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Signaling mechanisms of inhibition of phospholipase D activation by CHS-111 in formyl peptide-stimulated neutrophils

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ABSTRACT

A selective phospholipase D (PLD) inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) inhibited the $O_2^{\bullet-}$ generation and cell migration but not degranulation in formyl-Met-Leu-Phe (fMLP)-stimulated rat neutrophils. A novel benzyl indazole compound 2-benzyl-3-(4-hydroxymethylphenyl)indazole (CHS-111), which inhibited $O_2^{\bullet-}$ generation and cell migration, also reduced the fMLP- but not phorbol ester-stimulated PLD activity (IC_{50} 3.9 ± 1.2 μM). CHS-111 inhibited the interaction of PLD1 with ADP-ribosylation factor (Arf) 6 and Ras homology (Rho) A, and reduced the membrane recruitment of RhoA in fMLP-stimulated cells but not in GTPγS-stimulated cell-free system. CHS-111 reduced the cellular levels of GTP-bound RhoA, membrane recruitment of Rho-associated protein kinase 1 and the downstream myosin light chain 2 phosphorylation, and attenuated the interaction between phosphatidylinositol 4-phosphate 5-kinase (PIP5K) and Arf6, whereas it only slightly inhibited the guanine nucleotide exchange activity of human Dbs (DH/PH) protein and did not affect the arfaptin binding to Arf6. CHS-111 inhibited the interaction of RhoA with Vav, the membrane association and the phosphorylation of Vav. CHS-111 had no effect on the phosphorylation of Src family kinases (SFK) but attenuated the interaction of Vav with Lck, Hck, Fgr and Lyn. CHS-111 also inhibited the interaction of PLD1 with protein kinase C (PKC) α, βI and βII isoenzymes, and the phosphorylation of PLD1. These results indicate that inhibition of fMLP-stimulated PLD activity by CHS-111 is attributable to the blockade of RhoA activation via the interference with SFK-mediated Vav activation, attenuation of the interaction of Arf6 with PLD1 and PIP5K, and the activation of Ca^{2+}-dependent PKC in rat neutrophils.

1. Introduction

Neutrophils are known to be first-line defenders in the innate immune response system. To fulfill this role, neutrophils carry out biological processes, such as chemotaxis, phagocytosis, oxidative response and degranulation [1]. However, over-reactive neutrophils are also responsible for tissue destruction in inflammatory conditions. Thus, pharmacological interference with the function of key molecules in the neutrophil activation presents a promising strategy for therapeutic intervention aiming at decreasing the severity of inflammatory disorders in patients.

Phospholipase D (PLD) catalyses the hydrolysis of the membrane phosphatidylcholine to generate choline and the signaling lipid phosphatidic acid (PA) through the parallel reactions of phospholipid hydrolysis and transphosphatidylation. PA is a precursor of diacylglycerol and lyso-PA and is strategically located at the intersection of several major cell signaling and metabolic pathways. Aberrant PA signaling is observed in a number of disease states [2]. Two mammalian isoforms, PLD1 and PLD2, have been identified, with multiple splice variants of each. PLD1a is the major PLD isoform found in neutrophil membranes [3]. Despite structural similarities between the two isoforms, studies suggest distinct modes of activation and functional roles for PLD1 and PLD2 [4]. PLD1 has low basal activity that is highly regulated by protein kinase C (PKC) and several small GTPases and could provide

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stimulus-coupled control of cell function, whereas PLD2 has high basal activity and could play a housekeeper role. A combination of pharmacological, mainly utilizing primary alcohols to decrease PA formation by shunting phosphatidyl moieties into phosphatidylalcohols instead of PA, and molecular biological (such as utilizing inactive PLD mutants and RNA interference) approaches has indicated that PLD plays an important role in regulating chemotaxis, phagocytosis, degranulation, and superoxide anion ($O_2^{\bullet-}$) generation [1]. Despite the widespread utilization of primary alcohols over the past 20 years, concerns have been raised as to whether they fully block PA production at the concentrations used and whether primary alcohols and phosphatidylalcohols have other effects on cells that extend beyond inhibiting PA production. A potent dual PLD1/PLD2 inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), which inhibits both the hydrolytic and transphosphatidylation activities, was identified recently in vitro as well as in vivo [5]. Interestingly, several biological processes blocked by primary alcohols are not affected by FIPI, suggesting the need for re-evaluation of proposed roles for PLD. We found that FIPI inhibited $O_2^{\bullet-}$ generation and cell migration in neutrophils in the present study. A therapeutic agent, which inhibits the PLD activation, would preferentially block the over-reactive neutrophils and thus be an attractive pharmacological target for anti-inflammatory drugs.

In screening studies with the goal of identifying a potential anti-inflammatory benzyl indazole compound, 2-benzyl-3-(4-hydroxymethylphenyl)indazole (CHS-111) was recently found to have a potent inhibitory effect on $O_2^{\bullet-}$ generation and cell migration in formyl-Met-Leu-Phe (fMLP)-stimulated rat neutrophils [6]. In this study, we sought to determine whether PLD could be involved in the inhibition of neutrophil activation by CHS-111 and also evaluate the underlying mechanism of action.

2. Materials and methods

2.1. Materials

Dextran T500 was obtained from Pharmacosmos (Holbaek, Denmark). Ficoll-Paque, protein A sepharose and enhanced chemiluminescence reagent were purchased from GE Healthcare (Piscataway, NJ, USA). Hanks' balanced salt solution (HBSS), RPMI-1640 and calcein/AM were purchased from Invitrogen (Carlsbad, CA, USA). 1-*O*-[^{3}H]Octadecyl-*sn*-glycero-3-phosphocholine was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Antibodies against PLD1, RhoA, Arf6, arfaptin 1, phospho-Vav (Y174), G_β, Vav, Lck, Fgr, Hck, PKCα, PKCβI and PKCβII were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against PLD1, phospho-PLD1 (T147), ROCK1, phospho-MLC2 (T18/S19), MLC2, PIP5K1A, Src and phospho-Src family (Y416) were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA, USA). Antibodies against Lyn and RalA were obtained from BD Biosciences (Palo Alto, CA, USA). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Rhotekin-Rho binding domain (RBD) protein agarose beads and RhoGEF exchange assay biochem kit were purchased from Cytoskeleton (Denver, CO, USA). 4-Amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo-(3,4-d)pyrimidine (PP1) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). (R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) and silica gel 60 plates were purchased from Merck (Taipei, Taiwan). 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) was kindly supplied by Dr. M.A. Frohman (the Department of Pharmacology, Stony Brook University, NY, USA). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). CHS-111 (purity >99%) [6] was dissolved in dimethyl sulfoxide (DMSO). The final volume of DMSO in all reaction mixture was <0.5%.

2.2. Isolation of neutrophils

Rat (Sprague–Dawley) blood was collected from the abdominal aorta and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Paque, and hypotonic lysis of erythrocytes [7]. Purified neutrophils containing >95% viable cells were normally resuspended in HBSS containing 10 mM HEPES (pH 7.4), and 4 mM $NaHCO_3$, and kept in an ice bath before use. All experiments in the present study were performed under the guidelines of the Institutional Experimental Laboratory Animal Committee of Taichung Veterans General Hospital and were in strict accordance with the Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

2.3. Measurement of PLD activation

Neutrophils (4×10^7/ml) were incubated with 10 μCi 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine in HBSS at 37 °C for 75 min [7]. Cells were washed and then incubated with test drug in the presence of 0.5% (v/v) ethanol before stimulation. Lipids in the reaction mixture were extracted, dried, and separated on silica gel 60. The plates were developed halfway by using the solvent system consisting of hexane/diethyl ether/methanol/acetic acid (90:20:3:2, v/v/v/v), and then dried and developed again to the top using the upper phase of the solvent system consisting of ethylacetate/isooctane/acetic acid/water (110:50:20:100, v/v/v/v). The zones of [^{3}H] phosphatidylethanol (PEt) were detected and quantified by a PhosphorImager (Fujifilm FLA-5100) using MultiGauge software.

