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Inhibition of phospholipase D activation by CYL-26z in formyl peptide-stimulated neutrophils involves the blockade of RhoA activation

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Abstract

5-[4-Acridin-9-ylamino]phenyl]-5-methyl-3-methylenedihydrofuran-2-one (CYL-26z) inhibited the formyl-Met-Leu-Phe (fMLP)-stimulated phospholipase D (PLD) activity, which was assessed by the production of phosphatidylethanol (PEt) in the presence of ethanol, in rat neutrophils (IC_{50} 1.2 ± 0.2 μM). CYL-26z caused a slight but significant attenuation of the global protein tyrosine phosphorylation stimulated by fMLP only at concentrations of CYL-26z up to 30 μM. CYL-26z blocked the membrane recruitment of protein kinase C-α (PKC-α) at concentrations of CYL-26z ≥3 μM, but failed to affect the membrane association of PKC-βI and -βII. The translocation of RhoA to the membrane was attenuated by CYL-26z (IC_{50} 3.8 ± 0.8 μM) in fMLP-stimulated neutrophils, whereas CYL-26z caused no significant inhibition of the membrane recruitment of ADP-ribosylation factor (Arf). CYL-26z inhibited the activation of RhoA and dissociation of the RhoA-Rho guanine nucleotide dissociation inhibitor (GDI) complex in fMLP-stimulated neutrophils (IC_{50} 1.8 ± 1.0 μM and 1.8 ± 0.9 μM, respectively). In a cell-free system, CYL-26z effectively attenuated the membrane association of RhoA in response to GTPγS (IC_{50} 1.3 ± 0.5 μM). In contrast, the GTPγS-stimulated translocation of Arf to membrane was suppressed only at concentrations of CYL-26z up to 30 μM. CYL-26z inhibited the fMLP-stimulated membrane expression of CD11b, CD45 and CD63, and the release of lysozyme and β-glucuronidase. These results indicate that CYL-26z inhibited the fMLP-stimulated PLD activity, mainly through the blockade of RhoA activation, and degranulation in rat neutrophils.

Keywords: CYL-26z; Phospholipase D; RhoA; RhoGDI; Degranulation; Neutrophils

1. Introduction

Neutrophils play a critical role in the first-line defense against invading microbial pathogens via phagocytosis, generation of reactive oxygen species, and release of microbicidal substances. 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 promising strategies for therapeutic intervention aiming at decreasing the severity of inflammatory disorders in patients. The signal transduction

Abbreviations: Arf, ADP-ribosylation factor; CYL-26z, 5-[4-acridin-9-ylamino]phenyl]-5-methyl-3-methylenedihydrofuran-2-one; dhCB, dihydrocytochalasin B; DMSO, dimethyl sulfoxide; fMLP, formyl-Met-Leu-Phe; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; HBSS, Hanks' balanced salt solution; PA, phosphatidic acid; PEt, phosphatidylethanol; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; TK, tyrosine kinase

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events pertaining to these neutrophil functions remain elusive. Accumulating evidence has indicated that phospholipase D (PLD) plays an important role in regulating cell responses such as phagocytosis, degranulation, and superoxide anion generation in neutrophils [1–3]. 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. PLD primarily catalyses the hydrolysis of phosphatidylcholine to produce the second messenger phosphatidic acid (PA), which in turn is further metabolized to diacylglycerol and lysoPA.

In mammalian cells, two related genes encoding for differently regulated PLD enzymes, PLD1 and PLD2 have been characterized. Splice variants of PLD1 (PLD1a and PLD1b) [4] and PLD2 (PLD2a and PLD2b) [5] have also been characterized. PLD1 has a low basal activity and is highly regulated whereas PLD2 exhibits a high basal activity and is much less regulated. PLD1a has been found to be the major PLD isoform in neutrophil membranes [6]. Our previous report demonstrated that PLD1a, but not PLD1b, and PLD2 are expressed in rat neutrophils as assessed by reverse transcription-polymerase chain reaction [7]. Several mechanisms have been proposed for the activation of PLD in neutrophils, including the participation of Ca^{2+}-dependent protein kinase C (PKC), small GTPases RhoA and ADP-ribosylation factor (Arf), and protein tyrosine phosphorylation [8,9].

In screening studies with the goal of identifying a potential anti-inflammatory compound from the synthetic 9-anilinoacridine and 9-phenoxyacridine derivatives [10], 5-[4-acridin-9-ylamino]phenyl]-5-methyl-3-methylenedihydrofuran-2-one (CYL-26z) was found to have a potent inhibitory effect on the neutrophil functions that include degranulation and the production of superoxide anion (data not shown). In addition, CYL-26z has recently been reported to down-regulate the tumor necrosis factor-α-induced inflammatory genes expression through suppression of IκB kinase activity and NF-κB activation [11]. Since PLD plays an important role in the regulation of neutrophil biological functions, we first assessed the effects of CYL-26z on PLD activation as part of addressing the mechanism of action of CYL-26z. formyl-Met-Leu-Phe (fMLP) has been the most intensely studied formyl-peptide derived from bacterial proteins, whose specific receptor is identified on the neutrophil plasma membrane. The fMLP-receptor interaction with G_i protein activates multiple transduction pathways responsible for various neutrophil functions, which represent the physiological response to bacterial infection. One of the early signaling events upon fMLP stimulation is PLD activation. Therefore, we investigated the effect of CYL-26z on the signaling cascade that leads to PLD activation upon stimulation with fMLP.

