Archives of Biochemistry and Biophysics 540 (2013) 133–144

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

# Role of PKC-ζ in NADPH oxidase–PKCα–$G_i$α axis dependent inhibition of β-adrenergic response by U46619 in pulmonary artery smooth muscle cells

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ARTICLE INFO

*Article history:*
Received 16 July 2013
and in revised form 2 October 2013
Available online 30 October 2013

*Keywords:*
U46619
Adenyl cyclase
cAMP
PKC-ζ
PKC-α
$G_i$α

ABSTRACT

Treatment of bovine pulmonary artery smooth muscle cells (BPASMCs) with U46619 attenuated isoproterenol caused stimulation of adenyl cyclase activity and cAMP production. Pretreatment with SQ29548 (Tp receptor antagonist), apocynin (NADPH oxidase inhibitor) and Go6976 (PKC-α inhibitor) eliminated U46619 caused attenuation of isoproterenol stimulated adenyl cyclase activity. Pretreatment with SQ29548 and apocynin prevented U46619 induced increase in NADPH oxidase activity, PKC-α activity and $G_i$α phosphorylation. However, pretreatment with CZI, a PKC-ζ inhibitor, markedly, but not completely, inhibited U46619 induced increase in NADPH oxidase activity, PKC-α activity, $G_i$α phosphorylation and also significantly eliminated U46619 caused attenuation of isoproterenol stimulated adenyl cyclase activity. Pretreatment with Go6976 inhibited U46619 induced increase in $G_i$α phosphorylation, but not PKC-ζ activity and NADPH oxidase activity. Pretreatment with pertussis toxin eliminated U46619 caused attenuation of isoproterenol stimulated adenyl cyclase activity without any discernible change in PKC-ζ, NADPH oxidase and PKC-α activities. Transfection of the cells with Tp, PKC-ζ and PKC-α siRNA duplexes corroborate the findings observed with their respective pharmacological inhibitors on the responses produced by U46619. Taken together, we suggest involvement of PKC-ζ in U46619 caused attenuation of isoproterenol stimulated β-adrenergic response, which is regulated by NADPH oxidase–PKCα–$G_i$α axis in pulmonary artery smooth muscle cells.



## Introduction

Two major signal transduction pathways are those involved in the hydrolysis of inositol lipids by PLC-PKC and formation of cAMP by adenyl cyclase play important roles in a variety of physiological and pathological events [1–4]. Activation of one pathway has been shown to be stimulatory or inhibitory on the other pathway. For example, treatment of frog erythrocytes with PMA activates PKC[1], which enhances adenyl cyclase activity [4]. In contrast, PKC activation by a phorbol ester reduces the amplitude of isoproterenol induced cAMP production in different cell types, for example, pulmonary endothelial cells [5,6].

Oxidative stress has been implicated in many disease processes including reperfusion injury [7]. In ischemia–reperfusion, significant amount of superoxide $O_2^{\cdot-}$ is produced, which has been recognized as the important factor in regulating pulmonary vascular tone and function [8]. NADPH oxidase has been shown to be the most important source of $O_2^{\cdot-}$ production in the vasculature [9]. We have recently demonstrated a role of PKC in the generation of NADPH oxidase derived $O_2^{\cdot-}$ during treatment of BPASMCs with U46619 [9]. However, the involvement of exact isoform(s) of PKC in this scenario remains unresolved.