2.4. Immunoblot analysis

Reactions were terminated by boiling in a Laemmli sample buffer. Proteins were resolved by 10% (for PLD1, Vav and Src family) or 13% (for MLC2) SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dried milk in TBST buffer [6] and probed with anti-phospho-MLC2 (T18/S19), anti-phospho-Vav (Y174), anti-phospho-Src family (Y416) or anti-phospho-PLD1 (T147) antibody. The blots were then stripped and reprobed with anti-MLC2, anti-Vav, anti-Lyn or anti-PLD1 antibody, respectively, to standardize protein loading in each lane. Detection was performed with the enhanced chemiluminescence reagent. The band intensity was detected by a Luminescent Image Analyzer (Fujifilm LAS-3000) using MultiGauge software.

For the membrane translocation experiments, neutrophils were resuspended in a disruption solution [6]. After sonication, the lysate was centrifuged to remove the unbroken cells, and then further centrifuged to collect pellets as the membrane fraction. Proteins were resolved by 9% (for ROCK1) or 12% (for PLD1, RhoA, Vav and Arf6) SDS–PAGE, and then immunoblotted with anti-PLD1, anti-Arf, anti-RhoA, anti-Vav or anti-ROCK1 antibody, and also with anti-G_β antibody as a membrane marker to standardize the protein loading in each lane.

In the cell-free experiments, neutrophils were washed and suspended in HEPES buffer [7]. After sonication, the lysate was centrifuged to remove the unbroken cells. The supernatants were incubated with test drugs in the presence of 1 mM Ca^{2+} before stimulation with GTPγS. Reaction was terminated by the addition of five-fold excess ice-cold buffer, and then centrifugation was performed to collect pellets as the membrane fraction. Proteins were subjected to Western blot analysis using the anti-Arf6, anti-RhoA, or anti-G_β antibody.

2.5. RhoA activation and RhoGEF exchange assays

Neutrophils (2×10^7/ml) were preincubated with test drug before stimulation, and then washed twice with ice-cold HBSS containing 25 mM NaF and 1 mM Na_3VO_4, and then resuspended in Mg^{2+} lysis/wash buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% (v/v) Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 10 μg/ml each of aprotinin A and leupeptin, 25 mM NaF, and 1 mM Na_3VO_4 on ice for 15 min. After centrifugation (14,000 × *g* for 5 min at 4 °C), the cell lysate was incubated with Rhotekin-RBD protein agarose beads for 1 h at 4 °C with constant mixing. The beads were then washed three times with lysis buffer and eluted by boiling in a Laemmli sample buffer. RhoA was detected by immunoblotting with anti-RhoA antibody.

The mant fluorophore based GEF assay was performed according to the instructions provided by the manufacturer. Briefly, the reaction mixture containing exchange buffer, 2 μM RhoA-His protein and water was mixed well and then fluorescence was read once every 30 s with a fluorescence microplate reader at 460 nm with excitation at 360 nm. After 5 readings, 2 μM human Dbs-His protein or H_2O was added to respective wells, mixed well and then reading was resumed. Results are expressed in relative fluorescence units (RFU).

2.6. Immunoprecipitation

Neutrophils were suspended in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% (v/v) Nonidet P-40, 2 mM $MgCl_2$, 2 mM Na_3VO_4, 1 mM NaF, 1 μg/ml each of leupeptin, pepstatin and aprotinin A, and kept in an ice-bath for 30 min with occasional shaking. The lysates were centrifuged (12,000 × *g* for 10 min at 4 °C), and the supernatants (500 μg protein) were incubated overnight at 4 °C with anti-PLD1 antibody with constant mixing. Immunocomplexes were collected with protein A-agarose beads for 2 h at 4 °C with constant mixing. The beads were sedimented, washed, and then eluted by boiling in a Laemmli sample buffer. Proteins were resolved by SDS-PAGE and subjected to immunoblot analysis with the specific antibodies against small GTPases (Arf6, RhoA, and RalA), PKCs (α, βI and βII) and PLD1. In some experiments, cell lysates were immunoprecipitated with specific antibody against Lck, Hck, Fgr, Lyn or RhoA followed by immunoblot analysis of the proteins in immunocomplexes with anti-Vav, anti-Lck, anti-Hck, anti-Fgr, anti-Lyn or anti-RhoA antibody, or immunoprecipitated with anti-Arf6 antibody followed by immunoblot analysis of the proteins in immunocomplexes with antibodies against PIP5K1A and Arf6.

2.7. Measurement of $O_2^{\bullet-}$ generation, degranulation and cell migration

The generation of $O_2^{\bullet-}$ in neutrophils was assessed in superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction assay [7]. Briefly, the reaction mixture contained neutrophils (2×10^6 cells) and 40 μM ferricytochrome *c* in a final volume of 1.5 ml at 37 °C. Cells were pretreated with DMSO or test drug before stimulation with fMLP in the presence of dihydrocytochalasin B (dhCB). SOD (17.5 U/ml) was added to the reference cuvette at the beginning of the incubation. Absorbance changes were monitored continuously with a double-beam spectrophotometer at 550 nm.

For neutrophil degranulation, cells (1×10^7/ml) were pretreated with DMSO or test drug before stimulation with fMLP in the presence of dhCB. After centrifugation (2000 × *g* for 5 min at 4 °C), the supernatant was removed for measurement of the enzymatic activities of lysozyme and β-glucuronidase [8].

Cell migration was assessed by means of Fluoroblok inserts (Falcon). Briefly, 1 μM fMLP in 0.2 ml of RPMI-1640 medium containing 1 mM $CaCl_2$ and 0.5% (w/v) bovine serum albumin (RPMI/BSA) was added to a 24-well plate, and 0.2 ml of calcein-loaded neutrophils suspension was delivered to the inserts (3-μm pore size) and placed in the 24-well plate [6]. After incubation at 37 °C for 2 h, the cells in the lower compartment were removed and the fluorescence was monitored with a fluorescence microplate reader at 510 nm with excitation at 485 nm.

2.8. Statistical analysis

Statistical analyses were performed using ANOVA followed by the Bonferroni *t*-test for multigroup comparisons; $P < 0.05$ was considered significant for all tests. The curve estimation regression analysis with logarithmic model (SPSS) was used to calculate IC_{50} values.

3. Results

3.1. Effects of FIPI on fMLP-induced $O_2^{\bullet-}$ generation, degranulation and cell migration

A selective PLD inhibitor FIPI inhibited $O_2^{\bullet-}$ generation, the reactive oxygen product of NADPH oxidase, in response to fMLP in a concentration-dependent manner (about 30% inhibition at 100 nM FIPI) (Fig. 1A). This inhibition was not owing to the $O_2^{\bullet-}$ scavenging effect as assessed by conducting a simple experiment to trigger $O_2^{\bullet-}$ generation during dihydroxyfumaric acid autoxidation (data not shown). FIPI alone had negligible effect on $O_2^{\bullet-}$ generation in neutrophils. As expected, the $O_2^{\bullet-}$ generation was significantly attenuated by 0.5% ethanol and by a NADPH oxidase inhibitor diphenylene iodonium (3 μM) (about 45% and 80% inhibition, respectively).

Resting neutrophils released little lysozyme, a lytic enzyme found in azurophil and specific granules, and β-glucuronidase, an acidic hydrolase found in azurophil granule, into the medium, whereas, fMLP promoted a significant secretion of both enzymes. FIPI did not inhibit but increased the fMLP-induced responses (about 30% and 45% enhancement, respectively, at 100 nM FIPI) (Fig. 1B). A general tyrosine kinase inhibitor genistein diminished the fMLP-induced degranulation [9] as the positive control.

