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Activation of phosphoinositide 3-kinase and Src family kinase is required for respiratory burst in rat neutrophils stimulated with artocarpol A

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Abbreviations:
ART, artocarpol A
dhCB, dihydrocytochalasin B
fMLP, formyl-Met-Leu-Phe
HBSS, Hanks' balanced salt solution
MAPK, mitogen-activated protein kinase
MAPKAPK-2, MAPK-activated protein kinase-2
$O_2^{\bullet-}$, superoxide anion

ABSTRACT

Artocarpol A (ART), a natural product isolated from *Artocarpus rigida*, stimulated superoxide anion ($O_2^{\bullet-}$) generation, which was inhibited by 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002), a phosphoinositide 3-kinase (PI3K) inhibitor, in rat neutrophils. ART stimulated phosphorylation of protein kinase B (PKB/Akt) on both T308 and S473 residues, and LY 294002 inhibited these effects. Rat neutrophils expressed both class IA PI3K subunits (p85, p110α, p110β, and p110δ) and a class IB PI3K subunit (p110γ) as assessed by a combination of Western blotting and reverse transcription-polymerase chain reaction (RT-PCR) approaches. Stimulation of neutrophils with ART evoked phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) formation, which reached a maximal level at 2 min and was attenuated by LY 294002, as evidenced by immunofluorescence microscopy and by flow cytometry. Detectable membrane-association of class IA PI3Ks, class IB PI3K and Ras was seen as early as 1.5, 0.5 and 1.5 min, respectively, after stimulation with ART. The kinetics of ART-induced Ras activation paralleled the kinetics of class IA PI3Ks recruitment to membrane caused by ART, and the p85 and p110γ immunoprecipitates contain Ras. ART stimulated Src family kinase activation, which was detectable within 1.5 min of incubation with ART. Both Src kinase activity and PtdIns(3,4,5)P_3 formation in ART-stimulated neutrophils were inhibited by 4-amino-1-*tert*-butyl-3-(1′-naphthyl)pyrazolo[3,4-d]pyrimidine (PP1 analog). PP1 analog also attenuated the ART-stimulated $O_2^{\bullet-}$ generation in rat neutrophils. These results indicate that the stimulation of respiratory burst by ART in neutrophils implicates PI3K signaling.

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PDK1, phosphoinositide-dependent kinase 1
PI3K, phosphoinositide 3-kinase
PKB/Akt, protein kinase B
PKC, protein kinase C
PLC, phospholipase C
PtdIns(3,4,5)P_3, phosphatidylinositol-3, 4,5-trisphosphate
RT-PCR, reverse transcription-polymerase chain reaction

1. Introduction

Neutrophils play a critical role in the bactericidal host defense system. Production of superoxide anion ($O_2^{\bullet-}$) and its reactive metabolites is important in bacterial killing. However, over-reactive neutrophils are also responsible for tissue destruction under inflammatory conditions. The mechanism of activation of $O_2^{\bullet-}$ generation in neutrophils is complex and not completely understood. Previous reports demonstrated that the stimulation of neutrophils by receptor-binding ligands activates mitogen-activated protein kinase (MAPK) [1], which is involved in $O_2^{\bullet-}$ generation. Phospholipase C (PLC) is also activated upon cell activation, leading to the hydrolysis of phosphatidylinositol-4,5-bisphosphate to generated D-*myo*-inositol-1,4,5-trisphosphate, which increases in $[Ca^{2+}]_i$, and diacylglycerol, which activates protein kinase C (PKC) [2]. These signaling messengers act synergistically for assembly of the cytosol components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac2) with the membrane flavocytochrome b_{558} to form a functional NADPH oxidase complex, which catalyzes the reduction of oxygen to $O_2^{\bullet-}$ by using NADPH as the electron donor [3]. This oxygen consumption process is termed ''respiratory burst''.

Subsequent reports have indicated that phosphoinositide 3-kinase (PI3K) pathway is implicated not only in the direction movement but also the $O_2^{\bullet-}$ production in chemoattractant-stimulated neutrophils as evidenced from pharmacological and genetic approaches [4,5]. PI3K is a lipid kinase that phosphorylates the D-3 position of the inositol ring of phosphoinositides, which serve as membrane targeting signals to mediate membrane recruitment of selected proteins. PI3K-catalyzed lipid products bind to the PX domains of $p40^{phox}$ and $p47^{phox}$ and thus are probably critical for assembly of the NADPH oxidase [6]. In addition, PI3K regulates the phosphorylation of $p47^{phox}$ and thus the membrane translocation [7]. PI3Ks are categorized as class I (A and B), II, and III, depending on their subunit structure, regulation, and substrate selectivity [8]. The class IA PI3Ks are activated primarily by signaling pathways that involve tyrosine kinase activation, whereas the class IB PI3K is activated by βγ subunits of heterotrimeric G proteins [9]. Little is known about the functions of class II and III PI3Ks. There are also demonstrated that the PI3K-independent mechanisms for NADPH oxidase activation as seen using phorbol ester and arachidonic acid [4,10], thus different stimuli induce distinct patterns of intracellular signaling for $O_2^{\bullet-}$ generation.

A natural phenolic compound artocarpol A (ART) (Fig. 1A), isolated from the root bark of a moraceous plant *Artocarpus rigida* [11], was found to inhibit TNF-α formation in murine macrophages [11], and to stimulate $O_2^{\bullet-}$ generation through the activation of p38 MAPK, PLC/Ca^{2+}, and PKC signaling pathways in rat neutrophils [12]. In the present study, we have elucidated the role of class I PI3Ks in the ART stimulation of $O_2^{\bullet-}$ generation in neutrophils by a combination of pharmacological and immunological approaches, and found that class IA PI3Ks appear to play a pivotal role. Thus ART may serve as a new pharmacological probe for studies on the signal transduction involved in the inflammatory processes of leukocytes.

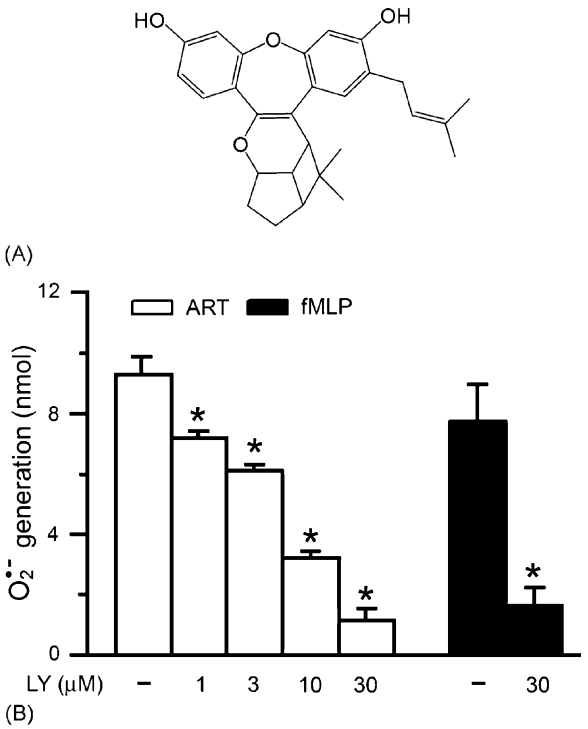

Fig. 1 – Effect of LY 294002 (LY) on ART-stimulated $O_2^{\bullet-}$ generation in rat neutrophils. (A) Chemical structure of artocarpol A. (B) Cells were pretreated with DMSO (as control) or the indicated concentrations of LY for 3 min at 37 °C before stimulation with 30 μM ART ($n = 5$) or 1 μM fMLP plus 5 μg/ml of dhCB ($n = 3$) for 10 min. Values are expressed as means ± S.D. $^*P < 0.05$, as compared with the corresponding control values.