2. Materials and methods

2.1. Materials

Dextran T-500, enhanced chemiluminescence reagent, 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine and protein A beads were purchased from Amersham Pharmacia Biotech. Hanks balanced salt solution (HBSS) was obtained from Invitrogen. Mouse monoclonal antibody to CD63 and FITC-conjugated antibody against CD11b and CD45 were purchased from BD Transduction Laboratories. Rabbit polyclonal antibodies to CD88 and Arf, mouse monoclonal RhoA and Rho guanine nucleotide dissociation inhibitor (GDI) antibodies were obtained from Santa Cruz Biotechnology. Polyvinylidene difluoride membrane was obtained from Millipore. Rho activation assay kit was purchased from Upstate. Other chemicals were purchased from Sigma–Aldrich. CYL-26z (purity >99%) was dissolved in dimethyl sulfoxide (DMSO). The final volume of DMSO in the 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 [12]. 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 and were in strict accordance with the Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institute of Health.

2.3. Measurement of PLD activity

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 [12]. 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]products were visualized with a PhosphorImager (Molecular Dynamics 445 SI) and the radioactivity of [^{3}H]phosphatidylethanol (PEt) was quantified with a Liquid Scintillation Analyzer.

2.4. Protein tyrosine phosphorylation

Neutrophils (2×10^7/ml) were incubated with test drugs before stimulation. Reactions were terminated by the addition of a stop solution containing 20% (w/v) trichloroacetic acid, 1 mM phenylmethylsulphonyl fluoride, 2 mM *N*-ethylmaleimide, 10 mM NaF, 2 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate, and 10 μg/ml each of leupeptin and pepstatin. Proteins (60 μg per lane) were electrophoresed on 10% SDS–PAGE, and then transferred to a polyvinylidene difluoride membrane [7]. The membranes were blocked with 1% (w/v) BSA, and then probed with anti-phosphotyrosine antibody. Detection was performed with enhanced chemiluminescence reagent. Quantification was by densitometry.

2.5. Membrane association of PKC, RhoA, and Arf

Neutrophils (2×10^7/ml) were preincubated with test drug before stimulation, and then resuspended in a disruption solution containing 0.34 M sucrose, 10 mM Tris–HCl (pH 7.0), 1 mM phenylmethylsulphonyl fluoride, 2 mM EGTA, 10 mM benzamidine, and 10 μg/ml each of leupeptin and pepstatin [7]. After sonication, the lysate was centrifuged ($800 \times g$ for 10 min at 4 °C) to remove the unbroken cells, and then further centrifuged ($100{,}000 \times g$ for 30 min at 4 °C) to collect pellets as the membrane fraction. Proteins were resolved by 7.5% (for PKC) or 15% (for RhoA and Arf) SDS–PAGE, and then transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% (w/v) non-fat milk (for PKC and RhoA) or 1% (w/v) gelatin (for Arf) in TBST buffer, and then probed with anti-PKC-α, anti-PKC-βI, anti-PKC-βII, anti-Arf or anti-RhoA antibody, and also with anti-CD88 antibody to standardize the protein loading in each lane.

In the cell-free experiments, neutrophils (2×10^7/ml) were washed and suspended in buffer B containing 25 mM HEPES (pH 7.4), 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 mM Mg–ATP, 1 mM EGTA, 5 mM dithiothreitol, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulphonyl fluoride, and 10 μg/ml of leupeptin. After sonication, the lysate was centrifuged to remove the unbroken cells. The supernatants were incubated with test drugs in the presence of 1 μM Ca^{2+} before stimulation with 10 μM GTPγS. Reaction was terminated by the addition of five-fold excess ice-cold buffer B, and then centrifugation was performed to collect pellets as the membrane fraction. Proteins were subjected to Western blot analysis using the anti-Arf or anti-RhoA antibody, and also with anti-CD88 antibody to standardize the protein loading in each lane.

2.6. RhoA activation assay

RhoA activation was determined by using a Rho activation assay kit according to the instructions of the manufacturer. 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% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μg/ml each of aprotinin and leupeptin, 25 mM NaF, and 1 mM Na_3VO_4 on ice for 15 min. After centrifugation ($14{,}000 \times g$ for 5 min at 4 °C), the cell lysate was incubated with Rhotekin-Rho-binding domain-agarose 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.

2.7. Immunoprecipitation

Neutrophils (2×10^7/ml) were incubated with test drugs before stimulation, and then resuspended 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, and kept in an ice-bath for 30 min with occasional shaking. The lysates were centrifuged ($12{,}000 \times g$ for 10 min at 4 °C), and the supernatants (500 μg protein) were incubated with anti-RhoGDI antibody for 2 h at 4 °C with constant mixing. Immunocomplexes were collected with protein A-agarose beads at 4 °C with constant mixing overnight. The beads were sedimented, washed, and then eluted by boiling in a Laemmli sample buffer. RhoA was detected by immunoblotting with anti-RhoA antibody. The later blots above were then stripped and reprobed with anti-RhoGDI (blocked with 5% (w/v) non-fat milk) antibody.

2.8. Surface expression of CD11b, CD45, and CD63

Neutrophils (2×10^7/ml) were incubated with test drug before stimulation, and then washed twice. Aliquot of cell suspensions was incubated with FITC-conjugated antibodies against CD11b or CD45, or with an isotype-matched FITC-conjugated irrelevant monoclonal antibody (as control) for 30 min in an ice-bath. Aliquot of cell suspensions was incubated with anti-CD63 antibody for 30 min in an ice-bath, and then washed twice and incubated with FITC-conjugated secondary antibody. Cells were thereafter analyzed with a FACSCalibur flow cytometry system in both side and forward scatter using Cell-FIT software (BD Biosciences). The mean fluorescence intensity (MFI) of the control group was subtracted from the MFI of the specific antibody-treated groups.

