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Inhibition of superoxide anion generation by YC-1 in rat neutrophils through cyclic GMP-dependent and -independent mechanisms

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Abstract

3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), a soluble guanylyl cyclase (sGC) activator, inhibited formyl-methionyl-leucyl-phenylalanine (fMLP)-induced superoxide anion ($O_2^{\bullet -}$) generation and O_2 consumption in rat neutrophils (IC_{50} values of 12.7 ± 3.1 and 17.7 ± 6.9 μM, respectively). Inhibition of $O_2^{\bullet -}$ generation by YC-1 was partially reversed by the cyclic GMP-lowering agent 6-anilinoquinoline-5,8-quinone (LY83583) and by the Rp isomer of 8-(4-chlorophenylthio)guanosine-3′,5′-monophosphorothioate (Rp-8-pCPT-cGMPS), a cyclic GMP-dependent protein kinase inhibitor. In cell-free systems, YC-1 failed to alter $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation, phorbol 12-myristate 13-acetate (PMA)-activated neutrophil particulate NADPH oxidase preparation, and arachidonic acid-induced NADPH oxidase activation. YC-1 increased cellular cyclic GMP levels through the activation of sGC and the inhibition of cyclic GMP-hydrolyzing phosphodiesterase activity. The plateau phase, but not the initial spike, of fMLP-induced $[Ca^{2+}]_i$ changes was inhibited by YC-1 (IC_{50} about 15 μM). fMLP- but not PMA-induced phospholipase D activation was inhibited by YC-1 (IC_{50} about 28 μM). Membrane-associated ADP-ribosylation factor and Rho A in cell activation was also reduced by YC-1 at a similar concentration range. Neither cytosolic protein kinase C (PKC) activity nor PKC membrane translocation was altered by YC-1. YC-1 did not affect either fMLP-induced phosphatidylinositol 3-kinase activation or p38 mitogen-activated protein kinase phosphorylation, but slightly attenuated the phosphorylation of extracellular signal-regulated kinase. Collectively, these results indicate that the inhibition of the fMLP-induced respiratory burst by YC-1 is mediated by cyclic GMP-dependent and -independent signaling mechanisms.
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Keywords: Neutrophil; YC-1; Superoxide anion; Cyclic GMP; Cellular free Ca^{2+}; Phospholipase D

1. Introduction

Microbicidal reactive oxygen species derived from neutrophil-generated $O_2^{\bullet -}$ have an important role in host defense against microbial infection. The enzyme responsible for $O_2^{\bullet -}$ generation is NADPH oxidase, which is inactive in resting cells and becomes active during the acute immune response. The oxidase consists of membrane cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$) and cytosolic components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac), which assemble into a functional complex upon activation [1,2]. Thus, activated neutrophils evoke a respiratory burst in which oxygen uptake from the extracellular medium is increased and large amounts of $O_2^{\bullet -}$ are generated. The signal transduction events pertaining to the respiratory burst remain elusive. The intracellular signals from the fMLP receptors are mediated by G_i protein. Phospholipase C is activated rapidly, leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate and diacylglycerol, resulting in an increase of $[Ca^{2+}]_i$ and activation of PKC, respectively [3]. These two

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Abbreviations: ARF, ADP-ribosylation factor; dhCB, dihydrocytochalasin B; ERK, extracellular signal-regulated kinase; fMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; MAPK, mitogen-activated protein kinase; $O_2^{\bullet -}$, superoxide anion; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine-3′,5′-monophosphate; Rp-8-pCPT-cGMPS, Rp-isomer of 8-(4-chlorophenylthio)guanosine-3′,5′-monophosphorothioate; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase.

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second messengers act synergistically in $O_2^{\bullet -}$ generation. PLD is also activated by fMLP in neutrophils and appears to be functionally linked to $O_2^{\bullet -}$ generation [4]. Moreover, activation of PI3K and MAPK by fMLP eventually leads to NADPH oxidase activation [5,6].