Moreover, inhibition of fMLP-induced neutrophil migration by FIPI (about 33% inhibition at 100 nM FIPI) (Fig. 1C) is consistent with the result of a previous study on differentiated HL-60 cells [5]. FIPI alone did not affect the cell migration.

3.2. Effect of CHS-111 on PLD activation

In 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine-loaded neutrophils, exposure to fMLP caused the production of [^{3}H]PEt in the presence of ethanol. CHS-111 attenuated this response in a concentration-dependent manner (Fig. 2A) with an IC_{50} value of about 3.9 ± 1.2 μM. The result that pretreatment of cells with FIPI (100 nM for 30 min) completely abolished the PEt production (data not shown) is compatible with a report that 75 nM FIPI could virtually abolish the PLD activity in CHO cells stably expressing PLD [5]. However, CHS-111 at the highest concentration tested (30 μM) had no effect on phorbol 12-myristate 13-acetate (PMA)-induced [^{3}H]PEt formation (Fig. 2B). These data suggest an indirect impact of CHS-111 on PLD activation.

3.3. Effects of CHS-111 on the association of PLD1 with small GTPase cofactors and on the recruitment of RhoA and Arf6 to the plasma membrane

PLD1 is a multimodule protein and requires interactions with several small GTPase cofactors, such as ADP-ribosylation factor

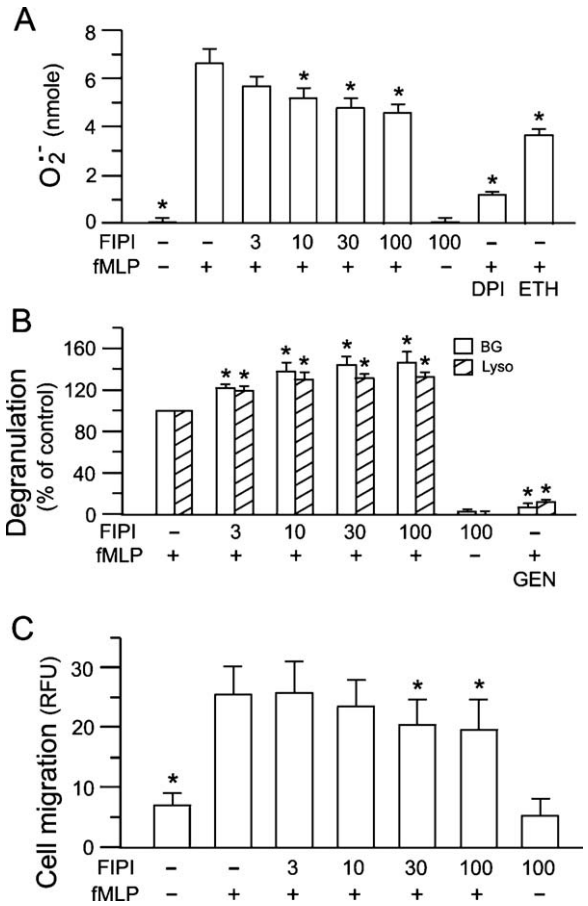

Fig. 1. Effects of FIPI on $O_2^{\bullet-}$ generation, degranulation and cell migration in fMLP-stimulated neutrophils. Cells were preincubated with DMSO, the indicated concentrations (nM) of FIPI or 100 μM genistein (GEN) for 30 min, or with 3 μM diphenylene iodonium (DPI), 0.5% ethanol (ETH) for 5 min before stimulation or no stimulation with 1 μM fMLP (in the presence of 5 μM dhCB for 3 min) for (A) 10 min to determine the $O_2^{\bullet-}$ generation ($^*P < 0.05$, compared with the control value (2nd column)) or (B) 45 min to determine the enzymatic activities of lysozyme and β-glucuronidase in the supernatant ($^*P < 0.05$, compared with the control value (1st column)). (C) Calcein-loaded neutrophils were pretreated with DMSO or the indicated concentrations of FIPI for 30 min, and then delivered to the inserts and placed in the 24-well plate which contained DMSO or fMLP (in the absence of dhCB) for another 2 h. The cell fluorescence in the lower compartment was monitored. $^*P < 0.05$, compared with the control value (2nd column). Values are expressed as means ± S.D. from four independent experiments.

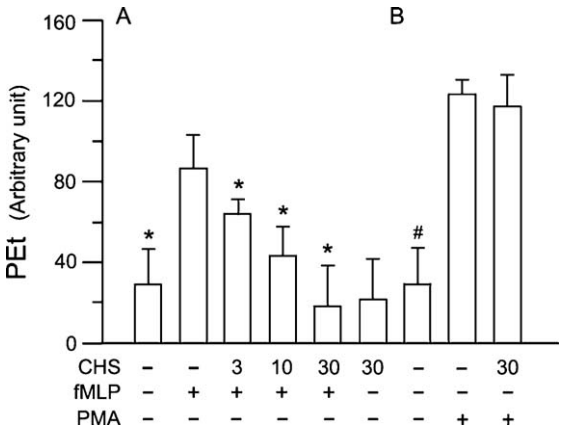

Fig. 2. Effect of CHS-111 (CHS) on PLD activation. 1-*O*-[^{3}H]Octadecyl-*sn*-glycero-3-phosphocholine-loaded neutrophils were incubated with DMSO or the indicated concentrations (μM) of CHS for 5 min at 37 °C, and 5 μM dhCB (for fMLP stimulation) and 0.5% (v/v) ethanol were added during the last 3 min before stimulation or no stimulation with (A) 1 μM fMLP for 0.5 min or with (B) 0.2 μM PMA for 30 min. Lipids in the reaction mixture were extracted and separated. [^{3}H]PEt generation was detected by PhosphorImager. Values are expressed as means ± S.D. of 4 independent experiments. $^*P < 0.05$, $^\#P < 0.05$, as compared with the corresponding control values (column 2).

(Arf), Ras homology (Rho) A and Ras-like (Ral) A, for activation [1]. As shown in Fig. 3A, Arf6 and RhoA were co-immunoprecipitated with PLD1 from the cell lysates of fMLP-stimulated rat neutrophils, and CHS-111 inhibited these responses in a concentration-dependent manner (IC_{50} values about 19.3 ± 1.1 μM and 12.4 ± 2.7 μM, respectively). The association of RalA with PLD1 was not significantly promoted by fMLP in comparison with the vehicle control and was not affected by CHS-111.

It is conceivable that the inactive Arf and Rho GTPases (GDP-bound state) are cytosolic proteins that translocates onto membranes in concert with their activation (GTP-bound state). The membrane anchoring of Arf and Rho GTPases is a prerequisite for PLD activity. As displayed in Fig. 3B, exposure to fMLP increased the breadth of immunoreactive bands of RhoA and Arf6 relative to that apparent with the vehicle-treated cells. This is compatible with experiments in human neutrophil [3]. CHS-111 decreased the amounts of RhoA recovered in membrane fractions following stimulation with fMLP in a concentration-dependent manner (IC_{50} value about 5.0 ± 0.5 μM), whereas it had no significant effect on the membrane association of Arf6. The result that fMLP stimulation increased the membrane expressed PLD1 is in line with the finding of a report in human neutrophils [10], and this effect was not attenuated by CHS-111 over the same concentration range to inhibit RhoA membrane association, excluding the change in amount of PLD1 mediated the decrease in membrane level of RhoA. None of the treatments in this study had any impact on membrane levels of G_β.

In a neutrophil cell-free system, the amounts of membrane-associated Arf6 and RhoA were significantly increased in response to GTPγS, a direct small GTPases activator, as visualized by Western blotting of cell membrane fractions (Fig. 3C) and confirmed our previous study [7]. CHS-111 had no inhibitory effect on either RhoA or Arf6 recovered in membrane fractions following stimulation with GTPγS.