2. Materials and methods

2.1. Materials

Dextran T-500, enhanced chemiluminescence reagent, and protein A beads were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Hanks' balanced salt solution (HBSS) was obtained from Invitrogen. Mouse monoclonal antibody to p110α was obtained from BD Transduction Laboratories. Mouse monoclonal PtdIns(3,4,5)P_3 antibody was obtained from Echelon Research Laboratories. Mouse monoclonal antibody to p38 MAPK, rabbit polyclonal antibodies to Akt1/2, CD88, p85, PKC-βI, p110β, and p110δ, and goat polyclonal p110γ antibodies were obtained from Santa Cruz Biotechnology. Rabbit polyclonal phospho-PKC (pan), phospho-MAPKAPK-2, phospho-Akt(S473) and phospho-Akt(T308) antibodies were purchased from Cell Signaling Technology. Mouse monoclonal GST antibody was obtained from Abcam (Cambridge, UK). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB 203580), 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X), 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione (U-73122), 4-amino-1-*tert*-butyl-3-(1′-naphthyl)pyrazolo[3,4-d]pyrimidine (PP1 analog) and 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) were obtained from Calbiochem-Novabiochem. Polyvinylidene difluoride membrane was obtained from Millipore. Ras activation assay kit and rabbit polyclonal p85 antibodies were purchased from Upstate. Src family kinase assay kit was obtained from Promega. Other chemicals were purchased from Sigma–Aldrich. ART (purity > 99%), extracted from the root barks of *A. rigida* with $CHCl_3$ and then purified by silica gel chromatography [11], 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 [13]. Purified neutrophils (>95% viable cell) were 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 Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

2.3. Measurement of $O_2^{\bullet-}$ generation

$O_2^{\bullet-}$ generation was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome c [13]. Briefly, the reaction mixture contained neutrophils (2×10^6 cells) and 40 μM of ferricytochrome *c* in a final volume of 1.5 ml. The reference cuvette also contained 17.5 U/ml of superoxide dismutase. Absorbance changes in the reduction of ferricytochrome *c* were monitored continuously at 550 nm in a double-beam spectrophotometer.

2.4. Immunoblot analysis of the phosphorylation of Akt, PKC, p38 MAPK, and MAPK-activated protein kinase (MAPKAPK)-2

After addition of Laemmli sample buffer to neutrophils (2×10^7 cells) suspension [12], the solution was boiled for 5 min. Proteins (60 μg per lane) were resolved by 10% SDS–PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dried milk in TBST buffer and probed with antibodies against phospho-Akt(T308), phospho-Akt(S473), phospho-PKC (pan) or phospho-MAPKAPK-2. The blots were then stripped and reprobed with anti-Akt, anti-PKC-βI or anti-p38 MAPK 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 Multi-Gauge software.

2.5. Detection of PtdIns(3,4,5)P_3 formation by immunofluoroscence microscopy

Neutrophils (5×10^5 cells) were fixed with 4% paraformaldehyde in TBS (150 mM NaCl, and 10 mM Tris–HCl, pH 7.5) and plated onto poly-L-lysine coated coverslips. Cells were then thoroughly washed twice and permeabilized with saponin solution (0.2% saponin, 100 mM NaCl, 10 mM Tris–HCl, pH 7.5, 2% fetal bovine albumin, and 1% bovine serum albumin) at room temperature for 30 min. After blocking with 10% bovine serum albumin, anti-PtdIns(3,4,5)P_3 antibody was added and incubated at 4 °C overnight. Cells were then washed three times with TBS, and rhodamine-labeled anti-mouse IgM antibody was added and incubated at room temperature for 30 min. The fluorescence in the cells was observed using a confocal laser scanning microscope (Leica TCS NT).

2.6. Quantitation of PtdIns(3,4,5)P_3 formation by flow cytometry

After fixation, permeabilization and blocking of neutrophils with 2% bovine serum albumin, anti-PtdIns(3,4,5)P_3 antibody was added and incubated for 2 h at room temperature. After being washed with TBS, the secondary antibody was added and incubated at room temperature for 1 h and counted on a FACSCalibur flow cytometry system in both side and forward scatter using Cell-FIT software. The mean fluorescence intensity (MFI) of the control group was subtracted from the MFI of the specific antibody-treated groups.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR) and electrophoresis of products

Total RNA was prepared and the PCR amplification was performed as previously described [14]. The sequences of the primer pairs used along with the predicted size of their expected fragments (shown in parentheses in base pairs) are as follows: 5′-gccagatttcatggatgctt-3′ and 5′-ccttggttttgccagatgtt-3′ (p110α,

261-bp) (AF395897), 5′-caggaaagcaggaaaagtgc-3′ and 5′-cgaagaccagctgtgcaata-3′ (p110β, 109-bp) (NM_053481), 5′-atgatgtcagttcccaagtt-3′ and 5′-cgtagatcgtcgccatgttt-3′ (p110γ, 247-bp) (XM_234053), 5′-gcactctatgctgtcgtgga-3′ and 5′-cgtactgtacccgcaggatt-3′ (p110δ, 203-bp) (XM_345606). The total RNA (5 μg) and oligo(dT)$_{15}$ were used for the first strand cDNA synthesis. PCR amplification was performed with initial heating for 5 min at 95 °C, followed by 28 cycles of 45 s denaturation at 94 °C, annealing for 30 s at 54–60 °C, extension for 1 min at 72 °C, and a final extension for 7 min at 72 °C. The PCR products were resolved using a 2% agarose gel, and the sequences of these products were confirmed using a CEQ 2000 capillary sequencer (Beckman Coulter) with Dye Terminator Cycle Sequencing kit.

2.8. Membrane association of p85, p110s and Ras

Neutrophils (2×10^7 cells/ml) were suspended in ice-cold extraction buffer (50 mM Tris–HCl, pH 7.5, 2 mM EGTA, 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, 10 μg/ml each of aprotinin, leupeptin and pepstatin A) [15]. After sonication, the lysates were centrifuged ($800 \times g$ for 10 min at 4 °C) to remove the unbroken cells and nuclei, and then further centrifuged ($150{,}000 \times g$ for 90 min at 4 °C) to collect pellets as membrane fractions. Proteins were resolved by 10% SDS-PAGE (for p85 and p110s) or 15% SDS-PAGE (for Ras), and probed with antibodies against p85, p110s and Ras, and also with CD88 antibody to standardize the protein loading in each lane.

2.9. Ras activation assay and immunoprecipitation

Ras activation was determined by using a Ras activation assay kit according to the instructions of the manufacturer. Neutrophils (2×10^7 cells) were 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 (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 GST-Ras-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 Laemmli sample buffer. Ras was detected by immunoblotting with anti-Ras antibody, and the blots above were also probed with antibody against GST to standardize the protein loading in each lane.

Cells (2×10^7 cells/0.5 ml) were suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Nonidet P-40 (v/v), 0.05% SDS, 0.5% sodium deoxycholate, 2 mM Na_3VO_4, 1 mM dithiothreitol, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin A) on ice for 30 min with constant mixing [12]. Cell debris was sedimented ($12{,}000 \times g$ for 10 min at 4 °C). Lysates (0.5 mg protein) were precleared by incubation with protein A-Sepharose (10 μl of a 1:1 slurry). Supernatants were then incubated with an anti-p85 or p110γ antibody for 2 h at 4 °C with constant mixing. Protein A-Sepharose was added and after overnight incubation at 4 °C with constant mixing, the immunocomplexes were sedimented and were washed twice with lysis buffer. The samples were then used in Western blot analysis.

2.10. Src family kinase activation assay

Neutrophils (2×10^6 cells/ml) were suspended in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 50 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, 10 μg/ml each of aprotinin, leupeptin and pepstatin A). Src family kinase activity was determined by using a Src family kinase assay kit according to the instructions of the manufacturer. Briefly, cell lysates were added to the 96-well plate in the presence of ATP and specific substrate in kinase solution, and then incubated for 1 h at room temperature before the addition of protease solution. After the addition of stabilizer solution, fluorescence intensity was measured at 485/530 nm via a fluorescent microplate reader.