3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), an sGC activator [7], has been introduced as an important research tool to characterize sGC and to probe for the involvement of cyclic GMP in various biological processes. YC-1 was shown to exert an anti-thrombotic effect *in vivo*, and to inhibit platelet aggregation and relax vascular smooth muscle *in vitro* [8,9]. Unlike the two well-known sGC activators, nitric oxide and carbon monoxide, YC-1 exerts an allosteric regulation without interacting with the heme moiety of sGC [10]. YC-1 acts synergistically in combination with nitric oxide and carbon monoxide. In general, an increase in cellular cyclic AMP levels inhibits chemoattractant-induced responses. However, the physiological role of cyclic GMP in neutrophils is still poorly understood. Both an inhibitory effect and a negligible effect of cyclic GMP on fMLP-induced responses have been reported [11,12]. The aims of this study were to characterize the effect of YC-1 on the respiratory burst in rat neutrophils and to determine a possible mechanism(s) of this action. The present data provide evidence that the inhibition of fMLP-induced respiratory burst by YC-1 is mediated only partly by cyclic GMP and is also attributed to the blockade of Ca^{2+} entry and the PLD signaling pathway.

2. Materials and methods

2.1. Materials

YC-1 (purity > 99%) was synthesized as described previously [13]. Dextran T-500, cyclic GMP enzyme immunoassay kit, enhanced chemiluminescence reagent, [8-^{3}H]cyclic GMP, and 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine were purchased from Amersham Pharmacia Biotech. HBSS was obtained from Gibco Life Technologies. Diphenylene iodonium and 6-anilinoquinoline-5,8-quinone (LY83583) were from RBI Laboratories. Rp-8-pCPT-cGMPS and 8-pCPT-cGMP were obtained from Biolog Life Science. 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro 201724) and zaprinast were obtained from Biomol Research Laboratories. Wortmannin, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF109203X), and fluo-3/AM were purchased from the Calbiochem-Novabiochem Co. ^{32}P and [γ-^{32}P]ATP were obtained from NEN Life Science Products. DE-52 cellulose was from Whatman International. AG 1-X8 resin was from Bio-Rad. Rabbit polyclonal antibodies to phospho-p44/42 MAPK, phospho-p38 MAPK, and p38 MAPK were purchased from New England Biolabs. Mouse monoclonal pan ERK antibody was purchased from BD Transduction Laboratories. Rabbit polyclonal ARF and mouse monoclonal Rho A antibodies were obtained from Santa Cruz Biotechnology. Polyvinylidene difluoride membrane was from the Millipore Co. Other chemicals were purchased from the Sigma Chemical Co. The final volume of DMSO in the reaction mixture was $\leq$0.5% (v/v).

2.2. Isolation of neutrophils

Rat blood was collected from the abdominal aorta, and neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Hypaque, and hypotonic lysis of erythrocytes [14]. Purified neutrophils containing >95% viable cells were resuspended in HBSS containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice bath before use.

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

The generation of $O_2^{\bullet -}$ in the neutrophil suspension was determined by the SOD-inhibitable reduction of ferricytochrome *c* [14]. For the determination of $O_2^{\bullet -}$ scavenging activity, $O_2^{\bullet -}$ generation during dihydroxyfumaric acid (2.5 mM) autoxidation in a cell-free system was assessed by measuring the reduction of nitroblue tetrazolium [15]. Absorbance changes of the reduction of ferricytochrome *c* and nitroblue tetrazolium at 550 and 560 nm, respectively, were monitored continuously in a double-beam spectrophotometer. The O_2 consumption in the neutrophil suspension was measured continuously with a Clark-type oxygen electrode using a YSI biological oxygen monitor (model 5300).

2.4. Measurement of NADPH oxidase activity in cell-free systems

Neutrophils were treated with 2.5 mM diisopropyl fluorophosphate, disrupted in Tris buffer by sonication, and fractionated by centrifugation [16]. Supernatants were pooled as the cytosolic fractions, and pellets were collected and resuspended in Tris buffer as the membrane fractions. Plasma membrane and cytosolic fractions were mixed in 1.5 mL of assay buffer (0.17 M sucrose, 2 mM NaN_3, 1 mM $MgCl_2$, 1 mM EGTA, 65 mM KH_2PO_4–NaOH, pH 7.0) supplemented with 10 μM FAD, 3 μM GTPγS, 0.25 mg/mL of ferricytochrome *c*, 50 μM NADPH, and activated by 100 μM arachidonic acid. PMA-activated NADPH oxidase was isolated, and its activity was determined as described previously [16]. The assay mixture contained 0.04% (w/v) sodium deoxycholate, 12.5 μM FAD, 0.25 mg/mL of ferricytochrome *c*, particulate protein solution, and 62.5 μM NADPH in a final volume of 1.6 mL. NADPH oxidase activity was measured spectrophotometrically by continuously detecting the absorbance changes of SOD-inhibitable ferricytochrome *c* reduction.