Activation of PKC is known to be an important mechanism of pulmonary hypertension and pulmonary vascular hypertrophy [10,11]. Pretreatment of parenchymal lung strips with isoproterenol has been shown to prevent contraction caused by the protein kinase C activator, PMA; but isoproterenol was unable to eliminate the contraction caused by PMA [12]. A previous report indicated that during development of pulmonary hypertension, the pulmonary circulation becomes less responsive to vasodilators and structural remodeling of pulmonary artery occurs, involving vascular cell proliferation and hypertrophy [13]. These observations are attributed to another report indicating that although inhaled β-AR agonists represent a first line of treatment of bronchial asthma, yet a reduced response to β-AR agonist has been observed in asthmatic subjects [14], which may be exemplified by the fact that

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[1] *Abbreviations used:* Tp, thromboxane receptor; PKC-ζ, protein kinase C-ζ, PKCα, protein kinase C-α; $G_i$α, alpha subunit of $G_i$; CZI, myristoylated PKC-ζ pseudosubstrate peptide (myr-RRGARRWRK) inhibitor; CEI, myristoylated PKC-ε pseudosubstrate peptide (VI-2) inhibitor.

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http://dx.doi.org/10.1016/j.abb.2013.10.018

constrictors that activate PKC attenuate the increase in cAMP accumulation and relaxation caused by β-adrenergic agonists [15].

Several compounds, for example, the thromboxane $A_2$ mimetic, U46619 caused pulmonary vasoconstriction have been shown to occur with the involvement of PKC [16]. Isoproterenol pretreatment significantly reduced the increase in pulmonary artery pressure caused by PKC activators; however, post treatment with isoproterenol was ineffective in reducing pulmonary vasoconstriction caused by PKC activators [17,18,15]. This suggests that PKC activation impairs the capacity of isoproterenol to stimulate adenyl cyclase activity and to increase cAMP production and subsequent inhibition of relaxation of the pulmonary artery.

In bovine pulmonary artery endothelial cells, isoproterenol induced increase in adenyl cyclase activity was found to be inhibited by pretreatment with the PKC activator, phorbol ester [5]; however, the role that PKC plays in this scenario in PASMCs remains unexplored. Herein, we investigated the mechanism by which the thromboxane $A_2$ mimetic, U46619 attenuates isoproterenol stimulated adenyl cyclase activity in bovine pulmonary artery smooth muscle cells.

## Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, nonessential amino acids, gentamycin sulfate, fetal calf serum, streptomycin sulfate, penicillin and phosphate buffered saline (PBS) without calcium and magnesium were the products of GIBCO laboratories (Grand Island, NY). Molecular wt. markers, U46619, SQ29548, apocynin. pertussis toxin, forskolin, NaF, Gpp(NH)p, isoproterenol, calphostin C, Go6976, rottlerin, 4-chloro-1-naphthol and protein A/G agarose beads were obtained from Sigma Chemical Co. St. Louis, MO. Myr-RRGARRWRK (CZI) and Myristoylated εPKC VI-2 peptide (CEI) were obtained from BIOMOL Research Laboratories, Plymouth, PA. Polyclonal anti-phospho-serine/threonine specific antibody, polyclonal $G_i\alpha$, PKC-α, PKC-ζ, PKC-ε, PKC-δ antibodies and cAMP assay kit were the products of Cell Signaling Technologies, San Diego, CA. cAMP assay kit was the product of New England Nuclear (Boston, MA). Horseradish peroxidase conjugated goat anti-rabbit secondary antibody was obtained from Transduction Laboratories (Lexington, KY). PKC assay kit was the product of Calbiochem, SanDiego, CA. Antigens and polyclonal antibody of $G_i\alpha$ were the products of Chemicon International Inc., Temelcula, CA. Goat polyclonal annexin II antibody was the product of Abcam Ltd., Cambridge, UK. BCA protein assay reagent was purchased from Pierce Biotechnology Inc., Rockford, IL. $TxA_2$ (Tp) receptor, PKC-ζ, PKC-α and also scrambled siRNA duplexes were obtained from Integrated DNA Technologies (IDT), San Jose, CA. Lipofactamine was the product of Invitrogen, Carlsbad, CA. All other chemicals and reagents used were of highest purity grade available commercially.

## Methods

### Cell Culture

Bovine pulmonary artery smooth muscle cells obtained from Cell Sciences (San Diego, CA) were studied between passages 4 and 12. Cells were maintained in DMEM supplemented with 20% FCS, L-glutamine and non essential amino acids. Cells were subcultured after treatment with 0.25% trypsin. All experiments were performed in serum free media.