2.11. Statistical analysis

Statistical analyses were performed using ANOVA followed by the Bonferroni *t*-test for multigroup comparisons test; $P < 0.05$ 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. ART stimulated $O_2^{\bullet-}$ generation and Akt phosphorylation

ART (30 μM)-stimulated $O_2^{\bullet-}$ generation was attenuated in cells pretreated with LY 294002, a selective PI3K inhibitor, in a concentration-dependent manner (Fig. 1B) with an IC_{50} value of 4.9 ± 0.4 μM. Our previous report demonstrated that cell viability was ≥90% during the incubation of neutrophils with 30 μM ART at 37 °C for 10 min [12]. Inhibition of formyl-Met-Leu-Phe (fMLP)-induced response by LY 294002 was consistent with the result of a previous report on human neutrophils [16]. The lack of difference in the absorbance change between the incubation of cytochrome *c* with DMSO and with 30 μM ART (data not shown), thus ART did not reduce ferricytochrome *c* directly. These results imply the involvement of PI3K signaling.

We therefore examined the phosphorylation of Akt, a downstream target of PI3K, as determined from Western blot analysis. ART stimulated Akt(T308) phosphorylation in a concentration- and time-dependent manner (Fig. 2A). Significant increase in band immunointensity was observed at concentrations of ART ≥3 μM for 6 min reaction time and 30 μM ART at reaction time ≥1 min, whereas, fMLP increased Akt phosphorylation at the time point ≥5 s (Fig. 2B). Phosphorylation of both T308 and S473 residues is required for full activation of Akt [17]. Pretreatment of cells with LY 294002 concentration-dependently attenuated the ART-induced phosphorylation of both T308 and S473 residues on Akt (Fig. 2C) with the IC_{50} values of 1.2 ± 0.4 μM and 16.3 ± 3.4 μM, respectively. As expected, LY 294002 attenuated the bis-phosphorylation of Akt in response to fMLP stimulation. These results support the involvement of PI3K signaling in ART-induced $O_2^{\bullet-}$ generation.

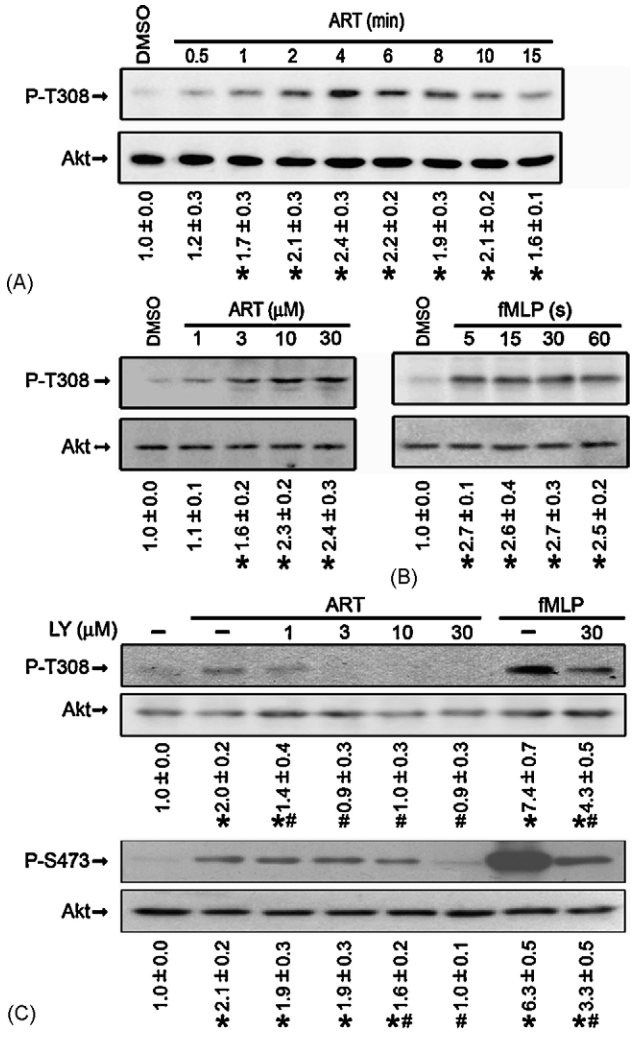

Fig. 2 – Effect of ART on Akt phosphorylation in neutrophils. Cells were incubated with DMSO, (A) 30 μM ART for the indicated time intervals ($n = 4$), or with the indicated concentrations of ART for 6 min ($n = 4$), or (B) with 1 μM fMLP plus 5 μg/ml of dhCB for the indicated time intervals ($n = 3$). Cell lysates were analyzed by immunoblotting with anti-phospho-Akt(T308) antibody. (C) Cells were preincubated with DMSO or the indicated concentrations of LY 294002 (LY) for 3 min before stimulation or no stimulation with 30 μM ART for 6 min or fMLP/dhCB for 30 s ($n = 4$). Cell lysates were analyzed by immunoblotting with anti-phospho-Akt(T308) or anti-phospho-Akt(S473) antibody. The blots above were then stripped and reprobed with anti-Akt antibody. The ratio of immunointensity between the phospho-Akt and Akt was calculated. The fold increase is expressed as means ± S.D. *$P < 0.05$ as compared with the corresponding vehicle control values (lane 1); #$P < 0.05$ as compared with the corresponding activated control values (lane 2).

3.2. ART stimulated the PtdIns(3,4,5)P_3 formation

The cellular PtdIns(3,4,5)P_3 levels, the major product of class I PI3Ks activation in vivo [8], serve as a convenient marker for these enzymes activity and reflect the level of lipid-dependent signaling inside cells. In our study, stimulation of neutrophils with fMLP resulted in a time-dependent increase in PtdIns(3,4,5)P_3 formation that rapidly reached a peak at around 15 s then declined rapidly as assessed by confocal microscopy and flow cytometry. These results reconcile earlier reports, which monitored analysis of [^{32}P]-labeled PtdIns(3,4,5)P_3 levels on HPLC, in murine and human neutrophils [18,19]. The rapid response is compatible with the results of Akt phosphorylation mentioned above. Stimulation with ART resulted in a time-dependent increase in PtdIns(3,4,5)P_3 formation, which was detectable within a 30 s reaction time interval, reached a maximal level at 2 min and then rapidly declined within 1 min to a new plateau at about 35% above baseline (Fig. 3A). The slow rate of PtdIns(3,4,5)P_3 formation is in line with the lag of Akt phosphorylation. In addition, ART revealed a concentration-dependent stimulation of PtdIns(3,4,5)P_3 formation (Fig. 3B), and this stimulatory effect of ART was concentration-dependently prevented by LY 294002 (62 ± 13 % inhibition at 1 μM LY 294002) (Fig. 3C). These results clearly confirm the activation of PI3K by ART.

3.3. Determination of PI3K isoforms protein and mRNA

Class I PI3Ks, which are heterodimers consisting of a catalytic subunit and an adaptor/regulatory subunit, are further subdivided into class IA and IB enzymes. Mammals have three class IA PI3K isoforms with adaptors (p55-85) and catalytic subunits (p110α, p110β, and p110δ), among which p110α and p110β are widely distributed, whereas p110δ is mainly found in leukocytes. The sole class IB PI3K (PI3Kγ), identified as the p110γ catalytic subunit complexed with a p101 adaptor, is abundant only in white blood cells [9]. A previous immunoblotting study using specific antibodies demonstrated the expression of p110α and p110δ in human neutrophils [20]. Moreover, the expression of p110γ and p110δ has been evidenced in neutrophils from mice lacking p110γ (p110γ$^{-/-}$) [19] and p110δ (p110δ$^{-/-}$) [21], respectively. By using specific antibodies to PI3K isoforms, we found five major immunoblot bands that correspond to p85, p110α, p110β, p110γ, and p110δ in rat neutrophils (Fig. 4A). Moreover, RT-PCR was performed using four specific primer pairs to screen for *p110s* gene products in rat neutrophils. A single PCR product band of the expected size for each product in agarose gel electrophoresis was observed (Fig. 4B), indicating the existence of p110α, p110β, p110γ, and p110δ mRNA. Comparison of sequences obtained with the GenBank database (data not shown) demonstrated 99%, 100%, 99% and 100% identity, respectively, between PCR product and the published catalytic subunits of rat class I PI3K isoforms. These results provide clear evidence for the presence of all members of class I PI3K isoforms in rat neutrophils.