3.4. Effects of CHS-111 on RhoA activation, membrane recruitment of ROCK1, phosphorylation of MLC2, the association of Arf6 with arfaptin 1 and PIP5K1A

Stimulation of neutrophils with fMLP increased the immunoreactivity of agarose-associated RhoA by using Rhotekin-RBD protein agarose, which specifically binds to GTP-bound Rho, and this effect was attenuated by CHS-111 in a concentration-dependent manner (IC_{50} value about 7.1 ± 2.4 μM) (Fig. 4A).

Upon translocation to the plasma membrane, GTP-bound RhoA recruits and activates Rho-associated protein kinase (ROCK) from cytosolic localization, leading to the phosphorylation of downstream targets including myosin light chain (MLC) [11]. To date, two ROCK isoforms, ROCK1 and ROCK2, have been described, but little is known about the expression of ROCK isoforms in neutrophils. Stimulation with fMLP resulted in the overlapping of the time dependencies of membrane recruitment of ROCK1 and phosphorylation of MLC2 (T18/S19) (Fig. 4B and D), which began at 0.5 min, reached a maximum at 1 min and declined thereafter. Both responses were attenuated by CHS-111 in a concentration-dependent manner with an IC_{50} value about 9.1 ± 5.6 μM against the recruitment of ROCK1 to membrane (Fig. 4C) and with 52.1 ± 7.1% inhibition of MLC2 phosphorylation at 30 μM CHS-111 (Fig. 4E). Y-27632, a cell-permeable inhibitor of ROCK, attenuated the phosphorylation of MLC2 to about 60% of control level at 3 μM.

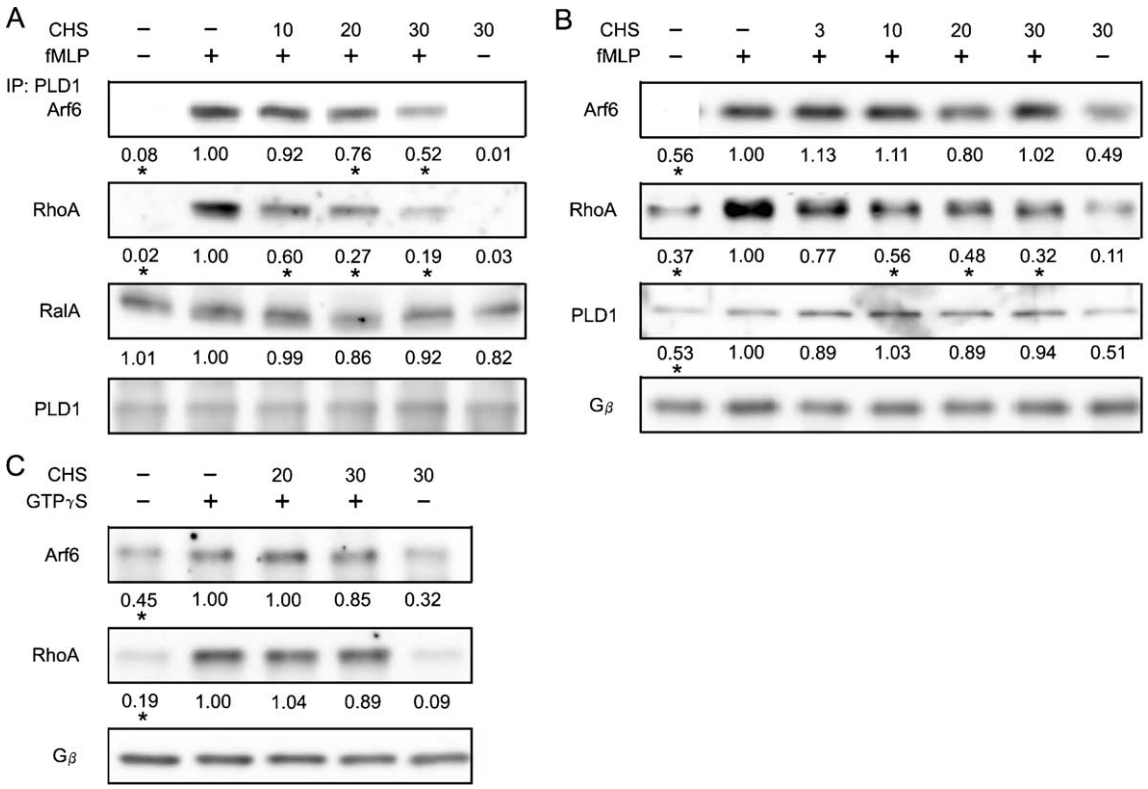

Fig. 3. Effects of CHS-111 (CHS) on the interaction of PLD1 with small GTPase cofactors and on the membrane association of Arf6 and RhoA. (A and B) Neutrophils or (C) cell lysates were pretreated with DMSO or the indicated concentrations (μM) of CHS for 5 min before stimulation or no stimulation with (A, B) 1 μM fMLP for 30 s (in the presence of 5 μM dhCB for 3 min) or (C) 10 μM GTPγS for 10 min. (A) PLD1 was immunoprecipitated from cell lysates, and then immunoblotted with the specific antibody against Arf6, RhoA, RalA or PLD1 (as loading control). (B and C) The membrane fractions were analyzed by immunoblotting with the specific antibody against Arf6, RhoA, PLD1 or G_β (as loading control). The ratio of immunointensity between the small GTPase proteins and the loading control was calculated. The numbers below blots are the mean fold change compared to the control values (2nd lane) from 3 to 4 independent experiments. $^*P < 0.05$ compared with the corresponding control values.

It has been reported that arfaptin binds specifically to GTP-bound Arf6 [12]. Both arfaptin 1 and 2 isoforms are known to be expressed in HL-60 cells [13]; however, whether they are expressed in primary neutrophil remains undefined. As shown in Fig. 4F, resting neutrophils had little interaction between Arf6 and arfaptin 1, and exposure to fMLP significantly enhanced this interaction. CHS-111 did not affect fMLP-induced response except at the highest concentration tested (about 35% inhibition at 30 μM CHS-111).

In mammalian cells, three type I phosphatidylinositol 4-phosphate 5-kinase (PIP5K) isozymes (PIP5K1A, B, and C) have been identified; however, the expression of PIP5K isozymes in neutrophils remain unclear. PIP5K directly interact with PA, leading to conformational change that allows binding of activated Arf6 at the plasma membrane to the kinase core domain [14] for stimulation of PIP5K to produce $PI(4,5)P_2$. Stimulation with fMLP increased the association between Arf6 and PIP5K1A in comparison with the vehicle control based on the immunoprecipitation experiment. However, this association was attenuated in cells pretreated with increasing concentrations of CHS-111 before fMLP exposure with an IC_{50} value about 9.3 ± 3.0 μM (Fig. 4G).

3.5. Effects of CHS-111 on the association of Vav with RhoA and on Vav activation

Rho guanine nucleotide exchange factors (GEFs) mediate the activation of Rho GTPases by promoting the release of GDP in exchange for GTP. Almost all RhoGEFs belong to the Dbl-RhoGEF family, in which the cell-specific differences in GEF activity of Vav towards each of RhoA, RhoG, Rac1 and Cdc42 in response to phosphoinositide 3-kinase (PI3K) activation has been reported [15]. The interaction of C-terminal PH domain with $PI(3,4,5)P_3$, the product of PI3K, recruits Vav to the plasma membrane for GEF activity. The results that Vav co-immunoprecipitated with RhoA from the cell lysates of fMLP-stimulated neutrophils and a PI3K inhibitor LY294002 (10 μM) completely inhibited this response (Fig. 5A) are consistent with the requirement of Vav for RhoA activation. Pretreatment of cells with CHS-111 attenuated the association of Vav with RhoA in a concentration-dependent manner (IC_{50} value about 12.9 ± 0.1 μM) suggesting the impairment of Vav-mediated RhoA activation.