2.5. Determination of cellular cyclic GMP levels

Neutrophils in HBSS were incubated with test drugs for 20 min at 37°, and then added to 0.05 M acetate buffer, pH 6.2, containing 50 μM zaprinast. After being boiled for 5 min, the suspension was sonicated, and then sedimented. Supernatants were acetylated by the addition of 0.025 mL of triethylamine:acetic anhydride (2:1, v/v). The cyclic GMP content of the samples was assayed using an enzyme immunoassay kit. Values are expressed as pmol cyclic GMP formation per 10^7 cells.

2.6. Measurement of sGC and PDE activities

Neutrophils were sonicated in buffer containing 25 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 100 μM phenylmethylsulfonyl fluoride, 2 mM EDTA, 5 mM $MgCl_2$, and 10 μM each of leupeptin and pepstatin; then they were sedimented at 100,000 *g* for 40 min at 4°. Supernatants were pooled and used as sources for sGC and PDE. For the sGC assay, the reaction mixture contained 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM 3-isobutyl 1-methylxanthine, 7.5 mM creatine phosphate, 3 units creatine phosphokinase, 1 mM GTP, and the sGC sample in a final volume of 0.1 mL. The reaction was carried out for 10 min at 37° [17] and terminated by adding 0.9 mL of ice-cold 50 mM acetate buffer, pH 4.0, and boiling for 3 min. The cyclic GMP content of the samples was assayed using an enzyme immunoassay kit. Values are expressed as fmol cyclic GMP formed/10 min per 10^6 cells. For the PDE assay, the reaction mixture contained 40 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 1 μM cyclic GMP (0.05 μCi [^{3}H]cyclic GMP/test), 0.1 mg/mL of BSA, and the PDE sample in a final volume of 0.2 mL. The reaction was carried out for 30 min at 37° and stopped by the addition of 0.2 M HCl, and incubation with 0.2 mg/mL of Crotalus atrox snake venom at 37° for 15 min [18]. The reaction mixture was applied to an AG 1-X8 resin (formate) column. The nucleoside product was eluted using 30 mM ammonium formate adjusted to pH 6.0 with formic acid and then was assessed for radioactivity. Values are expressed as pmol cyclic GMP degraded/30 min per 10^7 cells.

2.7. $[Ca^{2+}]_i$ measurement

Neutrophils were loaded with 5 μM fluo-3/AM at 37° for 45 min. After being washed, the cells were resuspended in HBSS to a concentration of 5×10^6 cells/mL. Fluorescence was monitored with a fluorescence spectrophotometer at 535 nm with excitation at 488 nm. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$, where F is the observed fluorescence intensity [19]. The values of F_{max} and F_{min} were obtained at the end of the experiments by the sequential addition of 0.33% (v/v) Triton X-100 and 50 mM EGTA. The K_d was 400 nM.

2.8. PKC activity assay

For the preparation of cytosolic and membrane PKC, neutrophils were disrupted by sonication [14]. After centrifugation at 100,000 *g* for 60 min at 4°, the supernatants (cytosolic fractions) and pellets were separated. Pellets were resuspended in the presence of 0.1% Triton X-100, and then sonicated. After centrifugation at 30,000 *g* for 15 min at 4°, supernatants were collected (membrane fractions). The cytosolic and membrane fractions were applied to DE-52 columns to obtain partially purified PKC. For the membrane PKC assay, the reaction mixture contained 50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.1 mM sodium pyrophosphate, 1 mM NaF, 1 mM EGTA, 0.1 mg/mL of myelin basic protein, 50 μM ATP (0.2 μCi [γ-^{32}P]ATP/test), and the PKC sample in a total volume of 75 μL. The reaction was performed at 25° for 15 min. For the cytosolic PKC assay, the reaction mixture also contained 1 mM $CaCl_2$, 80 μg/mL of phosphatidylserine, and 2 μM PMA.

