Biochemistry and Biophysics Reports 2 (2015) 75–86

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Insights into the inhibitory mechanism of triazole-based small molecules on phosphatidylinositol-4,5-bisphosphate binding pleckstrin homology domain

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ARTICLE INFO

Article history:
Received 22 April 2015
Received in revised form
18 May 2015
Accepted 19 May 2015
Available online 27 May 2015

Keywords:
Pleckstrin homology domains
Phosphatidylinositols
1,2,3-Triazol-4-yl methanol substituent
Inhibition of protein–lipid interactions
Surface plasmon resonance-based competitive binding assay
Cellular displacement assay

ABSTRACT

Background: Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] is an important regulator of several cellular processes and a precursor for other second messengers which are involved in cell signaling pathways. Signaling proteins preferably interact with PI(4,5)P_2 through its pleckstrin homology (PH) domain. Efforts are underway to design small molecule-based antagonist, which can specifically inhibit the PI(4,5)P_2/PH-domain interaction to establish an alternate strategy for the development of drug(s) for phosphoinositide signaling pathways.

Methods: Surface plasmon resonance, molecular docking, circular dichroism, competitive Förster resonance energy transfer, isothermal titration calorimetric analyses and liposome pull down assay were used.

Results: In this study, we employed 1,2,3-triazol-4-yl methanol containing small molecule (CIPs) as antagonists for PI(4,5)P_2/PH-domain interaction and determined their inhibitory effect by using competitive-surface plasmon resonance analysis (IC_{50} ranges from 53 to 159 nM for PI(4,5)P_2/PLCδ1-PH domain binding assay). We also used phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3], phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2], PI(4,5)P_2 specific PH-domains to determine binding selectivity of the compounds. Various physicochemical analyses showed that the compounds have weak affect on fluidity of the model membrane but, strongly interact with the phospholipase C δ1 (PLCδ1)-PH domains. The 1,2,3-triazol-4-yl methanol moiety and nitro group of the compounds are essential for their exothermic interaction with the PH-domains. Potent compound can efficiently displace PLCδ1-PH domain from plasma membrane to cytosol in A549 cells.

Conclusions: Overall, our studies demonstrate that these compounds interact with the PIP-binding PH-domains and inhibit their membrane recruitment.

General significance: These results suggest specific but differential binding of these compounds to the PLCδ1-PH domain and emphasize the role of their structural differences in binding parameters. These triazole-based compounds could be directly used/further developed as potential inhibitor for PH domain-dependent enzyme activity.

1. Introduction

In response to specific stimuli, various cytosolic proteins are reversibly recruited to the cellular membranes and form dynamic signaling complexes with specific lipid molecules, including phosphatidylinositols (PIPs). The PIP lipids have drawn considerable attention due to their various cellular functions through a plethora of effector proteins [1–4]. A range of human diseases which are directly or indirectly linked with PIP-binding/metabolism have been identified and become an exciting therapeutic target in biomedical research [2,3,5–8]. Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2) is the most abundant PIP in the plasma membrane (PM). Hydrolysis of PI(4,5)P_2 to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) by activated phospholipase C (PLC) enzymes is one of the crucial cellular signaling pathways. IP3 regulates Ca^{2+} release from

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http://dx.doi.org/10.1016/j.bbrep.2015.05.007

endoplasmic reticulum (ER) and DAG is the activator of protein kinase C (PKC) enzymes. Various membrane receptors including G-protein-coupled receptors (GPCRs) strongly interact with PLC enzyme and regulate $PI(4,5)P_2$ turnover and consequent downstream cell signaling pathways. $PI(4,5)P_2$ regulates several proteins including PLCδ, Ras GTPase activating protein (RasGAP) and pleckstrin, which also mediate a wide verity of cellular process [9–12]. Improper cellular functions of these effectors proteins are associated with disorder such as neurodegenerative, cardiovascular diseases and others [13,14]. The PI-kinase I/II and SH2-containing Inositol-5′-Phosphatase (SHIP), phosphatase and tensin homolog (PTEN) enzymes phosphorylate phosphatidylinositol-4-phosphate (PI(4)P)/phosphatidylinositol-5-phosphate (PI(5)P) and dephosphorylate phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$), respectively to generate $PI(4,5)P_2$ at the inner PM in responses to various stimuli [15,16]. $PI(4,5)P_2$ lipid is phosphorylated by class I PI3-kinase to form $PI(3,4,5)P_3$, which regulates cellular functions of several crucial signaling proteins including AKT.

The $PI(4,5)P_2$ generation recruits several proteins to the PM through their interaction with pleckstrin homology (PH)- or other PIP-binding modules. This $PI(4,5)P_2$-dependent membrane association of the lipid binding modules is necessary and sufficient for activation and proper functioning of the effector proteins; including PLCδ. Activation of PLCδ proceeds only after $PI(4,5)P_2$-binding of the PH domain at the inner PM that reorient the EF-hands–C2 domain–TIM barrel unit so that the catalytic domain is in a productive orientation relative to the membrane. Recent studies reported that in addition to PH domain, C2 domain of PLCδ also interacts with the membrane and plays an important role in PLCδ activation [17,18].