fMLP evoked the membrane recruitment of Vav in rat neutrophils and this effect was attenuated by CHS-111 in a concentration-dependent manner (IC_{50} value about 11.5 ± 4.6 μM) (Fig. 5B) with no changes in levels of G_β. Moreover, the phosphorylation of Vav Y174 having been shown to regulate Vav activity [16], however, the nature of tyrosine kinase involvement has yet to be identified in neutrophils. Fig. 5C shows fMLP-induced phosphorylation of Vav Y174 occurred in a time-dependent manner, with a significant increase at 5 s, a maximal at 30 s, and then a gradual decline to baseline, thus confirming the recent report that Vav Y174 is phosphorylated within seconds of receptor stimulation [17]. Preincubation of cells with CHS-111 resulted in concentration-dependent decrease in the maximal response of fMLP (IC_{50} value about 14.8 ± 5.5 μM) (Fig. 5D). The result that a Src family kinases (SFK) inhibitor PP1 strongly inhibited the phosphorylation of Vav is in accordance with the findings of previous reports about the phosphorylation of Vav Y174 by Src-related tyrosine kinase [16–18]. In the presence of DH/PH domain of human Dbs protein, the increase in fluorescence intensity of reaction mixture reflected the GEF activity based on the binding of mant (N-methylanthraniloyl)-GTP to RhoA (Fig. 5E). CHS-111 up to 30 μM slightly attenuated the GEF activity, whereas, addition of 10 μM GDP eliminated the fluorescence changes.

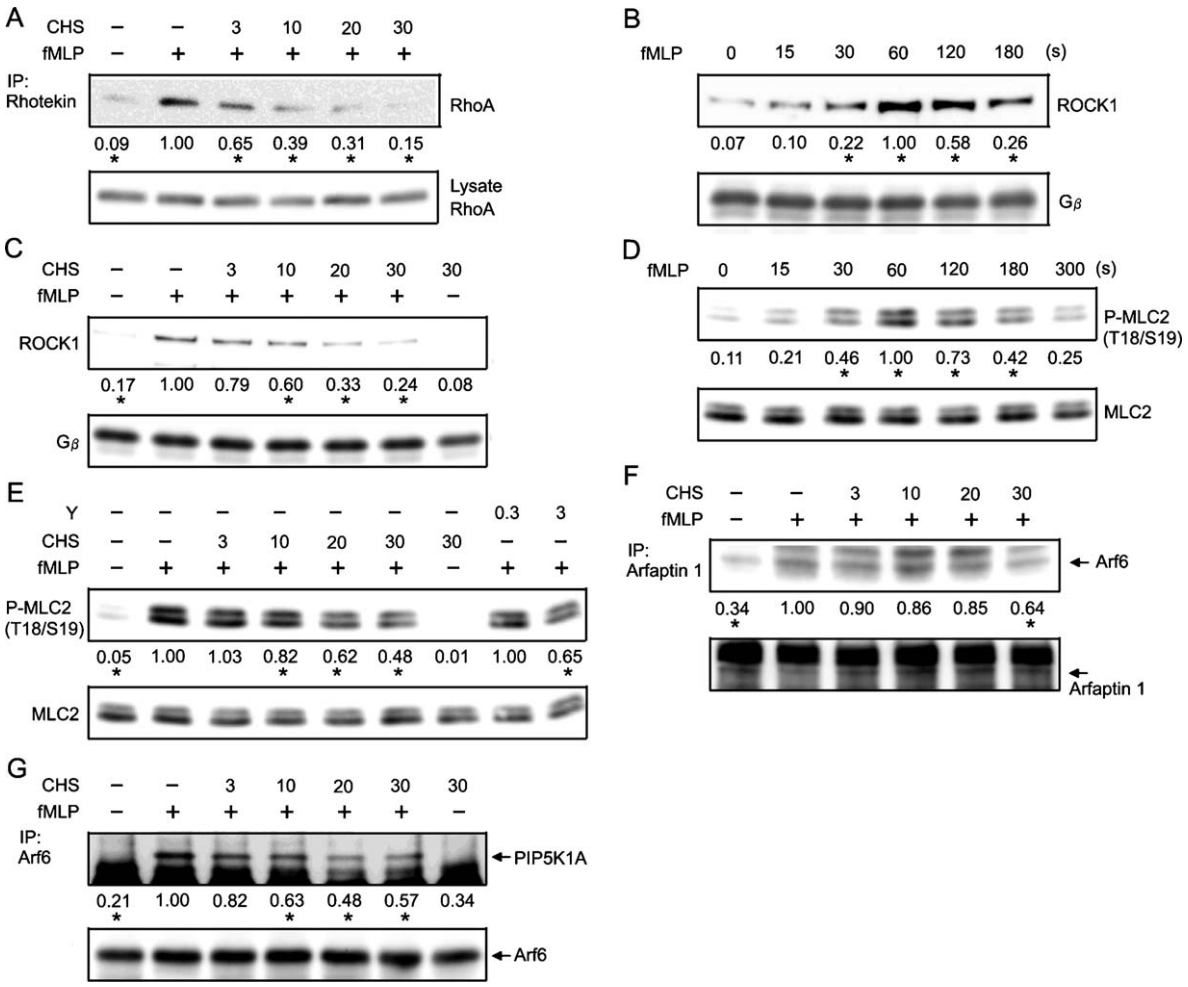

Fig. 4. Effects of CHS-111 (CHS) on RhoA activation, the interaction of Arf6 with arfaptin 1 and PIP5K1A. Neutrophils were pretreated with DMSO or the indicated concentrations (μM) of CHS for 5 min before stimulation or no stimulation with 1 μM fMLP for 30 s (in the presence of 5 μM dhCB for 3 min). (A) RhoA-GTP, (F) Arf6 or (G) PIP5K1A was immunoprecipitated from cell lysates, and then immunoblotted with anti-RhoA antibody, the specific antibody against Arf6 and arfaptin 1 (as loading control for F), or with anti-PIP5K1A antibody and anti-Arf6 antibody (as loading control for G). The relative densitometric unit for the ratio of small GTPase proteins to the loading control or the lysate RhoA was calculated. Cells were (B and D) stimulated with 1 μM fMLP for the indicated time periods (in the presence of dhCB for 3 min), or (C and E) pretreated with the indicated concentrations (μM) of CHS for 5 min or with Y-27632 (Y) for 10 min before stimulation or no stimulation with fMLP for 1 min. The (B and C) membrane fractions or (D and E) cell lysates were analyzed by immunoblotting with the specific antibody against ROCK1 or G_β (as loading control) and against phospho-MLC2(T18/S19) or MLC-2 (as loading control), respectively. The relative densitometric unit for the ratio of ROCK1 or phospho-MLC2 to the loading control was calculated. The numbers below blots are the mean fold change compared to the control values (4th lane for B and D; 2nd lane for A, C, E, F and G) from 3 to 4 independent experiments. $^*P < 0.05$ compared with the corresponding control values.

3.6. Effects of CHS-111 on SFK activation

The mechanism of G-protein-coupled receptor (GPCR)-mediated SFK activation is not completely understood. It is conceivable that the activity of SFK is upregulated by autophosphorylation of Y416 in the activation loop of the kinase [19]. As shown in Fig. 6A, phosphorylation of SFK Y416 from fMLP-activated neutrophils was detectable at 15 s, maximal after 30 s, and then gradually declines. CHS-111 exhibited no significant inhibition of SFK phosphorylation, whereas, PP1 virtually abolished the fMLP-induced response (Fig. 6B).

SFK comprise 11 structurally related, membrane-associated, nonreceptor tyrosine kinase. Of these, Lyn, Fgr, and Hck are known to be activated by fMLP in neutrophils [20]. Lck is also expressed in neutrophils [21] and exerts its effect through the phosphorylation of Vav Y174 [16]. Moreover, phosphorylation of Vav was markedly reduced in $hck^{-/-}fgr^{-/-}$ neutrophils [18]. A significant association of Vav with Lck, Hck, Fgr and Lyn was observed after stimulation of cells with fMLP as assessed in immunoprecipitation experiments, and CHS-111 attenuated these responses in a concentration-dependent manner with IC_{50} values about 12.0 ± 4.0, 16.1 ± 2.9, 15.2 ± 3.4 and 12.7 ± 4.0 μM, respectively (Fig. 6C).

