continued.

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] levels at the plasma membrane, releasing mammalian PLC-δ1 from the plasma membrane to increase the levels in the cytoplasm. The efficacy of mammalian PLC-δ1 binding to nuclear import machineries then increases and more nuclear import complexes are formed and transported into the nucleus [12, 15].

Taken together, our results suggest that elevated intracellular calcium levels would facilitate the nuclear import of PLC-δ1. Among the three isoforms of olive flounder PLC-δ1s, only GFP-tagged PoPLC-δ1A which contains the intact functional NES and the NLS sequences in the EF-hand domain and the C-terminus of the X domain and the XY-linker, was observed nucleocytoplasmic shuttling. That means PoPLC-δ1A is main role of cell localization and translocation in fish.

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초록 : 넙치 3가지 타입 인지질가수분해효소(PLC-δ1)의 세포 내 위치 및 이동

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본 연구의 목적은 넙치 인지질가수분해효소(PLC- δ1) 3가지 타입의 세포내 특성을 규명하고자 하였다. 일반적으로 인지질가수분해효소(PLC)의 신호전달경로는 핵, 세포막, 세포질에 분포한다고 알려져 있으나, 핵내 위치 메커니즘은 여전히 불분명하다. PoPLC-δ1A, PoPLC-δ1B (Sf)과 PoPLC-δ1B (Lf)의 3타입의 유전자들은 각각 핵위치신호(NLS)와 핵방출서열(NES)을 포함하고 있다. 본 연구에서는, 넙치 3가지 타입 인지질가수분해효소(PLC-δ1)의 세포내 위치이동 메커니즘 분석을 위해 GFP 벡터에 유전자를 삽입하여 ionomycin과 thasogargin처리 후 세포위치와 이동양상을 공초점 레이저 주사현미경으로 관찰하였다. PoPLC-δ1A는 PoPLC-δ1B (Lf)와 PoPLC-δ1B (Sf)가 원형질막에 국한되어 분포할때 세포질과 세포막보다 세포 소기관에 분포되어 있었다. PoPLC-δ1B (Lf) 및 PoPLC-δ1 (Sf)이 핵 세포질내 이동양상을 보이지 않을 때, PoPLC-δ1A은 ionomycin과 thapsigargin 처리에 의해 핵 내에 축적되는 양상을 나타냈다. 이런 결과는 손상되지 않은 기능적 NES 서열을 포함하는 PoPLC-δ1A가 어류에서 핵 세포질 내 왕복 및 이동의 주된 역할을 한다는 것을 보여주고 있다.