2.9. Neutrophil degranulation

Neutrophils (1×10^7/ml) were incubated with DMSO or test drug for 10 min at 37 °C, and dhCB (5 μg/ml) was added during the last 3 min before stimulation with 1 μM

fMLP for another 45 min. After centrifugation (2000 × *g* for 5 min at 4 °C), the supernatant was removed for lysozyme and β-glucuronidase measurement [10].

2.10. 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. Effect of CYL-26z on PLD activity in neutrophils

In 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine-loaded rat neutrophils, fMLP stimulated the production of [^{3}H]PEt in the presence of ethanol. This response was inhibited by CYL-26z in a concentration-dependent manner with an IC_{50} value of 1.2 ± 0.2 μM (Fig. 1). In addition, treatment of cells with 100 μM genistein, a general tyrosine kinase (TK) inhibitor, abolished the fMLP-induced [^{3}H]PEt formation. Cell viability was ≥95% during the incubation of cells with 30 μM CYL-26z for 20 min at 37 °C as assessed in a lactate dehydrogenase (LDH) release assay and in a trypan blue exclusion test. CYL-26z at the highest concentration tested (30 μM) did not inhibit the receptor-independent stimulus phorbol 12-myristate 13-acetate (PMA)-induced [^{3}H]PEt formation (data not shown).

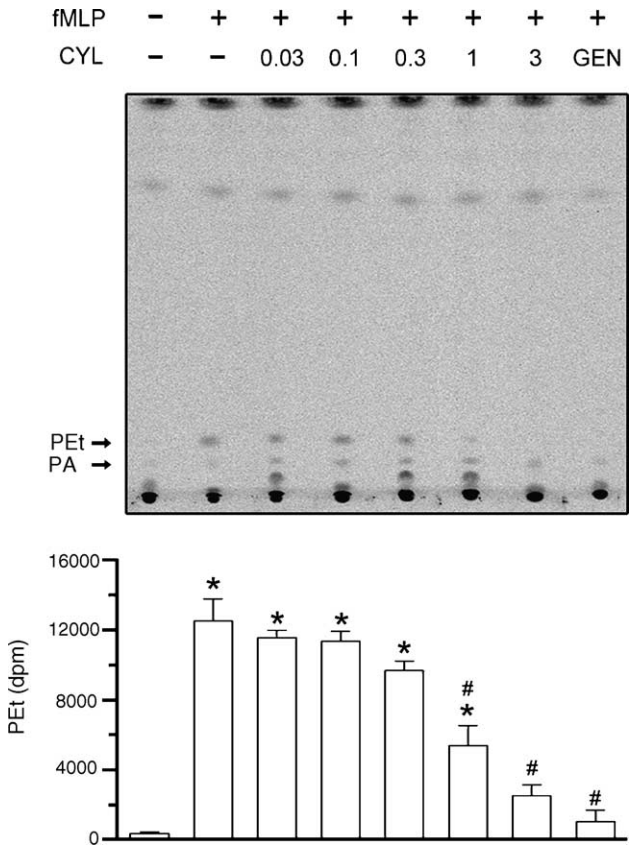

Fig. 1. Effect of CYL-26z (CYL) on PLD activation in fMLP-stimulated neutrophils. 1-*O*-[^{3}H]Octadecyl-*sn*-glycero-3-phosphocholine-loaded neutrophils were incubated with DMSO, the indicated concentrations (μM) of CYL or 100 μM genistein (GEN) for 10 min at 37 °C, and dhCB (5 μg/ml) and 0.5% (v/v) ethanol were added during the last 3 min before stimulation or no stimulation with 1 μM fMLP for another 0.5 min. Lipids in the reaction mixture were extracted and separated. The radioactivity of PEt was counted and expressed as means dpm ± S.D. of 4-6 independent experiments. $^*P < 0.05$, as compared with the vehicle control value (lane 1); $^\#P < 0.05$, as compared with the activated control value (lane 2).

3.2. Effect of CYL-26z on fMLP-stimulated protein tyrosine phosphorylation

To evaluate the underlying mechanisms, we first assessed the effects of CYL-26z on protein tyrosine phosphorylation. Addition of fMLP increased the phosphorylation of a variety of ill-defined substrates in the 50–60, 60–66, 77–85, 92–97 and 116–118 kDa range above basal levels. Treatment with genistein decreased the immunointensity of nearly all visible bands, the most prominent among the 108, 101, 48, 43, and 37 kDa regions (104.7 ± 6.9%, 85.5 ± 9.7%, 92.5 ± 2.9%, 147.2 ± 4.5%, and 93.4 ± 6.5% inhibition, respectively). CYL-26z (30 μM) effectively decreased the levels of tyrosine phosphorylation of bands apparent in the 118 and 108 kDa regions (54.8 ± 14.2% and 51.7 ± 11.1% inhibition, respectively). Like genistein, CYL-26z reduced stimulation of the global protein tyrosine phosphorylation profile induced by fMLP (Fig. 2), thus implying general TK inhibition. However, a significant inhibition ($P < 0.05$) of protein tyrosine phosphorylation was observed only at concentration of CYL-26z up to 30 μM (about 30-fold higher than that required for the inhibition of PLD activation).