3.7. Effects of CHS-111 on the association of PLD1 with PKC cofactors and on the phosphorylation of PLD1

More than ten isoenzymes of mammalian PKC have been identified, of which eight PKC isoenzymes (PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCθ, PKCι, and PKCζ) are expressed in rat neutrophils [6,22]. The Ca^{2+}-dependent PKCs (α, βI and βII) but not other PKC isoenzymes are PLD activators [23]. In the present study, fMLP stimulation resulted in the association of PKCα, PKCβI and PKCβII with PLD1 as assessed by using anti-PLD1 antibody for immunoprecipitation, and these interactions were attenuated by CHS-111 in a concentration-dependent manner with IC_{50} values about 13.6 ± 1.3 μM, 19.2 ± 3.8 μM, and 14.3 ± 4.1 μM, respectively (Fig. 7A).

Although PKC activates PLD1 without a phosphorylation process in vitro, a positive regulation of PLD activation by PKC kinase activity was reported in fMLP-stimulated neutrophils [24] and the phosphorylation of T147 residue in PLD1 was shown to be important in stimulation of PLD activity in vivo [25]. It is unclear at present whether phosphorylation of PLD1 T147 occurs in neutrophils upon fMLP stimulation. Fig. 7B shows the T147

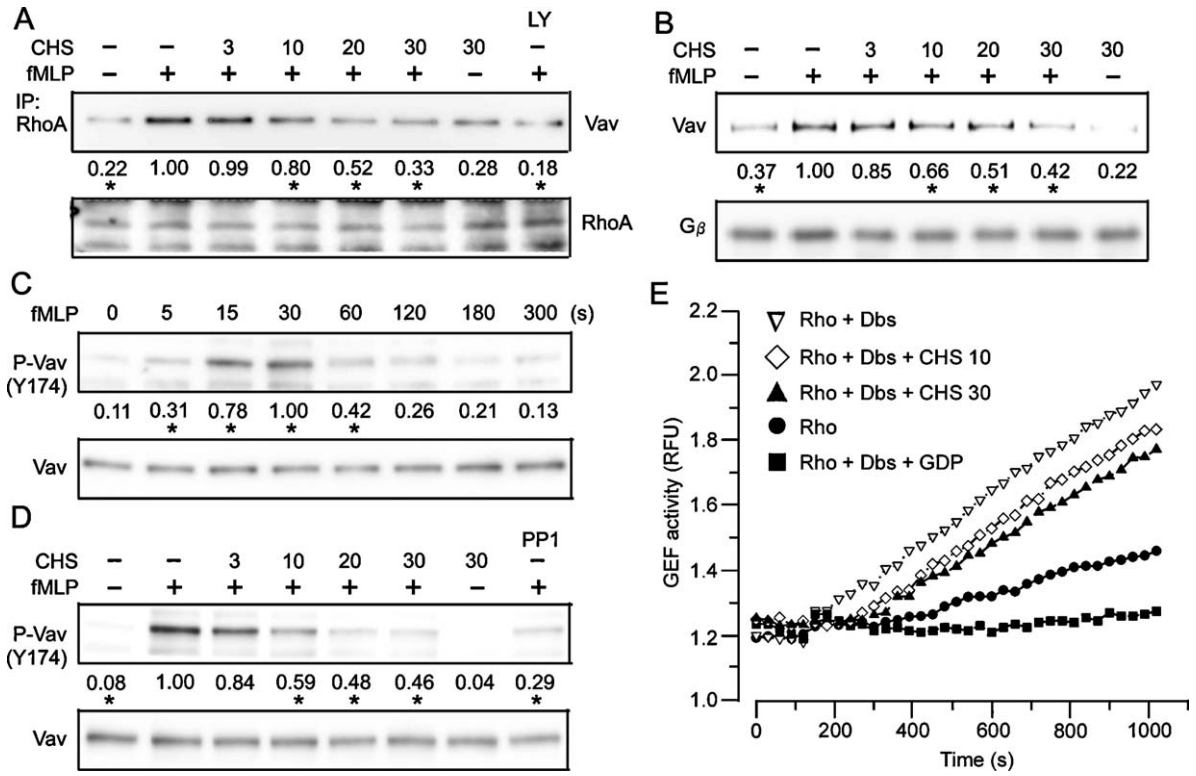

Fig. 5. Effects of CHS-111 (CHS) on the association of RhoA with Vav, Vav activation, and on RhoGEF activity. Neutrophils were pretreated with DMSO or the indicated concentrations (μM) of CHS for 5 min or with 10 μM LY 294002 (LY), 10 μM PP1 for 10 min before stimulation or no stimulation with 1 μM fMLP for 30 s (in the presence of 5 μM dhCB for 3 min). In some experiments, cells were stimulated with 1 μM fMLP for the indicated time periods. (A) RhoA was immunoprecipitated from cell lysates, and then analyzed by immunoblotting with anti-Vav antibody or anti-RhoA antibody (as loading control). (B) The membrane fractions were analyzed by immunoblotting with the specific antibody against Vav or G_β (as loading control). (C and D) Cell lysates were analyzed by immunoblotting with anti-phospho-Vav (Y174) antibody. The blots above were then stripped and reprobed with anti-Vav antibody (as loading control). The relative densitometric unit for the ratio of RhoA, Vav or phospho-Vav (Y174) to the loading control was calculated. The numbers below blots are the mean fold change compared to the control values (2nd lane for A, B and D; 4th lane for C) from 3 to 4 independent experiments. $^*P < 0.05$ compared with the corresponding control values. (E) Human Dbs protein was preincubated with DMSO, CHS (10 and 30 μM) or 10 μM GDP for 5 min before addition to the reaction mixture. Fluorescence changes were monitored at 360/460 nm.

phosphorylation time profile of PLD1, which was detectable within 5 s, reached a maximal level at 15 s then gradually declined, and was undetectable at times ≥1 min after exposure to fMLP. Pretreatment of cells with a PKC inhibitor GF109203X (1 μM) nearly abolished the phosphorylation of PLD1 T147, thus confirming the result of a previous study [24]. CHS-111 attenuated fMLP-induced response in a concentration-dependent manner with an IC_{50} value about 13.4 ± 5.5 μM (Fig. 7C).

4. Discussion

In this study we have used a recently developed potent and selective PLD inhibitor FIPI [5] to trace the relationship between PLD activation and several biological processes of neutrophil (respiratory burst, degranulation, and migration). Because the non-selective effects of widely utilized primary alcohols, it is difficult to evaluate the role of PLD in cellular functions and the short life span of neutrophils makes the current molecular biology approaches impracticable. We have shown PLD activation in rat neutrophils to be associated with fMLP-mediated respiratory burst and migration as assessed the effects of FIPI on these cellular functions at concentrations that completely abolished the PEt production, supporting the proposed functions for PLD, but not supporting degranulation [1]. The report that eliminating PLD activity by FIPI in Min6 pancreatic-cells does not affect glucose-stimulated insulin secretion [5] is in line with our observation. We also demonstrated that the blockade of signaling pathways leading to PLD activation by a novel benzyl indazole compound CHS-111 (Fig. 8) contributes, at least partly, to its anti-inflammatory activity.