2.9. PLD activity assay

Neutrophils were loaded with 10 μCi of 1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*rac*-glycero-3-PC; [^{3}H]octadecyl-SS) at 37° for 75 min, then washed, and resuspended in HBSS to a concentration of 5×10^7 cells/mL. The cells were incubated with test drugs in the presence of 1 mM $CaCl_2$ and 0.5% (v/v) ethanol for 3 min at 37° before stimulation with fMLP or PMA. Lipids in the reaction mixture were extracted, dried, and separated on silica gel 60 [16]. The plates were developed halfway using a system consisting of hexane:diethyl ether:methanol:acetic acid (90:20:3:2, by vol.), then dried, and developed again to the top using the upper phase of the solvent system consisting of ethyl acetate:isooctane:acetic acid:water (110:50:20:100, by vol.). The radioactivities of [^{3}H]phosphatidic acid and [^{3}H]phosphatidylethanol were quantified directly with a PhosphorImager (Molecular Dynamics 445 SI) using ImageQuaNT software.

2.10. PI3K activity assay

Neutrophils were incubated with 150 μCi $^{32}P_i$ in HEPES buffer (30 mM HEPES, pH 7.4, 110 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, and 10 mM glucose) supplemented with 2 mg/mL of BSA at 37° for 90 min, then washed, and resuspended in HEPES buffer to a concentration of 1×10^7 cells/mL. The cells were incubated with test drugs in the presence of 1 mM $CaCl_2$ for 3 min at 37° before stimulation with fMLP. Reactions were terminated by the addition of chloroform:methanol:8% $HClO_4$ (50:100:5, by vol.). Lipids in the reaction mixture were extracted, dried, and separated on silica gel 60 [20], which had been impregnated with 1.2% (w/v) potassium oxalate. The plates were developed with a solvent system containing chloroform:acetone:methanol:acetic acid:water (80:30:26:24:14,

by vol.), then dried, and visualized for the radioactivity of [^{32}P]phosphatidylinositol trisphosphate with a PhosphorImager using ImageQuaNT software.

2.11. Immunoblot analysis

For MAPK activation, cells were preincubated with test drugs for the indicated time before stimulation with fMLP. Reactions were terminated by the addition of stop solution [20% (w/v) trichloroacetic acid, 1 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide, 10 mM NaF, 2 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate, 7 μg/mL each of leupeptin and pepstatin]. Proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% (w/v) non-fat dried milk in TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and probed with anti-phospho-p44/42 MAPK or anti-phospho-p38 MAPK antibody. To standardize protein loading in each lane, blots were stripped with buffer containing 62.5 mM Tris–HCl, pH 6.8, 100 mM 2-mercaptoethanol, and 2% SDS at 50° for 30 min. Then the blots were washed thoroughly, followed by reprobing with anti-pan ERK or anti-p38 MAPK antibody. For the membrane association of ARF and Rho A, cells were disrupted by sonication, and membrane fractions were isolated. Proteins were resolved by 13% SDS-PAGE, then transferred to polyvinylidene difluoride membrane, probed with anti-ARF or anti-Rho A antibody, and revealed using the enhanced chemiluminescence reagent. Quantification was by densitometry.

2.12. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. $P \leq 0.05$ was considered significant for all tests. Analysis of the regression line test was used to calculated IC_{50} values. Data are expressed as means ± SD.

3. Results

3.1. Effect of YC-1 on respiratory burst

Addition of 0.3 μM fMLP to rat neutrophil suspensions in the presence of 5 μg/mL of dhCB evoked a rapid and transient production of $O_2^{\bullet -}$. In the presence of 1 mM NaN_3 and 5 μg/mL of dhCB, 0.1 μM fMLP induced non-mitochondrial O_2 consumption in neutrophil suspensions. YC-1 inhibited the fMLP-induced generation of $O_2^{\bullet -}$ and O_2 consumption in rat neutrophils in a concentration-dependent manner with IC_{50} values of 12.7 ± 3.1 and 17.7 ± 6.9 μM, respectively (Fig. 1A). The viability was about 95% when cells were incubated with 100 μM YC-1 for 15 min at 37° (the cells released 5.1 ± 1.6% lactate dehydrogenase in comparison with the Triton X-100-treated value).