3.4. ART-mediated membrane association of PI3K isoforms

Upon activation, an important function of the class I regulatory subunits is to recruit p110 catalytic subunits into contact with membrane, where their lipid substrates reside [9]. ART stimulated the translocation of p85, p110α,

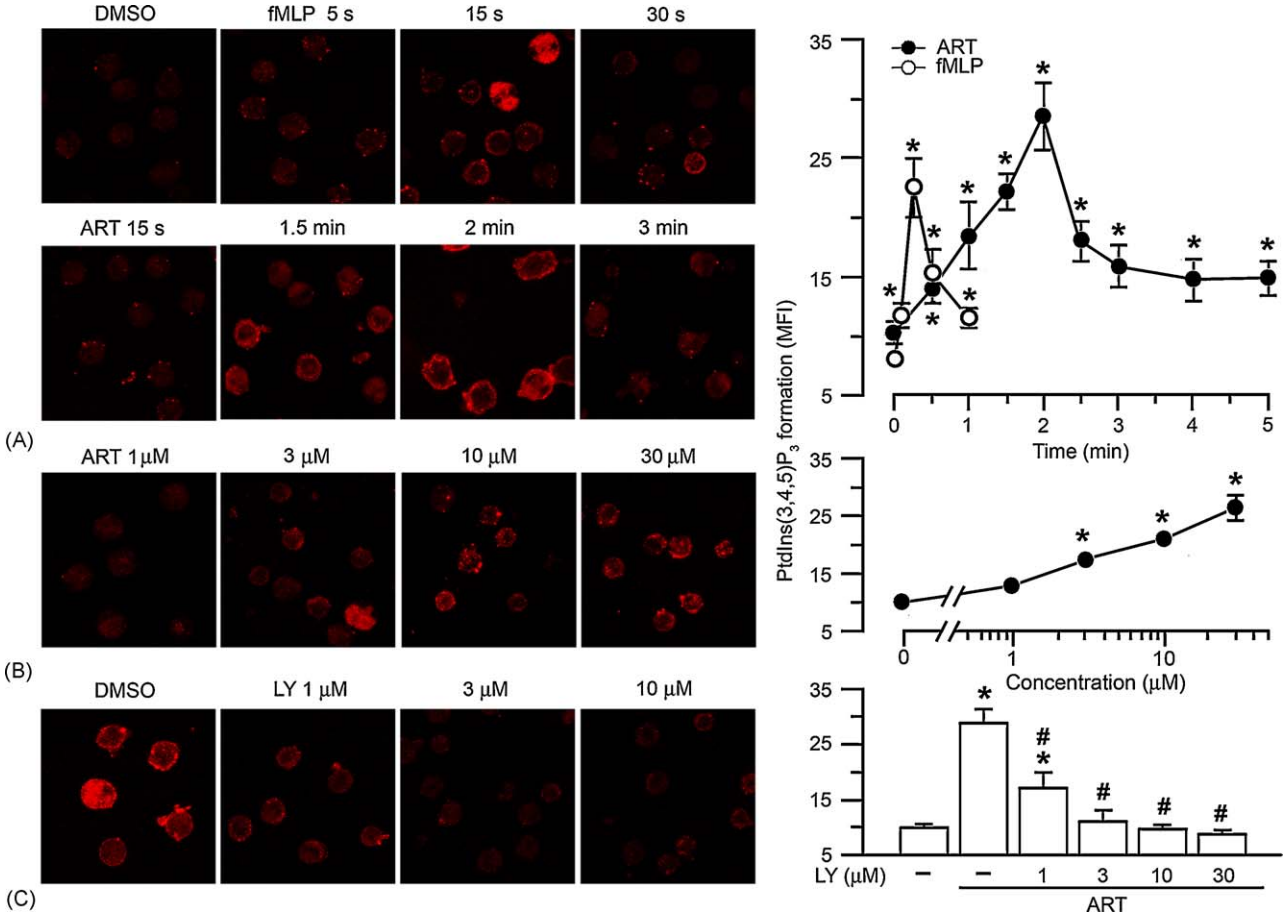

Fig. 3 – Effect of ART on PtdIns(3,4,5)P_3 formation in neutrophils. Cells were incubated with DMSO, (A) 1 μM fMLP plus 5 μg/ml of dhCB or 30 μM ART for the indicated time intervals ($n = 4$), or (B) with the indicated concentrations of ART for 10 min ($n = 4$). (C) Cells were pretreated with the indicated concentrations of LY 294002 (LY) for 3 min before stimulation with 30 μM ART for 2 min ($n = 3$). After fixation and permeabilization, cells were probed with anti-PtdIns(3,4,5)P_3 antibody and then stained with rhodamine-labeled secondary antibody. Samples were observed by immunofluorescence confocal microscopy (left panels) and counted in flow cytometry (right panels). Values are expressed as means ± S.D. $^{*}P < 0.05$ as compared with the corresponding vehicle control values; $^{\#}P < 0.05$, as compared with the activated control value.

p110β, and p110δ to membrane in a parallel time-dependent manner as evidenced by Western blotting of cell membrane. The association between these bands and membrane was detectable within 1.5 min, which remained on the membrane for, at least, the following 3.5 min, of incubation with 30 μM ART (Fig. 5A). In addition, the redistribution of p110γ from the cytoplasm toward the membrane occurred as early as 0.5 min, reached maximum over the next 2.5 min, and was undetectable at 5 min after ART stimulation. A concentration-dependent recruitment of PI3K subunits to membrane was also observed, and the membrane-associated PI3Ks were evident at concentrations as low as 3 μM ART (Fig. 5B). Detectable membrane association of class IA PI3Ks occurred within 15 s and extended to 60 s after stimulation of cells with fMLP, whereas the membrane-associated p110γ was observed as early as 5 s, reached a maximal level within 15 s, then declined to baseline levels (Fig. 5C). These results indicate the membrane recruitment of both classes IA and IB PI3Ks in response to fMLP and to ART.