Studies into the signaling mechanisms of GPCR-PLD coupling have implicated multiple pathways leading to PLD activation, that include phosphoinositides, Ca^{2+}, PKC, tyrosine kinase, and several small GTPases (such as Arf, Rho and Ral) [1]. Rho and Arf exhibit a GTP-dependence for direct binding to PLD and the subsequent stimulation of PLD activity in a range of cell types [4]. In vitro experiments have not demonstrated any great distinction between the six members of Arf GTPases family in their ability to stimulate PLD activity, however, the regulation of in vivo PLD activity is likely linked to the activation of Arf6 [26]. Rho GTPases family consists of 20 distinct members, and each member appears to activate PLD1, but RhoA activates PLD approximately four to five times better than does Rac1 or Cdc42 [27]. The interactions of both RhoA and Arf6 with PLD1 were suppressed by CHS-111 as evidenced from the co-immunoprecipitation experiments. The results that CHS-111 attenuated the membrane recruitment of RhoA in fMLP-stimulated cells, but not to GTPγS in a cell-free system, implies that the blockade of RhoA activation may be attributable to the decrease in PLD activity. CHS-111 had no effect on the recruitment of Arf6 to the plasma membrane, thus confirming it is not involved in the blockade of Arf6 activation. Both RhoA pulldown activation and arfaptin binding assays confirmed these observations. The blockade of membrane recruitment of ROCK1 and the downstream MLC2 phosphorylation by CHS-111 reinforce the involvement of RhoA activation. The local increase of the $PI(4,5)P_2$ level by type I PIP5K at the plasma membrane [28] appears crucial for the regulation of signal transduction and cell events including PLD activation [1]. The decrease in the interaction between PIP5K1A and Arf6 supports the inhibition of PLD by CHS-111. However, the

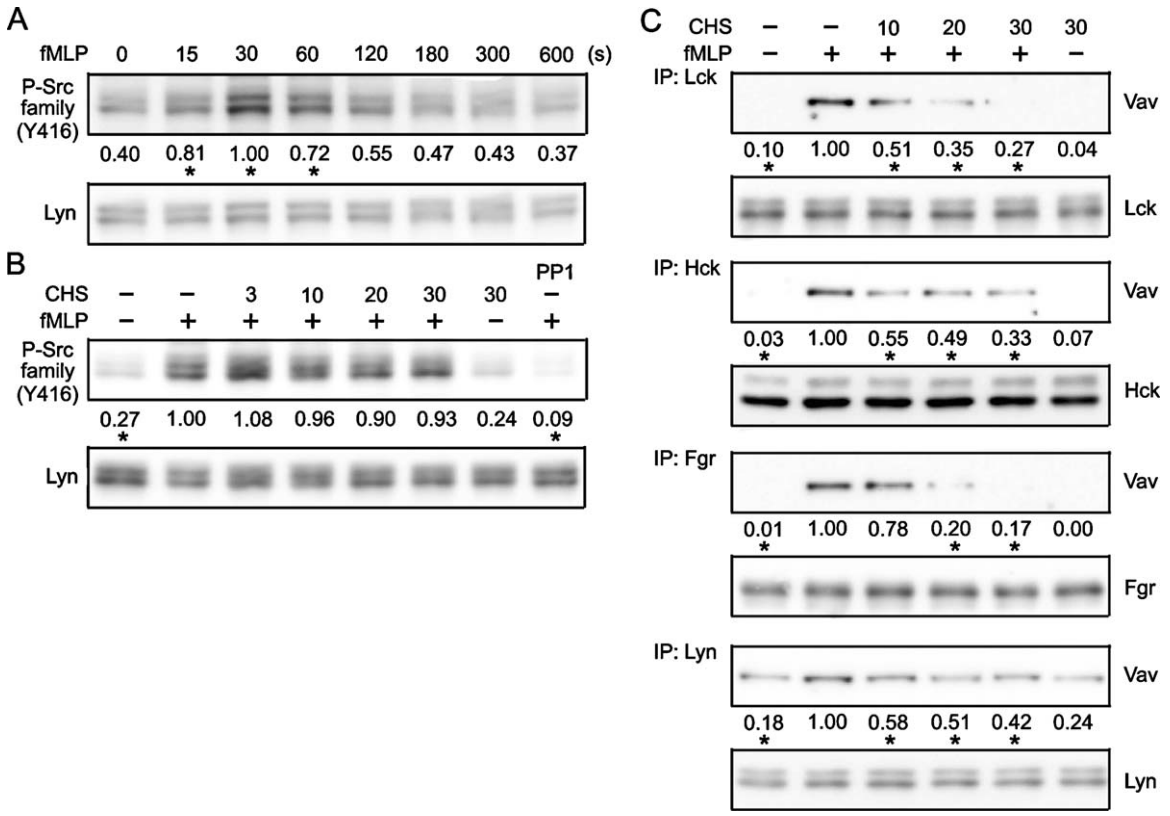

Fig. 6. Effect of CHS-111 (CHS) on Src activation. Neutrophils were (A) stimulated with 1 μM fMLP for the indicated time periods (in the presence of 5 μM dhCB for 3 min), or (B) pretreated with the indicated concentrations (μM) of CHS for 5 min or with 10 μM PP1 for 10 min before stimulation or no stimulation with fMLP for 30 s. Cell lysates were analyzed by immunoblotting with anti-phospho-Src family (Y416) antibody. The blots above were then stripped and reprobed with anti-Lyn antibody (as loading control). (C) Lck, Hck, Fgr, Lyn were immunoprecipitated from cell lysates, and then analyzed by immunoblotting with anti-Vav antibody or with the specific antibody against Lck, Hck, Fgr or Lyn as loading control. The relative densitometric unit for the ratio of phospho-Src or Vav to the loading control was calculated. The numbers below blots are the mean fold change compared to the control values (3rd lane for A; 2nd lane for B and C) from 3 to 4 independent experiments. $^*P < 0.05$ compared with the corresponding control values.

mechanism responsible for the interference with the recruitment of Arf6 to PLD1, which also account for the decrease in PLD activity by CHS-111, remains ill defined and awaits further investigation.

Although PLD1 associates directly with RalA, there is no gross difference in the binding efficiency of PLD to GTP- and GDP-bound RalA [29]. This is in accordance with our finding that the association of RalA with PLD1 was not significantly promoted by fMLP in comparison with the vehicle control. RalA alone is not sufficient to activate PLD1 [29]. Activated RalA leads to membrane recruitment of Arf [30] and synergistically enhances the Arf-

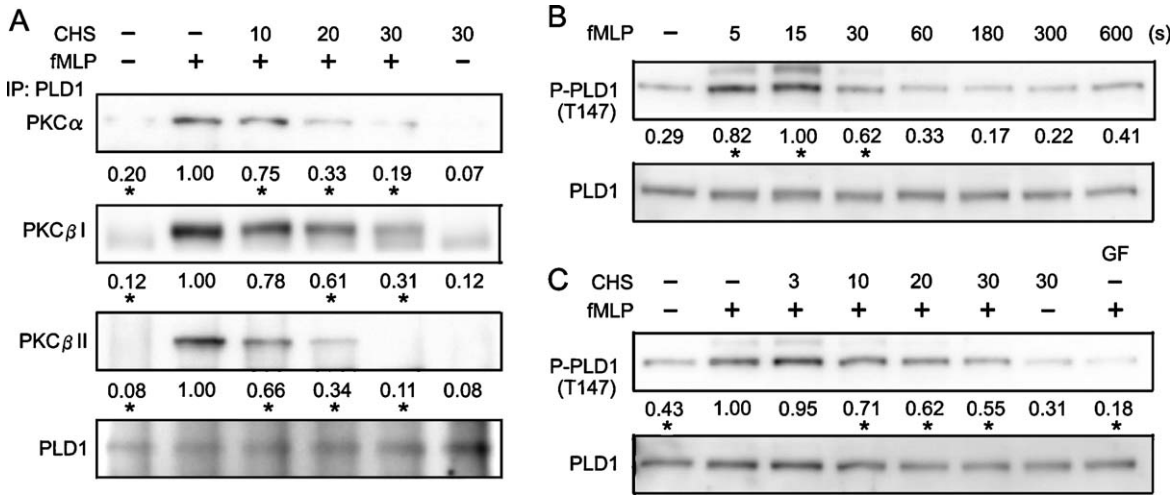

Fig. 7. Effects of CHS-111 (CHS) on the interaction of PLD1 with PKC cofactors and the phosphorylation of PLD1. (A) Neutrophils were pretreated with DMSO or the indicated concentrations (μM) of CHS for 5 min before stimulation or no stimulation with 1 μM fMLP for 30 s (in the presence of 5 μM dhCB for 3 min). PLD1 was immunoprecipitated from cell lysates, and then analyzed by immunoblotting with the specific antibody against PKCα, PKCβI, PKCβII or PLD1 (as loading control). Cells were (B) stimulated with fMLP for the indicated time periods or (C) pretreated with DMSO, the indicated concentrations of CHS for 5 min or with 1 μM GF109203X (GF) for 10 min before stimulation or no stimulation with fMLP for 15 s. Cell lysates were then immunoblotted with anti-phospho-PLD1(T147) antibody or anti-PLD1 antibody (as loading control). The relative densitometric unit for the ratio of PKC or phospho-PLD1 to the loading control was calculated. The numbers below blots are the mean fold change compared to the control values (2nd lane for A and C; 3rd lane for B) from 3 to 4 independent experiments. $^*P < 0.05$ compared with the corresponding control values.