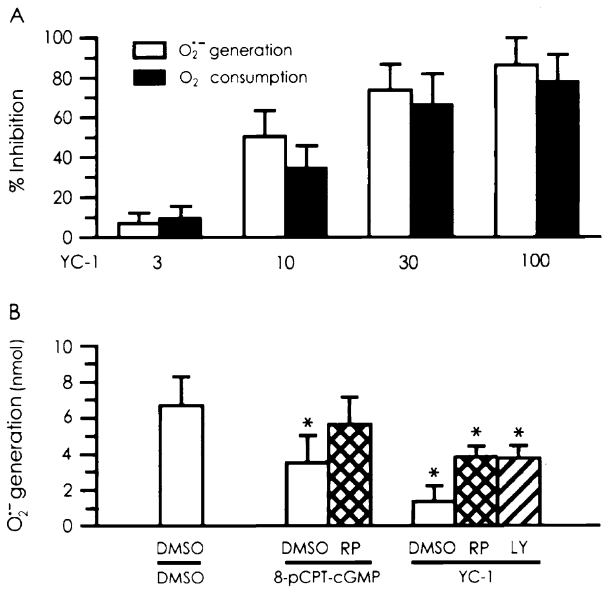

Fig. 1. Effect of YC-1 on the generation of $O_2^{\bullet -}$ and O_2 consumption in fMLP-stimulated neutrophils. (A) Cells were preincubated with DMSO (as control) or 3–100 μM YC-1 in the presence of 5 μg/mL of dhCB for 3 min at 37° before stimulation with 0.3 μM fMLP for $O_2^{\bullet -}$ generation or with 0.1 μM fMLP for O_2 consumption measurements. Results were calculated as the percent inhibition of control values (6.3 ± 1.2 nmol $O_2^{\bullet -}$/10 min per 10^6 cells and 8.3 ± 1.5 nmol O_2/5 min per 6×10^6 cells). Values are means ± SD of 3–5 separate experiments. (B) Cells were preincubated with DMSO, 100 μM Rp-8-pCPT-cGMP (RP), or 10 μM LY83583 (LY) in the presence of 5 μg/mL of dhCB for 3 min at 37°, and then incubated with DMSO, 50 μM 8-pCPT-cGMP, or 30 μM YC-1 for another 3 min before stimulation with 0.3 μM fMLP for the measurement of $O_2^{\bullet -}$ generation. Values are means ± SD of 4–6 separate experiments. (*) $P < 0.01$, compared with the control value (first column).

Cells treated with 50 μM 8-pCPT-cGMP, the selective activator of cyclic GMP-dependent protein kinase [21], significantly attenuated the fMLP-induced generation of $O_2^{\bullet -}$ (48.3 ± 9.7% inhibition). This effect was reversed by 100 μM Rp-8-pCPT-cGMPS, a cell membrane permeant cyclic GMP-dependent protein kinase antagonist [22]. Rp-8-pCPT-cGMPS (at 1 mM) has been shown to antagonize the activation of cyclic GMP-dependent protein kinase by 8-pCPT-cGMP without affecting cyclic AMP-dependent protein kinase or cyclic GMP-regulated PDE in platelets. Pretreatment of cells with 100 μM Rp-8-pCPT-cGMPS or 10 μM LY83583, an agent that lowers cellular cyclic GMP [23], partially reversed the inhibition by 30 μM YC-1 (Fig. 1B).