3.3. Effects of CYL-26z on PKC-α, -βI, and -βII recruitment to the plasma membrane

It has been reported that fMLP-activated PLD via Ca^{2+}-dependent PKC, but not other PKC isoenzymes, in an activity-independent manner in human neutrophils [8,13]. Our previous report indicated that Ca^{2+}-dependent PKC isoenzymes (PKC-α and -β) are expressed in rat neutrophils [14]. We, therefore, next examined the effect of CYL-26z on the recruitment of PKC to the plasma membrane by Western blot analysis of the neutrophil membrane fractions using anti-PKC-α, -βI and -βII antibodies. PKC isoenzymes were found predominantly in the cytosol fractions of resting cells, but translocation to the membrane fractions of cells occurred in response to fMLP (Fig. 3). A detectable inhibition of membrane recruitment of PKC-α was observed at concentrations ≥3 μM CYL-26z (about 40 ± 10% inhibition at 3 μM CYL-26z), whereas, CYL-26z up to 30 μM failed to alter the membrane association of PKC-βI and -βII.

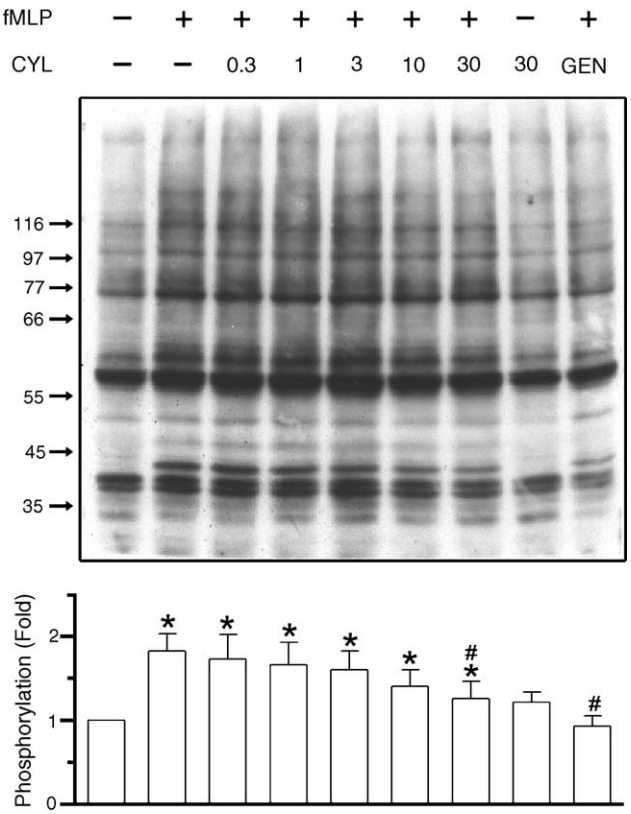

Fig. 2. Effect of CYL-26z (CYL) on protein tyrosine phosphorylation in fMLP-stimulated neutrophils. Cells were incubated with DMSO or the indicated concentrations (μM) of CYL for 10 min, or with 50 μM genistein (GEN) for 30 min at 37 °C, and dhCB (5 μg/ml) was added during the last 3 min before stimulation or no stimulation with 1 μM fMLP for another 1 min. Cell lysates were analyzed by immunoblotting with anti-phosphotyrosine antibody, and then quantified by densitometry. Values are expressed as means ± S.D. of the fold increase in the immunointensity as compared with the vehicle control value (lane 1) from 4 independent experiments. $^{*}P < 0.05$, as compared with the vehicle control value; $^{\#}P < 0.05$, as compared with the activated control value (lane 2).

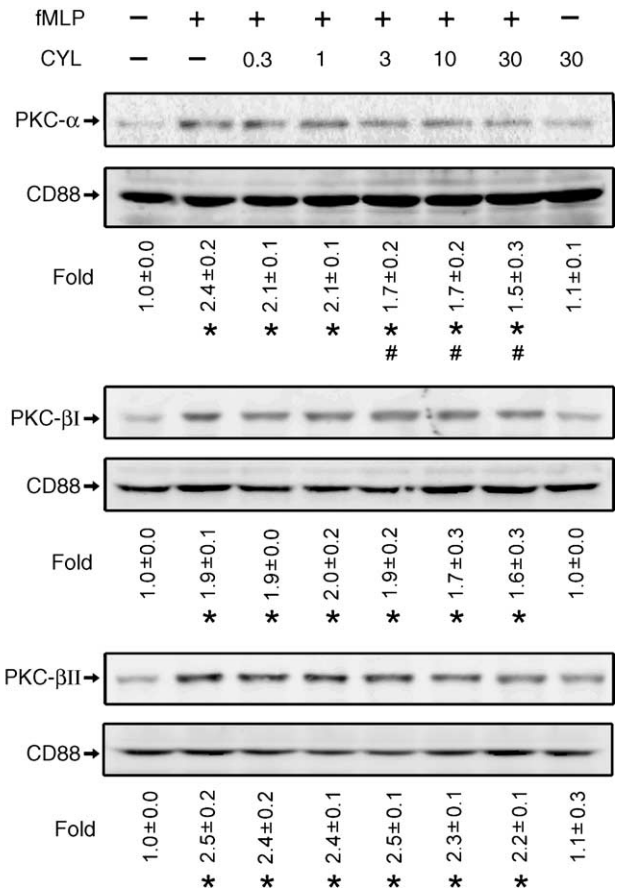

Fig. 3. Effect of CYL-26z (CYL) on the membrane association of PKC in fMLP-stimulated neutrophils. Cells were incubated with DMSO or the indicated concentrations (μM) of CYL for 10 min at 37 °C, and dhCB (5 μg/ml) was added during the last 3 min before stimulation or no stimulation with 1 μM fMLP for another 1 min. After sonication, the membrane fractions were analyzed by immunoblotting with anti-PKC-α, -βI or -βII antibody, and then quantified by densitometry. Values are expressed as means ± S.D. of the fold increase in the immunointensity as compared with the corresponding vehicle control values (lane 1) from 4 independent experiments. $^{*}P < 0.05$, as compared with the corresponding vehicle control values. $^{\#}P < 0.05$, as compared with the corresponding activated control values (lane 2).