3.5. *Membrane association and activation of Ras induced by ART*

The p110s of class I PI3Ks contain Ras-binding domain for GTP form of activated Ras, and signal downstream of Ras [9]. The translocation of Ras from cytosol to membrane was detectable within 1.5 min and maintained for at least 3.5 min after stimulation with 30 μM ART (Fig. 6A). ART also resulted in a concentration-dependent increase in the recruitment of Ras to membrane, and a visible band was detected at concentrations ≥10 μM. Similar patterns were obtained regarding the ART-induced Ras activation as assessed by precipitating Ras-GTP with GST-Ras-binding domain agarose (Fig. 6B). Under the same experimental conditions, fMLP-mediated Ras activation followed the same kinetics as the Ras recruitment to membrane. Detectable bands were observed as early as 5 s and extended to 60 s after stimulation of cells with fMLP. When the lysates were subjected to immunoprecipitation and Western blotting with the anti-p85 or anti-p110γ antibody, a band of the expected Mr was seen. Moreover, the blots of

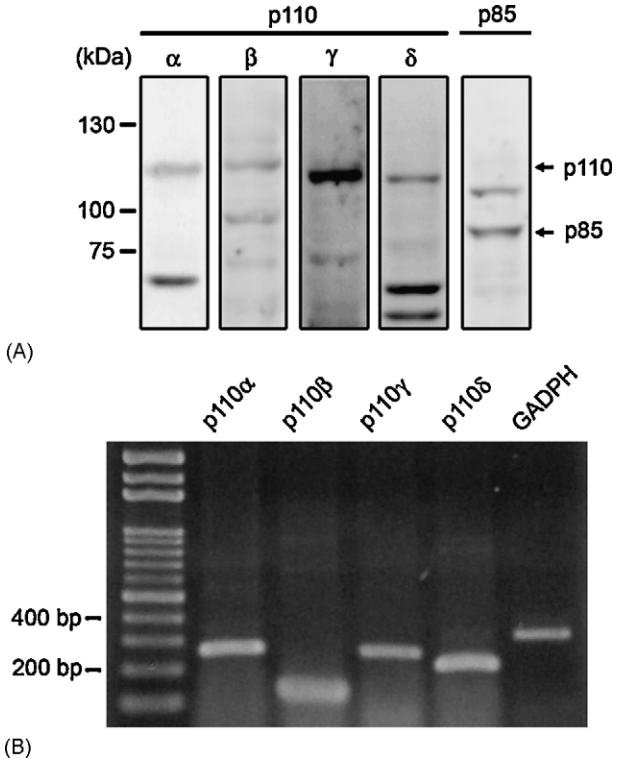

Fig. 4 – Expression of class I PI3K isoforms in rat neutrophils. (A) Cell lysates were immunoblotted with antibodies against p110α, p110β, p110γ, p110δ and p85. (B) The agarose gel electrophoresis of RT-PCR products of GAPDH (as an internal standard) and the catalytic subunits of class I PI3K isoforms. Result presented is representative of three independent experiments with similar results.

immunocomplexes of p85 and p110γ from fMLP- as well as ART-stimulated neutrophils showed the presence of Ras (Fig. 6C). The results that ART induced PtdIns(3,4,5)P_3 formation and the recruitment of PI3Ks to membrane within the relevant time-frame of Ras activation and the interaction between PI3K and Ras imply the upstream Ras signal leading to PI3K activation.

3.6. *Role of Src in ART-induced PtdIns(3,4,5)P_3 formation and $O_2^{\bullet-}$ generation*

Activation of PI3K by Src family kinase through binding of the proline-rich region of p85 to the Src-SH3 domain has been documented [22]. The Src kinase activity increased significantly at 1.5 min and gradually elevated with the time of exposure of neutrophils to 30 μM ART. ART also exerted a concentration-dependent activation on Src kinase (Fig. 7A). In contrast, a rapid rise in Src kinase activity commenced upon addition of fMLP and reached a peak within 30 s. Both ART- and fMLP-induced Src kinase activation was attenuated by a Src family kinase inhibitor PP1 analog with the IC_{50} values of 9.9 ± 1.3 and 7.0 ± 0.8 μM, respectively (Fig. 7B). The PP1 analog also resulted in a concentration-dependent decrease in PtdIns(3,4,5)P_3 formation ($55.4 \pm 2.8\%$ and $67.1 \pm 6.4\%$ inhibition at 10 μM PP1 analog, respectively, after subtraction from basal value; and no further inhibitory effect even at the higher concentration tested) (Fig. 7C) and $O_2^{\bullet-}$ generation (IC_{50} values of 14.3 ± 1.3 and 13.4 ± 1.2 μM, respectively) (Fig. 7D) in response to ART and fMLP. Moreover, fMLP- but not ART-induced PtdIns(3,4,5)P_3 formation and $O_2^{\bullet-}$ generation were attenuated by pretreatment of cells with pertussis toxin, a $G_{i/o}$ inhibitor. PP3, an inactive PP1 analogue, had no inhibitory effect on ART- and fMLP-induced $O_2^{\bullet-}$ generation. The ART-induced PtdIns(3,4,5)P_3 formation was substantially decreased in the duration of PP1 analogue treatment (Fig. 7E). These results suggest the involvement of Src/class IA PI3Ks signaling in ART- and fMLP-induced respiratory burst in rat neutrophils.

3.7. *Role of p38 MAPK, PLC/Ca^{2+} and PKC signaling pathways in ART-induced PI3K activation*

Pharmacological approaches were employed to elucidate the upstream intracellular signals and the downstream targets of ART-activated PI3K. Pretreatment of cells with 30 μM LY 294002, 30 μM SB 203580 (the p38 MAPK inhibitor), 1 μM GF 109203 X (a broad PKC inhibitor) or 1 μM U-73122 (a PLC-coupled processes inhibitor), the ART-stimulated PtdIns(3,4,5)P_3 formation was effectively attenuated only by LY 294002 (Fig. 8A). Moreover, LY 294002 suppressed the phosphorylation of MAPKAPK-2 (Fig. 8B), the downstream target of p38 MAPK, and blocked the $[Ca^{2+}]_i$ elevation in fluo 3-loaded cell (data not shown) in response to ART stimulation. LY 294002 had no effect on the phosphorylation of PKC (Fig. 8C), as assessed by immunoblotting with anti-phospho-PKC (pan) antibodies, which was raised against a number of phospho-PKCs including PKC-α, -βI, -βII, -γ, -δ, -ε, -η, and -ζ. Our previous report demonstrated that rat neutrophils express PKC-α, -β, -δ, -ε, -θ, -μ, -ι/λ, and ζ, although λ and ζ are barely detected [23].

4. Discussion

In the present study, we found that ART-induced $O_2^{\bullet-}$ generation involved PI3K signaling. Supporting this conclusion is the evidence that (1) a selective PI3K inhibitor LY 294002 diminished the ART-induced $O_2^{\bullet-}$ generation. The concentration dependency of this inhibition closely matched the concentration for the inhibition of fMLP-induced respiratory burst by LY 294002 in human neutrophils [4]; (2) ART stimulated the membrane recruitment of PI3K subunits (p85, p110α, p110β, p110γ, and p110δ) and $O_2^{\bullet-}$ generation [12] within the same concentration range; (3) ART stimulated the PI3K product PtdIns(3,4,5)P_3 formation and the phosphorylation of PI3K downstream target Akt. LY 294002 inhibited both responses in concentrations that blocked the $O_2^{\bullet-}$ response; (4) a Src kinase inhibitor PP1 analog at the effective concentrations that blocked the ART-induced Src kinase activation also inhibited the PtdIns(3,4,5)P_3 formation and $O_2^{\bullet-}$ generation in response to ART. The result that LY 294002 nearly abolished the ART-stimulated $O_2^{\bullet-}$ generation does not contradict the findings of our previous report that the pretreatment with SB 203580, U-73122, and with GF 109203X all completely blocked ART-stimulated $O_2^{\bullet-}$ generation [12] since the neutrophils from PLC-β2/-β3- or PI3Kγ-null mice also completely lost the ability to produce $O_2^{\bullet-}$ in response to fMLP