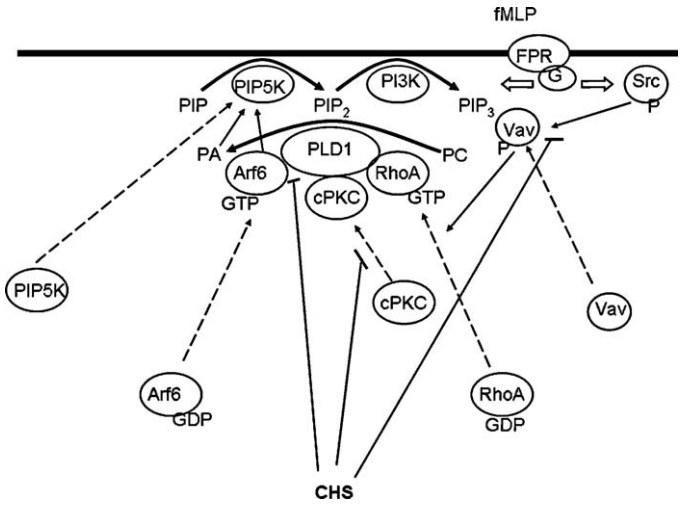

Fig. 8. Schematic diagram showing the mechanisms underlying the inhibition by CHS-111 (CHS). The signaling molecules generally employed in mediating activation of PLD1 are indicated. Some additional signaling molecules in these pathways have been omitted for the sake of clarity. Broken lines indicated the membrane translocation. Blunt lines indicated the possible sites of action of CHS.

dependent PLD activity. The results that CHS-111 did not affect the interaction of RalA with PLD1 and had no effect on the recruitment of Arf6 to membrane indicate the minor role of RalA in the inhibition of PLD activity by CHS-111.

The GTP/GDP cycling of Rho is tightly controlled through guanine nucleotide exchange and intrinsic GTPase activity. Before Rho activation, the GEF activity of Vav is autoinhibited by binding of a helix from the C-terminus of the acidic domain into the active site of the Dbl homology (DH) domain, blocking access to substrate [31]. This core inhibitory interaction is relieved by phosphorylation of Y174 in the inhibitory helix. The result that a SFK inhibitor PP1 strongly attenuated the phosphorylation of Vav Y174 supports a central role for SKF in governing Vav activation in fMLP-stimulated rat neutrophils. GDP could virtually abolish the GEF activity of Dbs (DH/PH) domain, whereas CHS-111 up to 30 μM exhibited weak activity, implying the minor role of direct inhibition of GEF domain; whether CHS-111 has direct inhibitory effect on the full-length of RhoGEF awaits further investigation.

Before cell activation, the autoinhibition of SFK is maintained by the intramolecular interactions of the tail phosphotyrosine (homologous to Y527 in c-Src) with the SH2 domain and of the SH3 domain with the SH2-catalytic domain linker [19]. SFK can be activated by disruption of the intramolecular restraints on the kinase domain through binding of high-affinity ligands. However, the mechanisms of SFK activation by chemoattractant receptors in neutrophils are totally unknown and it remains unclear which members of SFK have links the GPCR-PLD coupling. It is conceivable that autophosphorylation of Y416 (within the activation loop) stimulates Src activity [19]. Indeed, fMLP stimulated SFK activation in rat neutrophils as assessed by immunoblotting with phospho-Src family (Y416) antibody, which cross-reacts with SFK members when phosphorylated at equivalent sites. Although CHS-111 had no inhibitory effect on SFK activation, the blockade of SFK members (Lck, Hck, Fgr and Lyn) interaction with Vav by CHS-111 over a similar concentration range to inhibit the phosphorylation of Vav Y174 could account for the down-regulation of Vav signaling.

There are conflicting reports on the phosphorylation effect on PLD activity in vivo. Phosphorylation of PLD1 occur in cells upon exposure to PMA and its activity decreased as phosphorylation increased with time [32], and a positive regulation of PLD activation by PKC kinase activity was reported in fMLP-stimulated neutrophils [24]. The reasons for this discrepancy may arise from the use of different stimulators. Stimulation of neutrophils by fMLP, through the activation of GPCR, induced a rapid PA production, which peaked at 30–60 s, then declined [24], whereas when PA production was triggered with PMA, it bypassed the membrane receptor, and reached a maximum at 3 min [33]. Thus the different kinetics of PA formation between fMLP and PMA indicates distinct regulatory mechanisms. Our recent report demonstrated that CHS-111 decreased PKC autophosphorylation (homologous to S660 in PKCβII), the membrane recruitment of PKCs (α, βI, βII, δ and ζ) and reduced the total PKC kinase activity in fMLP-stimulated rat neutrophils, but failed to alter the PKC activity in cell-lysates from the activated cells [6]. Because the specific substrates have not been identified for the PKC isoenzymes, cellular inhibition of specific PKC isoenzymes is difficult to assess at this time. Alternatively, activation of Ca^{2+}-dependent PKCs is characterized by recruitment of cytosolic PKC to the membrane fraction in intact cells by immunoblotting with specific PKC isoenzyme antibodies. In the present study, CHS-111 attenuated both the association of PKCs (α, βI and βII) with PLD1 and the phosphorylation of PLD1 T147 over a similar concentration range in response to fMLP. These results, together with our recent evidence of inhibitory effects on PKC [6] noted above, likely account for the decrease in PLD activity by CHS-111 through the blockade of signaling pathway leading to PKC activation. More studies will be necessary to elucidate the underlying mechanisms; however, inhibition of extracellular Ca^{2+} entry by CHS-111 [6] could be one signaling mechanism that interferes with the activation of Ca^{2+}-dependent PKC. Although CHS-111 inhibited the recruitment of Arf6, RhoA and Ca^{2+}-dependent PKC to PLD1 at effective concentrations higher than those required to perform PLD inhibition, these effects are probably contributed to the inhibition of PLD activation by CHS-111 because Arf, RhoA and PKC act synergistically to regulate fMLP-stimulated PLD activity [34].

In conclusion, a potent and selective PLD inhibitor FIPI was employed to probe the involvement of PLD in fMLP-induced neutrophil activation, and our results showed that PLD is necessary for activation of respiratory burst and migration but not for promoting degranulation. Inhibition of PLD activation by CHS-111 is attributable to the interference with 1) RhoA activation through the blockade of interaction between SFK and Vav, and Vav activation, 2) interaction of Arf6 with PLD1 and PIP5K, and 3) the activation of Ca^{2+}-dependent PKC probably through the decrease in cytoplasmic free Ca^{2+} concentration in rat neutrophils. Since PLD plays an important role in neutrophil activation and neutrophils are relatively insensitive to the conventional anti-inflammatory agents, one may speculate that the inhibition of PLD activity by CHS-111 may have beneficial anti-inflammatory effect.

Acknowledgements

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