3.4. Effect of CYL-26z on the recruitment of RhoA and Arf to the plasma membrane

In addition to PKC, the membrane anchoring of small GTPases, Arf and Rho proteins, is a prerequisite for PLD activity. Since the vast majority of Arf and RhoA proteins reside in the cytosolic fraction of resting cells, the translocation of Arf and RhoA was monitored by immunoblotting that amount of Arf and RhoA present in the membrane fractions of neutrophils. As displayed in Fig. 4, the band detected upon Western blotting of RhoA and Arf was weak in vehicle-treated cells, whereas a visible band was detected in response to fMLP. CYL-26z decreased the amounts of RhoA recovered in membrane fractions following stimulation with fMLP in a concentration-dependent manner (IC_{50} value of 3.8 ± 0.8 μM), whereas it had no significant effect on the membrane association of Arf.

In a cell-free neutrophil system, the amounts of membrane-associated Arf and RhoA were significantly increased (both $P < 0.05$) in response to GTPγS as seen from Western blot (Fig. 5). CYL-26z attenuated the amounts of RhoA recovered in membrane fractions following stimulation with GTPγS in a concentration-dependent manner with an IC_{50} value of 1.3 ± 0.5 μM. A significant inhibition of Arf translocation was observed only at concentration of CYL-26z up to 30 μM.

3.5. Effect of CYL-26z on RhoA activation and the association of RhoGDI with RhoA

To further determine whether CYL-26z affected the fMLP-induced RhoA activation, we used Rhotekin-Rho-binding domain-agarose to precipitate the GTP-bound form of RhoA from cell lysates. The immunoreactivity of agarose-associated RhoA in fMLP-stimulated cell lysate was attenuated by pretreatment of cells with CYL-26z in a

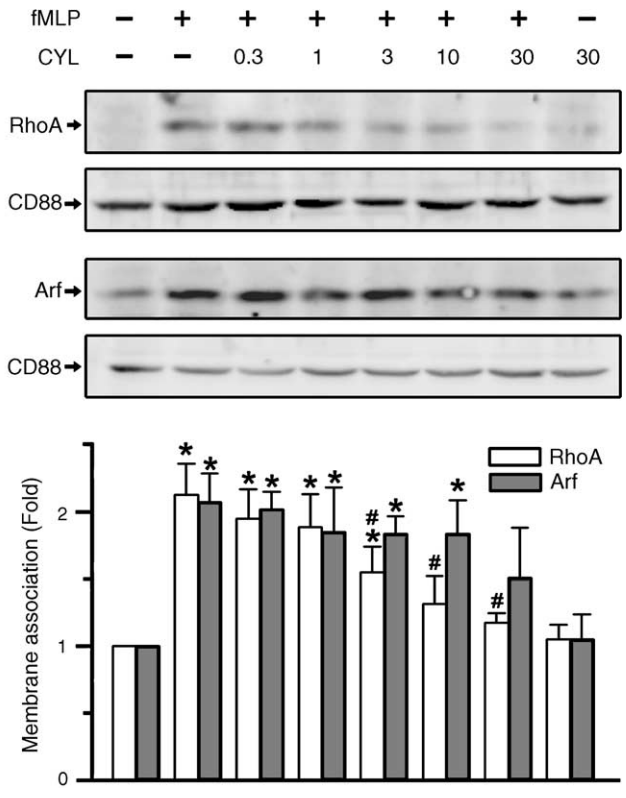

Fig. 4. Effect of CYL-26z (CYL) on the membrane association of RhoA and Arf in fMLP-stimulated neutrophils. Cells were incubated with DMSO or the indicated concentrations (μM) of CYL for 10 min at 37 °C, and dhCB (5 μg/ml) was added during the last 3 min before stimulation or no stimulation with 1 μM fMLP for another 1 min. After sonication, the membrane fractions were analyzed by immunoblotting with anti-RhoA or anti-Arf antibody, and then quantified by densitometry. Values are expressed as means ± S.D. of the fold increase in the immunointensity as compared with the corresponding vehicle control values (lane 1) from 4–5 independent experiments. $^{*}P < 0.05$, as compared with the corresponding vehicle control values; $^{\#}P < 0.05$, as compared with the corresponding activated control values (lane 2).