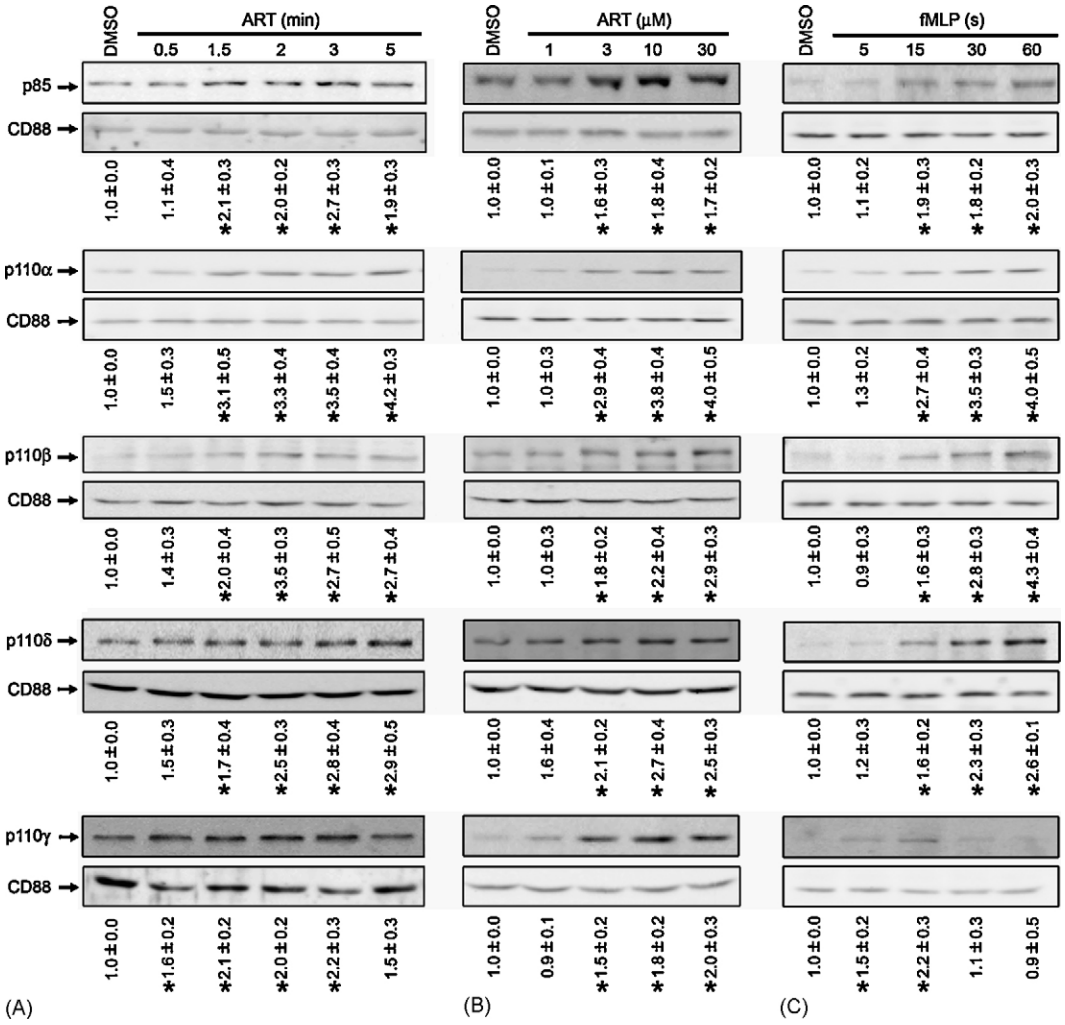

Fig. 5 – The membrane recruitment of PI3K in ART-stimulated neutrophils. Cells were treated with DMSO, (A) 30 μM ART for the indicated time intervals (n = 5), (B) the indicated concentrations of ART for 2 min (n = 4), or (C) with 1 μM fMLP plus 5 μg/ml of dhCB for the indicated time intervals (n = 6) at 37 °C. The cell membrane fractions were collected and immunoblotted with the specific antibody against p110α, p110β, p110γ, p110δ or p85. The blots above were also probed with anti-CD88 antibody as the loading control. The ratio of immunointensity between the PI3K and CD88 was calculated. The fold increase is expressed as means ± S.D. $^{*}P < 0.05$ as compared with the corresponding vehicle control values.

[5]. Thus, besides the activation of p38 MAPK, PLC/Ca^{2+}, and PKC signaling pathways [12], PI3K signaling is also responsible for ART-induced $O_2^{\bullet-}$ generation in rat neutrophils.

The lack of inhibitory effect on ART-stimulated PtdIns(3,4,5)P_3 formation by treatment of cells with SB 203580, GF 109203X and U-73122, thus indicating that neither of these signaling pathways is upstream of PI3K activation. However, LY 294002 attenuated the phosphorylation of MAPKAPK-2 and $[Ca^{2+}]_i$ elevation in ART-stimulated cells. These data imply that p38 MAPK and PLC/Ca^{2+} signaling pathways are downstream of PI3K. Our previous reports demonstrated that the PLC/Ca^{2+} is the upstream signaling of p38 MAPK activation in fMLP- and arachidonic acid-stimulated neutrophils [24,25]. To clarify whether PI3K, PLC/Ca^{2+} and p38 MAPK in a linear signaling pathway or alternatively that all of these signaling pathways together with PKC are converged on NADPH oxidase needs further investigation.

Besides the recruitment of $p40^{phox}$ and $p47^{phox}$ to the membrane and thus assembly of the active NADPH oxidase [6], the activation of Rac2 is also dependent on PI3K activity [26]. In addition, class IA PI3K may, through the activation of PKC-ζ, activate NADPH oxidase [15]. Class I PI3Ks in resting cells are cytoplasmic proteins [18]. The class IA PI3Ks are the downstream target of tyrosine kinase and Ras. The mechanism of activation involves the binding of SH2 domains in the adaptors to phosphorylated tyrosine residues on tyrosine kinase substrates at the plasma membrane and through binding of the proline-rich region of adaptors to the Src-SH3 domain [22], bringing PI3K to membrane-associated signaling complexes and, allowing further activation by binding of GTP-bound Ras to catalytic subunits [8]. Tyrosine phosphorylation of p85 by Src relieves the inhibitory effect of p85 on p110 catalytic activity [27]. On the other hand, the Gβγ-p101 interaction serves to recruit PI3Kγ to the cell membrane, and PI3Kγ