3.2. Effect of YC-1 on $O_2^{\bullet -}$ generation in cell-free systems

To address the question of whether YC-1 acts as an $O_2^{\bullet -}$ scavenger, the effect of YC-1 on $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation in a cell-free system was examined. Unlike SOD (data not shown), YC-1 (30 μM) had no scavenging activity (0.24 ± 0.02 for control vs. 0.23 ± 0.02 ΔA_{560}). We next determined whether YC-1 directly inhibits the activity of NADPH oxidase in

cell-free systems. In PMA-activated neutrophil particulate NADPH oxidase preparations, addition of NADPH induced $O_2^{\bullet -}$ generation. In an arachidonic acid-stimulated cell-free system, 100 μM arachidonic acid induced the assembly of the components of NADPH oxidase from the cytosolic and membrane fractions and generation of $O_2^{\bullet -}$ in the presence of NADPH. Addition of the NADPH oxidase inhibitor diphenylene iodonium [24] greatly attenuated the $O_2^{\bullet -}$ generation in both systems (0.89 ± 0.03 for control vs. 0.14 ± 0.07 nmol/10 min per 6×10^6 cells for PMA-activated NADPH oxidase, and 2.09 ± 0.14 for control vs. 0.44 ± 0.23 nmol/10 min per 10^7 cells for arachidonic acid-stimulated NADPH oxidase, both at $P < 0.01$). However, YC-1 up to 100 μM failed to alter $O_2^{\bullet -}$ generation significantly (1.16 ± 0.13 nmol/10 min per 6×10^6 cells and 2.11 ± 0.32 nmol/10 min per 10^7 cells, respectively, both at $P > 0.05$).

3.3. Effect of YC-1 on cellular cyclic GMP levels

YC-1 elicited a concentration-dependent increase in cellular cyclic GMP levels in rat neutrophils (up to 9-fold at 100 μM), which was abolished by preincubation with 10 μM LY83583 (Fig. 2A). YC-1 also activated sGC of the cytosolic fraction in a concentration-dependent manner (up to 10-fold at 100 μM), whereas the nitric oxide donor sodium nitroprusside at 100 μM stimulated cytosolic sGC activity up to 25 times the basal level (Fig. 2B). To determine PDE activity in the neutrophil cytosolic fraction, cyclic GMP breakdown in the presence of [^{3}H]cyclic GMP was determined. The addition of 100 μM zaprinast, the selective inhibitor of PDE5 [25], attenuated the degradation of cyclic GMP. A significant inhibition of cyclic GMP degradation by YC-1 was observed only at high concentration (100 μM) (Fig. 2C), whereas 30 μM Ro 201724, a specific inhibitor of PDE4 [26], had no inhibitory effect.

3.4. Effects of YC-1 on $[Ca^{2+}]_i$ and PKC activity

Addition of fMLP to the fluo-3/AM-loaded cells evoked an initial spike, followed by a plateau phase of $[Ca^{2+}]_i$ changes in the presence of extracellular Ca^{2+}. When YC-1 was added simultaneously with fMLP, the initial spike was not affected (up to 50 μM YC-1); however, the plateau phase of the fMLP-induced response was inhibited by YC-1 in a concentration-dependent manner with an IC_{50} value of about 15 μM (assessed using the maximum $[Ca^{2+}]_i$ level at the plateau phase) (Fig. 3A). In the absence of extracellular Ca^{2+}, fMLP induced a small spike of $[Ca^{2+}]_i$ change. This response was not affected by YC-1 up to 30 μM but was partially inhibited by 50 μM YC-1 (Fig. 3B).

Addition of 2 μM PMA and [γ-^{32}P]ATP to the PKC preparation from the cytosolic fraction of unstimulated neutrophils (in the presence of Ca^{2+} and phosphatidylserine) resulted in the incorporation of ^{32}P into myelin basic protein. Addition of 10 μM GF109203X, a PKC inhibitor