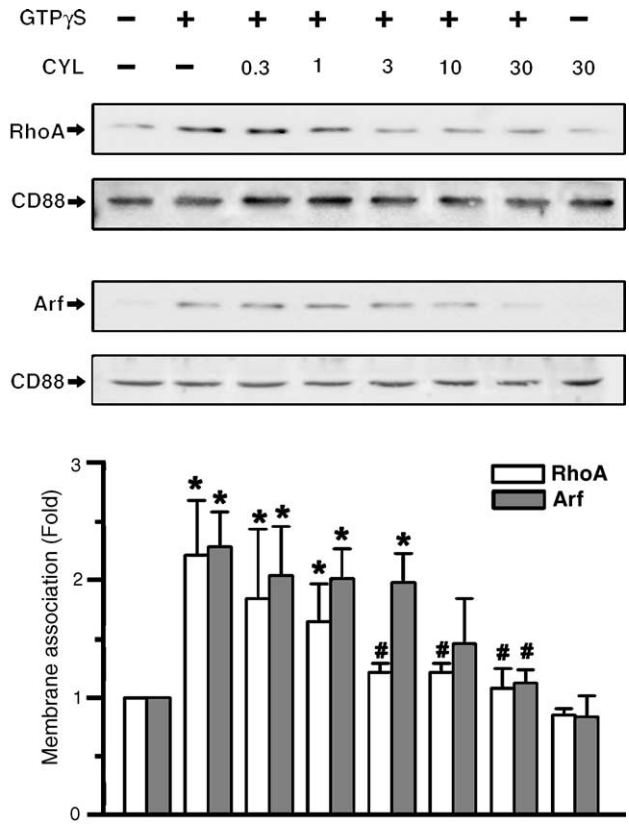

Fig. 5. Effect of CYL-26z (CYL) on the membrane association of RhoA and Arf in a cell-free system. Cell lysates were incubated with DMSO or the indicated concentrations (μM) of CYL for 10 min at 37 °C before stimulation or no stimulation with 10 μM GTPγS for 10 min. After sedimentation, the membrane fractions were analyzed by immunoblotting with anti-RhoA or anti-Arf antibody, and then quantified by densitometry. Values are expressed as means ± S.D. of the fold increase in the immunointensity as compared with the corresponding vehicle control values (lane 1) from 4–5 independent experiments. $^{*}P < 0.05$, as compared with the corresponding vehicle control values; $^{\#}P < 0.05$, as compared with the corresponding activated control values (lane 2).

concentration-dependent manner with an IC_{50} value of 1.8 ± 1.0 μM (Fig. 6A). Moreover, immunoprecipitation of vehicle-treated cell lysates with RhoGDI antibody pulled down RhoA, whereas this RhoA immunoreactive band was greatly attenuated in fMLP-stimulated cells. CYL-26z inhibited the dissociation of the RhoA-RhoGDI complex in fMLP-stimulated cells in a concentration-dependent manner with an IC_{50} value of 1.8 ± 0.9 μM (Fig. 6B).

3.6. CYL-26z attenuated fMLP-induced degranulation

Since PLD activation has been implicated in the release of granules in activated neutrophils, we, therefore, examined the effect of CYL-26z on neutrophil degranulation. Four different granules are formed in neutrophils, including azurophil granules, specific granules, gelatinase granules, and secretory vesicles. Flow cytometry has been a useful tool in elucidating the granules' fusion with the plasma membrane during exocytosis by using the antibody selectively against the specific membrane marker of granules. Azurophil granules contain CD63 in their membrane. CD11b is a membrane constituent of specific granules, gelatinase granules and secretory vesicles, whereas, CD45 resides primarily in the membrane of secretory vesicles [15]. Resting neutrophils expressed little CD11b, CD45 or CD63 on the plasma membrane, whereas fMLP promoted a significant rise in the CD11b, CD45 and CD63 levels of cells. CYL-26z inhibited the fMLP-stimulated expression of CD11b, CD45 and CD63 in a concentration-dependent manner (IC_{50} values of 6.0 ± 0.2 μM, 6.5 ± 0.1 μM and 2.5 ± 0.4 μM, respectively) (Fig. 7). The report that genistein blocked the exocytosis in human neutrophils in response to fMLP [16] is in line with our observation. Moreover, the release of lysozyme, a lytic enzyme found in the azurophil and specific granules, and β-glucuronidase, an acidic hydrolase found in the azurophil granules, from neutrophils in response to fMLP was concentration-dependently attenuated by CYL-26z (IC_{50} values of 4.0 ± 0.9 and 4.9 ± 0.9 μM, respectively).

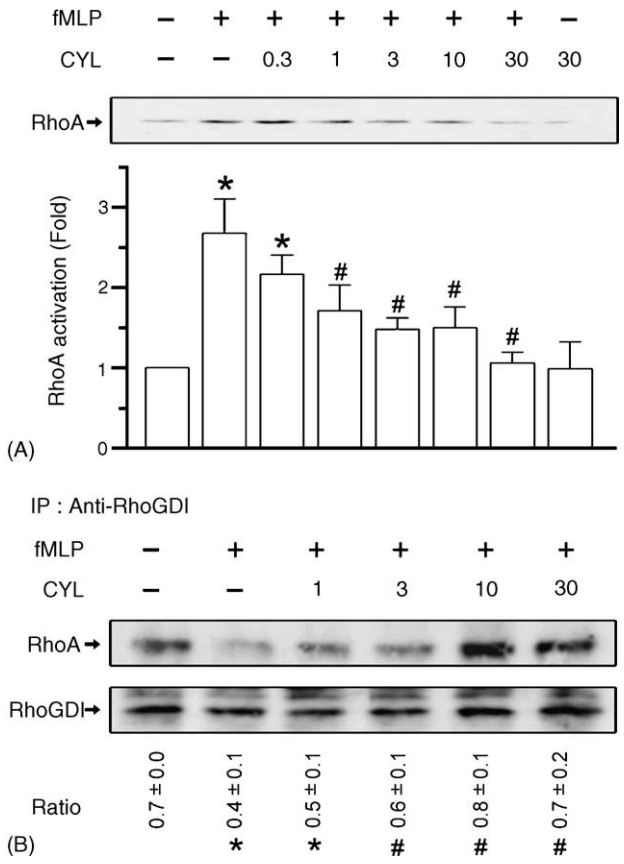

Fig. 6. Effect of CYL-26z (CYL) on RhoA activation and the association of RhoA with RhoGDI in fMLP-stimulated neutrophils. Cells were incubated with DMSO or the indicated concentrations (μM) of CYL for 10 min at 37 °C, and dhCB (5 μg/ml) was added during the last 3 min before stimulation or no stimulation with 1 μM fMLP for another 1 min. (A) Rhotekin-Rho-binding domain-agarose was precipitated or (B) RhoGDI was immunoprecipitated from cell lysates, and then analyzed by immunoblotting with anti-RhoA antibody. The later blots above were then stripped and reprobed with anti-RhoGDI antibody. The ratio of immunointensity between the RhoGDI and the RhoA is shown. Values are means ± S.D. from 4–5 independent experiments. $^{*}P < 0.05$, as compared with the corresponding vehicle control values (lane 1). $^{\#}P < 0.05$, as compared with the corresponding activated control values (lane 2).