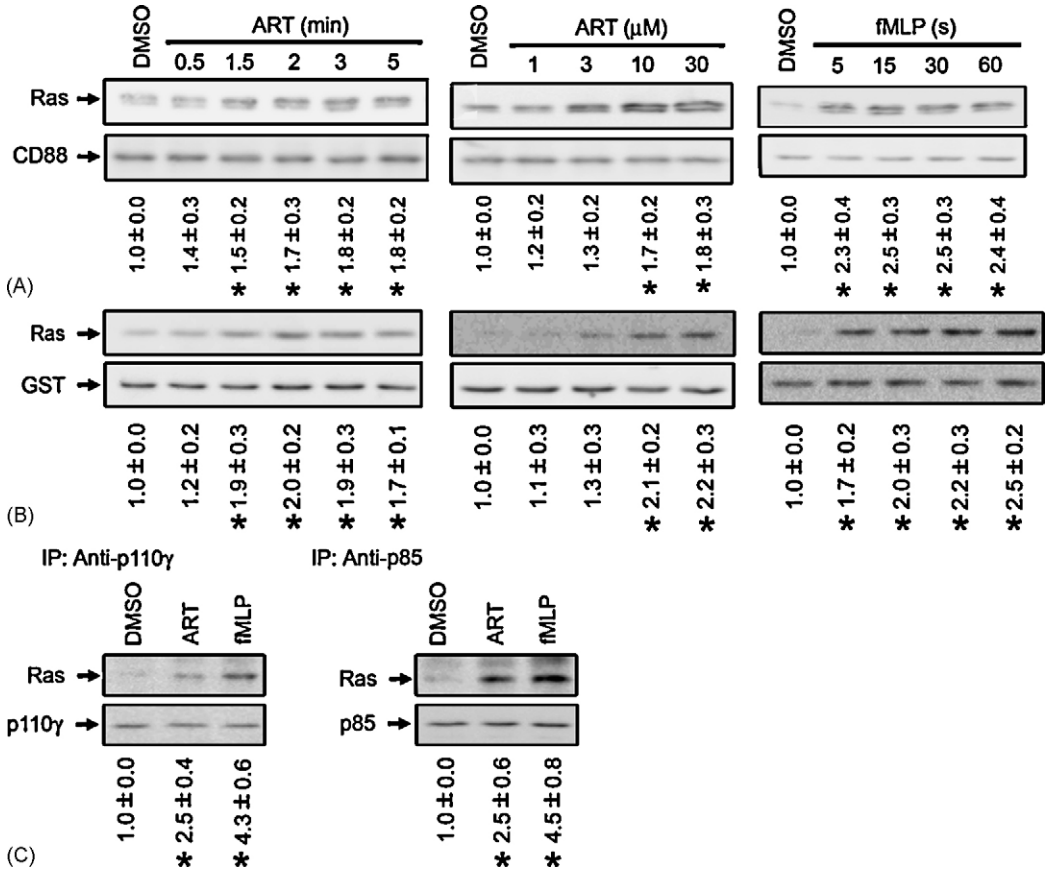

Fig. 6 – Effect of ART on Ras activation in neutrophils. Cells were treated with DMSO, (A) 30 μM ART or 1 μM fMLP plus 5 μg/ml of dhCB for the indicated time intervals, or with the indicated concentrations of ART for 2 min at 37 °C. The cell membrane fractions were immunoblotted with anti-Ras antibody. The blots above were also probed with anti-CD88 antibody as the loading control. The ratio of immunointensity between the Ras and CD88 was calculated ($n = 6$). (B) Cell lysates were precipitated with GST-Ras binding domain-agarose, and then immunoblotted with the specific antibody against Ras. The blots above were also probed with anti-GST antibody as the loading control. The ratio of immunointensity between the Ras and GST was calculated ($n = 4$). (C) Cell lysates were immunoprecipitated with anti-p110γ or anti-p85 antibody, and then analyzed by immunoblotting with anti-Ras antibody. The blots above were also probed with anti-p110γ or anti-p85 antibody as the loading control. The ratio of immunointensity between the Ras and p110γ or p85 was calculated ($n = 4$). The fold increase in the immunointensity is expressed as means ± S.D. $^{*}P < 0.05$ as compared with the corresponding vehicle control values.

activation is the consequence of the interaction of p110γ with Gβγ and active Ras [28,29]. In the Ras bound structure, the catalytic domain undergoes significant molecular rearrangement that affects the conformation of the phosphoinositide-binding site. There is accumulating evidence that the activation of class IA PI3K is also implicated in G-protein-coupled receptor-mediated signaling [30,31] and a dynamic interplay between class IA and IB isoforms in maintaining PtdIns(3,4,5)P_3 levels in neutrophils in response to stimulation of G-protein-coupled receptor [21].

The PtdIns(3,4,5)P_3 product mediated the recruitment of inactive Akt and constitutively active 3'-phosphoinositide-dependent kinase 1 (PDK1) through a PH domain from the cytosol to the plasma membrane, and Akt was subsequently phosphorylated on T308 residue by PDK1. Maximal activation requires phosphorylation of S473 in the hydrophobic motif by an unidentified kinase called PDK2, the nature of which is controversial [32]. The compelling candidates for PDK2 include rictor-mTOR complex, PDK1, MAPKAPK-2, integrin-linked kinase, PKC-βII, and DNA-dependent protein kinase [33,34]. The nature of PDK2 in regulating phosphorylation of S473 in response to ART is unclear at present. However, the relevance of Akt activation to $p47^{phox}$ phosphorylation is controversial [7,35,36].

We have demonstrated that both class IA and IB PI3Ks are expressed in rat neutrophils. However, an attempt to uncover the roles for specific PI3K isoforms might be difficult in neutrophils based on two points. First, the lack of small molecule inhibitors of the specific PI3K isoforms limits the pharmacological approaches. LY 294002, in general, lacks selectivity against different classes and/or isoforms of PI3K [37,38]. Second, the short life span of neutrophils makes the current molecular biology approaches (such as transfect dominant negative PI3K isoforms or antisense transfection) impracticable. fMLP utilizes G_i-protein-mediated transduction pathways to stimulate very rapid and large accumulations of