In general catalytic domains of the proteins are considered as drug target to down/up-regulate the cellular activities by direct inhibition/activation mechanism. However, the catalytic activities of the proteins should be effectively regulated in the cells, because direct inhibition/activation could also induce side-effects by disrupting the other up/down-stream of the cellular pathways [19–21]. Detailed mechanistic studies demonstrated that highly specific PIP/PH-domain interactions can regulate activities of the effector proteins. It is also important to note that the PH-domains contain a conserved structure with well-defined binding site for “prototypic” small molecule–protein interaction studies [22]. It is also depicted that protein–lipid interactions are readily targetable by small molecules due to the small and defined binding site of the proteins for specific lipid molecules, whereas protein–protein interactions normally need extended flat protein surfaces, that are difficult to disrupt by small molecules. It is also demonstrated that development of small molecules based inhibitors for lipid–protein interactions is advantageous over typical approaches in the aspect of side-effect and rational design because of relatively simple structures and functions [5,20,23,24]. For these reasons, the PH-domains of the effector proteins can be considered as an attractive alternate target in designing selective inhibitors for its interaction with PIPs.

However, development of inhibitors for PIP/PH-domain interactions to regulate enzyme activities had not been substantially described yet. Using the similar hypothesis, we recently demonstrated that development of DAG-responsive C1 domain based activator can be considered as an alternative target to regulate the activities of the PKC enzymes [25–27]. Recently developed PH-domain targeting lipid-based compound, 3-deoxy phosphatidylinositol ether lipid (DPIEL) and PHT-427 were described as potential drug candidates for the treatment of cancer and other human diseases [21,28]. Small molecules like PITENINs were also demonstrated as the inhibitor of $PI(3,4,5)P_3$ binding of AKT1/PDK1-PH-domains and down-regulator of PI3-Kinase/PDK1/AKT1 pathways [8]. There are only a few PLC regulators that play a significant role in understanding the $PI(4,5)P_2$ mediated cellular signaling pathways [17,29]. Neomycin is known as a regulator of $PI(4,5)P_2$–PLC enzyme interactions and PLC induced $PI(4,5)P_2$ hydrolysis at the cellular membranes. However neomycin directly interacts with the $PI(4,5)P_2$ molecules present at the membrane through an electrostatic interaction and blocks PLCδ enzyme activity [29]. However, there is no report of PH domain specific PLC regulator.

In this regard our current study describes the development of 1,2,3-triazol-4-yl methanol-based antagonists, **CIP**s for $PI(4,5)P_2$/PLCδ1-PH domain binding. These compounds at lower concentrations showed certain degree of selective inhibitory effect towards different PH-domains used for the current study. The 1,2,3-triazol-4-yl methanol moiety and nitro group of the compounds play a crucial role in distinguishing the PH-domains. Potent compound, **CIP-4**, can competitively interact with the PLCδ1-PH domain and displace it from PM to cytoplasm. We believe that these non-lipid potent compounds would be able to inhibit $PI(4,5)P_2$ targeted PIP-binding proteins/enzyme under cellular environment and regulate its cellular signaling pathways.

2. Experimental methods

2.1. General Information

All chemicals and reagents were purchased from Sigma (St. Louis MO), SRL (Mumbai, India), Merck (Mumbai, India) and used for the synthesis without further purification. Phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$), phosphatidylinositol-3,4 -bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) were purchased from Cayman Chemicals (Ann Arbor, MI). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (PS), 1,2- dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (dPE) were purchased from Avanti Polar Lipids. Octyl glucoside was purchased from Fisher. The Pioneer L1 sensor chip was purchased from Biacore AB (Piscataway, NJ). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers. Compounds were first dissolved in DMSO and then diluted in working buffer so that overall DMSO concentration was $< 5\%$ (v/v).

2.2. Protein purification

The AKT1 (homo sapiens; 1-121 amino acids), GRP1 (mus musculus, 1-127 amino acid), TAPP1 (homo sapiens; 180-305 amino acid) and PLCδ1 (rattus norvegicus, 1-131 amino acid) PH-domains were expressed in *Escherichia coli* cells (BL21-DE3) and purified using methods similar to those reported earlier [22]. The plasmids were generous gift from Prof. Wonhwa Cho (University of Illinois at Chicago, IL, USA).

2.3. Surface plasmon resonance (SPR) assay

All surface plasmon resonance (SPR) measurements were performed (at 25 °C, in 20 mM HEPES buffer, pH 7.4, containing 0.16 M KCl, flow rate of 30 μL/min) using a lipid-coated L1 sensorchip in the Biacore-X100 (GE Healthcare) system as described earlier [22,30]. Vesicles for SPR analysis were prepared at a concentration of 0.5 mg/ml in 20 mM HEPES buffer, pH 7.4, containing 0.16 M KCl, and were vortexed vigorously and passed through a 100-nm polycarbonate filter using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL) according to the manufacturer's protocol. After washing the sensor chip surface with the running buffer (20 mM HEPES, pH 7.4, containing 0.16 M KCl) PC/PE/PS/$PI(4,5)P_2$