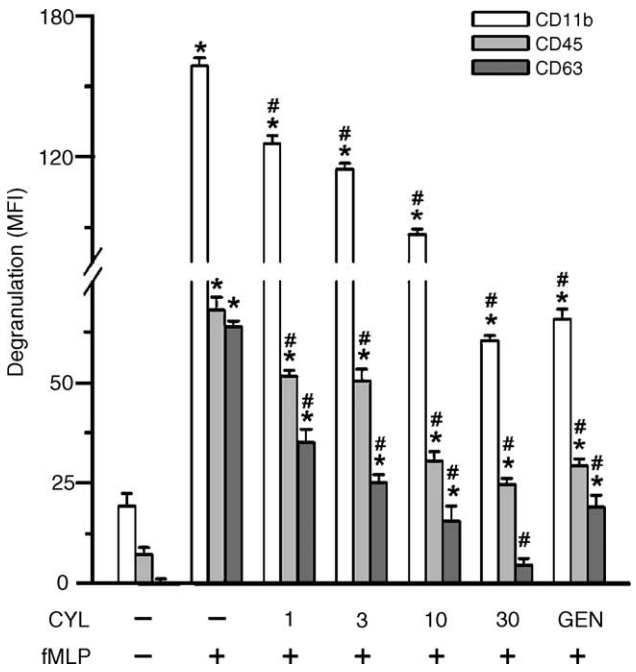

Fig. 7. Effect of CYL-26z (CYL) on the surface expression of CD11b, CD45, and CD63 in fMLP-stimulated neutrophils. Cells were incubated with DMSO, the indicated concentrations (μM) of CYL or 50 μM genistein (GEN) for 10 min at 37 °C, and dhCB (5 μg/ml) was added during the last 3 min before stimulation or no stimulation with 1 μM fMLP for another 1 min. After centrifugation, cells were incubated with anti-CD11b, anti-CD45 or anti-CD63 antibody at 4 °C for 30 min, and then analyzed by flow cytometry. Values are means MFI ± S.D. from 4–5 independent experiments. $^{*}P < 0.05$, as compared with the corresponding vehicle control values (group 1 columns). $^{\#}P < 0.05$, as compared with the corresponding activated control values (group 2 columns).

4. Discussion

In general, mammalian PLD appear to be membrane-bound enzymes, where PLD catalyses the hydrolysis of PC to produce PA. In the presence of ethanol, PEt instead of PA was produced via transphosphatidylation. PEt is produced only by PLD [17] and thus, has been widely used as a marker for PLD activity. The results that CYL-26z inhibited the fMLP- but not PMA-induced PEt formation and the high cell viability during the reaction time, it is reasonable to obviate the possibility that CYL-26z has a direct inhibitory effect on PLD, or that it disrupts the cell membrane integrity, more probably it interrupts the receptor-coupled signaling pathways in rat neutrophils. The observation that genistein abolished the fMLP-induced PEt formation is consistent with the previous report that implicated TK in receptor coupling to PLD activation in human neutrophils [8].

The steps that follow the activation of G protein-coupled receptors and link to the modulation of TK activity remain unclear. In the present study, the fact that visible bands were detected by Western blot analysis using the anti-phosphotyrosine antibody in untreated cells is indicative of tyrosine phosphorylation under basal conditions. Addition of fMLP increased the phosphorylation of a variety of ill-defined substrates. Due to the lack of receptor TK activity in the fMLP receptor, the protein tyrosine phosphorylation in response to fMLP via the non-receptor TK has been proposed in this mechanism. Although neutrophils express different types of non-receptor TKs, including the SRC family [18], FAK family [19], JAK family [20] and TEC family [21], the nature of the specific TKs involved in PLD activation has yet to be identified. SRC has been implicated in PLD activation in Caco-2 cells [22] and the $G_{\beta\gamma}$ subunit derived from the pertussis toxin-sensitive G protein has been shown to activate SRC [23]. The finding that a much higher concentration of CYL-26z was required to attenuate the global protein tyrosine phosphorylation profile than the inhibition of PLD activation, leads us to believe it is plausible that protein tyrosine phosphorylation plays a minor role in the CYL-26z inhibition of PLD activation.

The N-terminal PKC regulatory domain is required for interaction and activation of PLD by PKC [24]. In resting cells, PKC isoenzymes were found predominantly in cytosol fractions. The recruitment of PKC to the plasma membrane as assessed by Western blot analysis demonstrated that CYL-26z effectively inhibited PKC-α but failed to alter the membrane association of PKC-βI and -βII. These results suggest that the PKC-α, but not PKC-βI or -βII, signaling is more probably involved in the CYL-26z inhibition of PLD activity. Since CYL-26z had no effect on PMA-stimulated PLD activity, one possibility is that inhibition of membrane recruitment of PKC-α by CYL-26z in response to fMLP might not occur in PMA-stimulated cells, or alternatively that PKC-α might not play an important role in PMA-stimulated PLD activity in neutrophils. Previous studies demonstrated that the overexpression of PKC-βI but not PKC-α enhances PMA-induced PLD activity in fibroblasts [25,26]. Further study will be required to clarify these possibilities. As phorbol esters activate PLD via PKC in an activity-dependent manner [27], it would be reasonable to suggest that PKC activity is unaffected by CYL-26z.