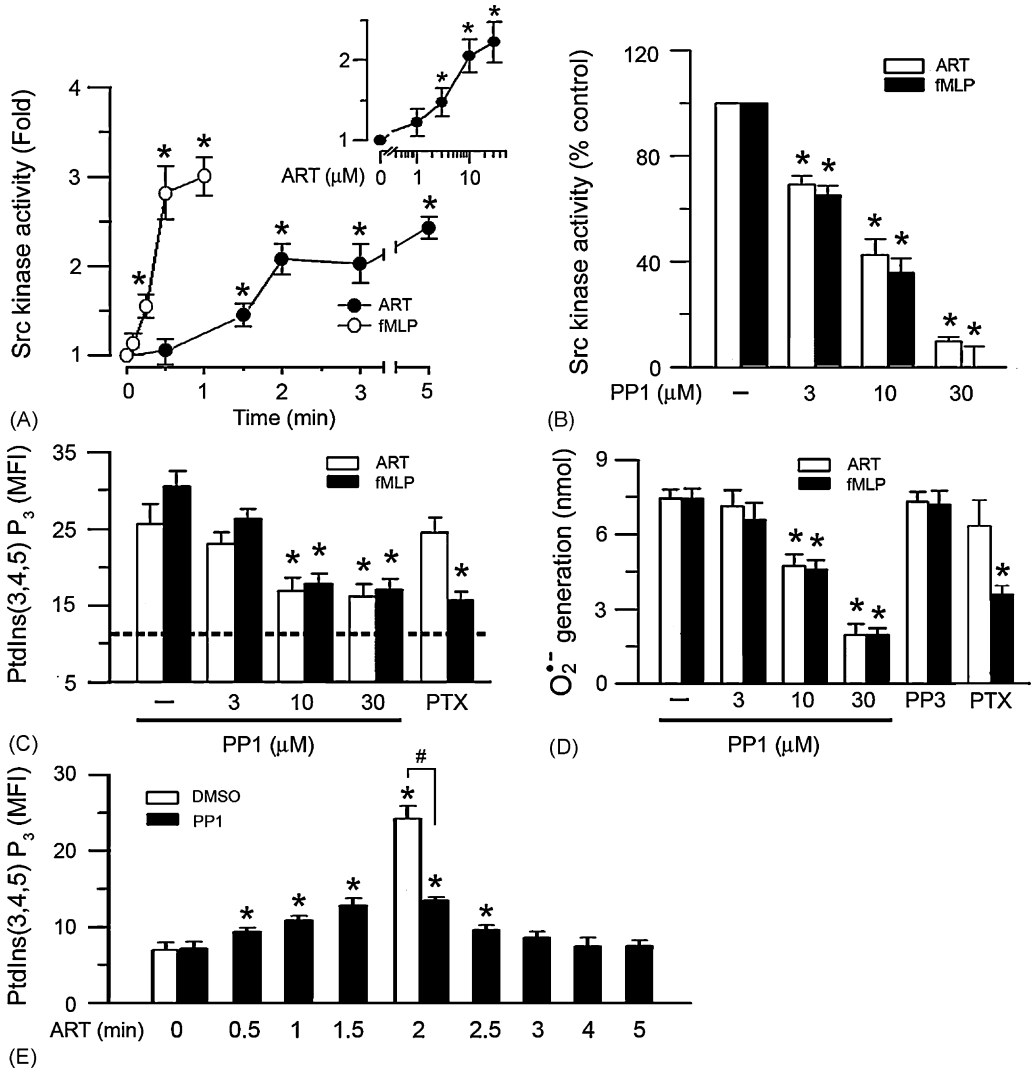

Fig. 7 – Effects of PP1 analog on Src family kinases activation, PtdIns(3,4,5)P_3 formation and $O_2^{\bullet-}$ generation in ART-stimulated neutrophils. (A) Cells were treated with DMSO, 30 μM ART or 1 μM fMLP plus 5 μg/ml of dhCB for the indicated time intervals, or with the indicated concentrations of ART for 2 min (inset) at 37 °C. Cellular Src family kinases activity was then determined. Values are expressed as means ± S.D (n = 4). $^*P < 0.05$ as compared with the vehicle control value. Cells were pretreated with DMSO (as control), the indicated concentrations of PP1 analog or 30 μM PP3 for 3 min, or with 1 μg/ml of pertussis toxin (PTX) for 60 min before stimulation (B) with ART for 2 min or fMLP/dhCB for 30 s (for cellular Src family kinases activation, n = 4), (C) with ART for 2 min or fMLP/dhCB for 15 s (for PtdIns(3,4,5)P_3 formation, n = 4; the dashed line represents the vehicle control value), and (D) with ART or fMLP/dhCB for 10 min (for $O_2^{\bullet-}$ generation, n = 5). Values are expressed as means ± S.D. $^*P < 0.05$ as compared with the corresponding control values (group 1 columns). (E) Cells were pretreated with DMSO or 30 μM PP1 analog for 3 min before stimulation with 30 μM ART for the indicated time intervals (for PtdIns(3,4,5)P_3 formation). Values are expressed as means ± S.D (n = 4). $^*P < 0.05$ as compared with the corresponding control values (group 1 columns); $^{\#}P < 0.05$.

PtdIns(3,4,5)P_3 [39], thus fMLP had no effect in pertussis toxin-treated cells. Considering the parallelism of time courses for both fMLP-induced PtdIns(3,4,5)P_3 formation and membrane recruitment of PI3Ks, this implies the regulation of cellular PtdIns(3,4,5)P_3 levels initially by PI3Kγ followed by class IA PI3Ks activation. However, fMLP rapidly induced Src activation and PP1 analog attenuated PtdIns(3,4,5)P_3 formation by 65% and $O_2^{\bullet-}$ generation by 73%, respectively, at a concentration that completely blocked the Src activation in rat neutrophils, indicating that the activation of class IA PI3Ks via a Src-mediated mechanism plays an important role. This interpretation is in accordance with the literature on human neutrophils [16,40,41]. It has been reported that fMLP-induced PtdIns(3,4,5)P_3 production and $O_2^{\bullet-}$ generation are both blocked in neutrophils isolated from PI3Kγ-deficient mice [19]. However, the responses in mouse neutrophils are substantially largely dependent on PI3Kγ alone [41].

ART-induced PtdIns(3,4,5)P_3 formation was characterized by a gradual increase, reaching a peak after about 2 min stimulation. The apparent slower rate taken to onset of PtdIns(3,4,5)P_3

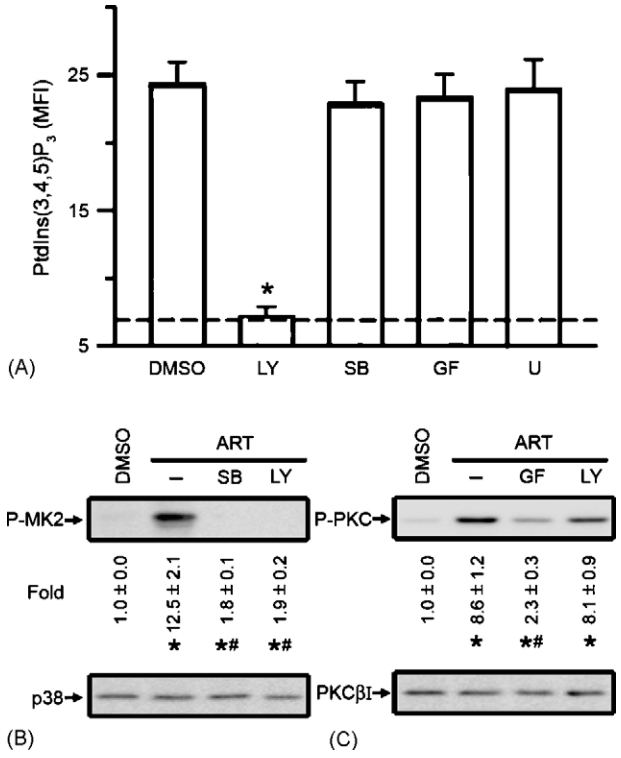

Fig. 8 – Effects of LY 294002 (LY), SB 203580 (SB), GF 109203X (GF) and U-73122 (U) on PtdIns(3,4,5)P_3 formation in ART-stimulated neutrophils. Cells were pretreated with DMSO, 30 μM LY, 30 μM SB, 1 μM GF or 1 μM U for 3 min before stimulation or no stimulation with 30 μM ART for 2 min (for PtdIns(3,4,5)P_3 formation) or 10 min (for Western blot). (A) After fixation and permeabilization, cells were probed with anti-PtdIns(3,4,5)P_3 antibody followed by the staining with rhodamine-labeled secondary antibody, and then counted in flow cytometry (n = 4). The dashed line represents the vehicle control value. Values are expressed as means ± S.D. $^*P < 0.05$ as compared with the activated control value (column 1). For Western blot analysis, cell lysates were probed with (B) anti-phospho-MAPKAPK-2 (P-MK2) or (C) anti-phospho-PKC antibody. The blots above were then stripped and reprobed with anti-p38 MAPK and anti-PKC-βI antibody, respectively. The ratio of immunointensity between the phospho-MAPKAPK-2 and p38 MAPK or the phospho-PKC and PKC-βI was calculated. The fold increase is expressed as means ± S.D. (n = 3). $^*P < 0.05$ as compared with the corresponding vehicle control values (lane 1); $^\#P < 0.05$ as compared with the corresponding activated control values (lane 2).

formation could preclude an important role for G-protein in ART-induced response. The data that pertussis toxin had no inhibitory effect on ART-stimulated PtdIns(3,4,5)P_3 formation is consistent with such an interpretation. However, the early detectable membrane-association p110γ and PtdIns(3,4,5)P_3 formation overlap in time, implying the contribution of PI3Kγ signaling in the initial PtdIns(3,4,5)P_3 formation. The following PtdIns(3,4,5)P_3 formation is probably attributable to the combination of class IA PI3Ks and PI3Kγ based on the kinetics of PI3Ks recruitment to membrane. Because the membrane-associated PI3Ks did not decrease after maximum PtdIns(3,4,5)P_3 level, the decline in cellular PtdIns(3,4,5)P_3 levels at that moment could be due to the dephosphorylation by phosphatase. The new plateau (about 35% above baseline) cellular PtdIns(3,4,5)P_3 level after the peak appears to be solely attributable to class IA PI3Ks activation. Both Src and Ras downstream regulate the ART-induced class IA PI3Ks activation as evidenced from the parallelism of kinetics of membrane recruitment of Ras and PI3Ks, Ras co-precipitated with p85, and the attenuation of cellular PtdIns(3,4,5)P_3 level by PP1 analog. However, the mechanism by which ART mediated the membrane recruitment of p110γ is still unclear and awaits further investigation. The data that ART-induced PtdIns(3,4,5)P_3 formation and $O_2^{\bullet-}$ generation were attenuated by PP1 analog (by 57% and 72% inhibition, respectively) at a concentration that completely blocked the Src activation in rat neutrophils indicate that the activation of class IA PI3Ks signaling might also play an important role.

In conclusion, rat neutrophils express class IA PI3Ks (p85, p110α, p110β, and p110δ) and class IB PI3K (p110γ). Stimulation with fMLP and ART activates both class IA and IB PI3Ks as assessed by immunoblotting analysis of PI3Ks recruitment to membrane. Both class IA and IB PI3Ks contribute to the PtdIns(3,4,5)P_3 formation and class IA PI3Ks activation might play a major role in $O_2^{\bullet-}$ generation in rat neutrophils in response to ART as well as to fMLP.

Acknowledgements

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