### Preparation of the cytosolic and cell membrane fraction

The cytosolic and cell membrane fractions were isolated by following the method described by Chakraborti et al. [19] with some modification. In brief, the cells were grown in T150 flasks. After attaining about 80% confluence, the cells were washed twice with PBS. The cells were then scraped from the flasks and suspended in the homogenizing medium containing 0.25 M sucrose, 1.5 mM $MgCl_2$, 0.01% Nonidet P-40 and 5 mM Tris/HCl buffer (pH 7.4), and homogenized using a Dounce homogenizer with a tight-fitting pestle. The homogenate was centrifuged at 1000*g* for 15 min. The resulting supernatant was centrifuged at 15,000*g* for 30 min and subsequently at 100,000 g for 1 h. The supernatant obtained was used as cytosolic fraction. The pellet was suspended in 10% (w/v) sucrose containing 10 mM Tris/HCl buffer, pH 7.4, and layered on a discontinuous sucrose gradient consisting of 40% (w/v) and 20% (w/v) sucrose both in 10 mM Tris/HCl buffer, pH 7.4. The gradient was centrifuged at 105,000*g* for 2 h. The fraction collected at the 20–40% sucrose interface was considered as the cell membrane fraction. All steps were performed at 4 °C. The cytosolic and membrane fractions were aliquoted, stored under liquid nitrogen, and thawed before use.

### Assay of cAMP

After reaching confluence, the media was replaced with DMEM containing 1 mM IBMX. After 15 min, varied concentrations of different pharmacological inhibitors were added for 10 min, then U46619 (10 nM) was added for 10 min followed by treatment with isoproterenol. cAMP was extracted from the cells by the addition of 0.25 ml of 0.1 N HCl, and the samples were neutralized with 50 mM NaOAc. cAMP was assayed with cAMP assay kit as per instructions of the manufacturer (New England Nuclear, Boston, MA). Briefly, aliquots were acetylated with triethylamine and acetic anhydride prior to the assay. Each assay tube contained 15 μCi $^{125}$I-2′-O-succinyl cAMP tyrosine methyl ester, NaOAc buffer, and the cAMP samples were incubated overnight, and the antibody bound radioactivity was separated from tracer using specific antiserum, and the radioactivity was measured.

### Adenyl cyclase activity

Adenyl cyclase activity was measured by following a previously described method [19]. Briefly, bovine pulmonary artery smooth muscle cell membrane (~50 μg) was incubated for 20 min at 30 °C in 50 mM Tris–HCl buffer (pH 7.4) containing 3 mM $MgCl_2$, and 0.3 mM KCl, 20 μM cAMP, 0.1 DTT, and 5 mM creatine phosphate, 2.8 U creatine phosphokinase, 0.1 mM [α-$^{32}$P]ATP and 1 mM IBMX in a total reaction volume of 100 μl. Assay tubes were placed on ice during preparation and agonists were added as indicated in the Figure legends. GTP (10 μM) was present under basal and isoproterenol stimulated conditions. The reaction was terminated by the addition of 100 μl of ice-cold 50 mM HEPES buffer (pH 7.5) containing 2 mM ATP, 0.5 mM [$^3$H] cAMP (0.8 Ci/mmol) and heating the mixture to 95 °C for 3 min. The [$^{32}$P]cAMP synthesized during the incubation was fractionated using Dowex–alumina chromatography [5]. Recovery of cAMP averaged 70%–80%.

### Measurement of NADPH oxidase activity

The cells grown to confluence were washed twice with PBS. The cells were then scraped from the flasks with a buffer containing 100 mM Tris–HCl buffer (pH 7.4), then the cell membrane fraction was isolated and NADPH oxidase activity was measured by monitoring $O_2^{\cdot-}$ generation by SOD inhibitable cytochrome C reduction assay [20].