In addition to PKC, the optimal activation of PLD also requires the recruitment of small GTPases, Arf and Rho proteins, to the plasma membrane [9]. Thus, most small GTPases cycle between cytosol and membrane. Activation of PLD by PKC-α is synergistic with Arf and Rho proteins, suggesting that these three regulators interact with different sites on PLD [4]. The Rho family consists of 20 distinct members, of which three major Rho proteins (Rho, Rac, and Cdc42) are capable of binding and stimulating PLD activity [28]. RhoA activates PLD approximately four to five times better than does Rac1 or Cdc42. Arf have been divided into three classes: class I (Arf1, 2, and 3), class II (Arf4 and 5), and class III (Arf6) [29]. Arf1 and Arf6 have been reported to recruit to granulocyte membranes in response to fMLP [9,30], but only the latter has been implicated in PLD activation [31]. The results that CYL-26z decreased the membrane association of RhoA, but not Arf, and the PLD activity in a similar concentration range imply the critical role of RhoA. It has been reported that persistent cAMP elevation inhibited fMLP-induced PLD activity and the recruitment of Arf, RhoA, and PKC to the membrane fraction of human neutrophils [32]. The decrease instead of increase in cellular cAMP levels (0.64 ± 0.10 pmol versus 0.32 ± 0.06 pmol 2×10^6/cells in the absence or the presence of 30 μM CYL-26z, respectively, for 9.5 min prior to stimulation with fMLP for 0.5 min, $P < 0.05$) and the lack of effect on Arf by CYL-26z preclude the involvement of this mechanism of action.

In a cell-free human neutrophil system, Arf and RhoA are direct activators of GTPγS-stimulated PLD [33]. When GTPγS is used, the guanine nucleotide exchange factor (GEF) must be bypassed [9]. Thus, the GDP/GTP exchange is followed by the activation of small GTPases in the resistance of nucleotide hydrolysis, which results in the stable interaction of Arf and RhoA with membrane fractions. The findings that CYL-26z effectively attenuated the amounts of RhoA, but not Arf, recovered in membrane fractions in a cell-free system imply direct interference with RhoA activation, and preclude the enhancement of intrinsic GTPase activity of RhoA by CYL-26z.

It is conceivable that activation of small GTPases depend on GEFs. GEF accelerate the rate of exchange of bound GDP for GTP, resulting in a conformational change and association with membrane fractions. The notion that CYL-26z affects RhoA activation is supported by the observation that CYL-26z diminished the amount of GTP-bound form of RhoA. The GTP/GDP cycling of Rho proteins is tightly controlled by a RhoGDI, which captures GDP-bound Rho proteins and maintains it in an inactive cytosolic complex [34]. Thus, the dissociation of GDI from RhoA is a prerequisite for membrane association and activation of RhoA by GEFs [35]. To date, three RhoGDIs (RhoGDI-1, RhoGDI-2 and RhoGDI-3) have been described, of which both RhoGDI-1 and -2 are cytosolic proteins; however, only the former has been isolated from the cytosol of neutrophils [36]. The identical concentrations of CYL-26z in the inhibition of both RhoA activation and the dissociation of the RhoA-RhoGDI complex have clearly confirmed the inhibition of RhoA activation by CYL-26z. These results also suggest that blockade of the RhoA-RhoGDI complex dissociation is the possible site of action of CYL-26z inhibition of RhoA activation. It has been reported that phosphorylation of RhoGDI by Ser/Thr kinases stabilizes the interaction of RhoA and RhoGDI in the neutrophil cytosol, whereas alkaline phosphatase treatment destabilizes the RhoA-RhoGDI complex [37]. Although the cAMP-dependent phosphorylation of RhoA enhanced the interaction with RhoGDI [38], this is not implicated in CYL-26z actions. The interaction between the PH domain of BMX, a member of the TEC family TK, and RhoA resulting in the dissociation of the RhoA-RhoGDI complex has been reported in C2C12 cells [39]. Nevertheless, the precise mechanisms underlying the inhibitory effect of CYL-26z on the dissociation of the RhoA-RhoGDI complex remain to be determined. Studies have shown that RhoA plays an important role in the regulation of cytoskeletal dynamic, actin stress fiber formation, and cell motility [40]. It would be of interest to investigate the effect of CYL-26z on neutrophil motility in future studies.

PLD activation has been implicated in the release of granules in activated neutrophils [1]. Granules constitute an important reservoir not only of antimicrobial proteins and proteases, but also of a wide range of membrane-associated receptors, adhesion molecules, and components of the NADPH oxidase. Thus, exocytosis of granules and secretory vesicles play a pivotal role in most neutrophils functions from early activation to the destruction of phagocytosed microorganisms. The notion that CYL-26z inhi-

bits PLD activity is in line with the observation that it inhibits neutrophil degranulation.

In conclusion, CYL-26z inhibited the fMLP-stimulated PLD activity in rat neutrophils mainly through the blockade of RhoA activation and probably partly through inhibition of membrane recruitment of PKC-α, whereas the attenuation of protein tyrosine phosphorylation probably only plays a minor role. Moreover, CYL-26z inhibited neutrophil degranulation, which has been implicated with PLD activation. Previous reports have demonstrated that the activation of PLD by fMLP is also, at least in part dependent on the activity of phosphoinositide 3-kinase [41] and cytoplasmic Ca^{2+} concentration [42]. Investigation of these possibilities will require further research.

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