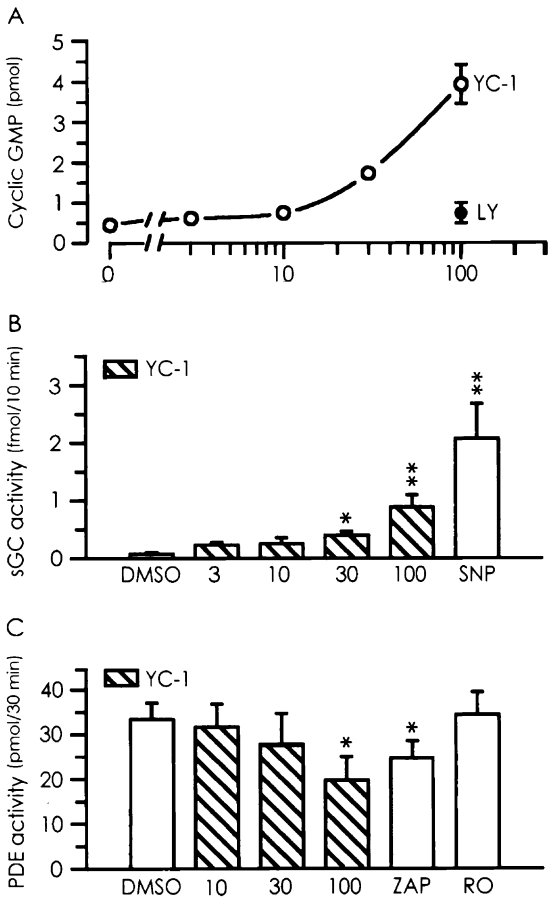

Fig. 2. Effect of YC-1 on cellular cyclic GMP levels and the activities of sGC and PDE. (A) Cells were preincubated with DMSO or 10 μM LY83583 (LY) for 3 min at 37° before stimulation with 3–100 μM YC-1 for 20 min. Values are means ± SD of 4–6 separate experiments. (B) Cell cytosolic fractions were incubated with DMSO (as control), 3–100 μM YC-1, or 100 μM sodium nitroprusside (SNP) for 10 min at 37° in the presence of 1 mM GTP. Values are means ± SD of 4–6 separate experiments. (*) $P < 0.05$ and (**) $P < 0.01$, compared with the control value. (C) Cell cytosolic fractions were incubated with DMSO (as control), 10–100 μM YC-1, 100 μM zaprinast (ZAP), or 30 μM Ro 201724 (RO) for 30 min at 37° in the presence of 1 μM cyclic GMP (0.05 μCi [^{3}H]cyclic GMP). Values are means ± SD of 5–6 separate experiments. (*) $P < 0.01$, compared with the control value.

[27], greatly reduced the phosphorylation of myelin basic protein (2.90 ± 0.19 for the PMA-stimulated control without inhibitor vs. 0.13 ± 0.02 nmol/min per mg protein in the presence of inhibitor, $P < 0.01$). However, YC-1 had no effect on the PMA-induced phosphorylation of myelin basic protein (2.78 ± 0.37 nmol/min per mg protein, $P > 0.05$). Moreover, pretreating cells with 50 μM YC-1 did not affect the subsequent stimulatory effect of PMA on membrane-associated PKC activity (2.66 ± 0.19 for the PMA-stimulated control vs. 2.61 ± 0.39 nmol/min per mg protein for YC-1 pretreatment, $P > 0.05$).

3.5. Effect of YC-1 on PLD activity

PLD catalyses the hydrolysis primarily of phosphatidylcholine to produce phosphatidic acid [28]. In the presence

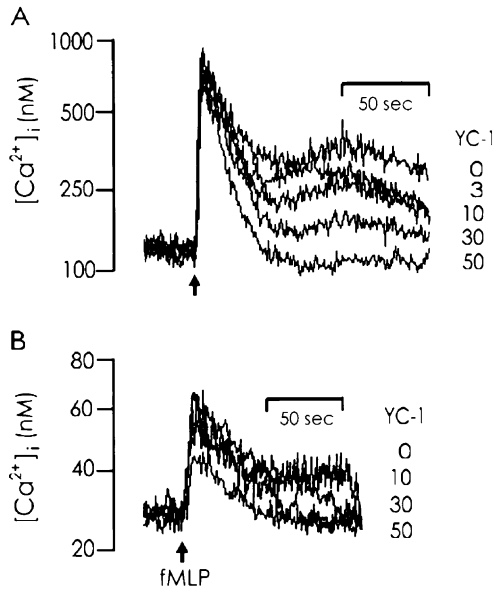

Fig. 3. Effect of YC-1 on $[Ca^{2+}]_i$. Fluo-3/AM-loaded cells in (A) 1 mM Ca^{2+}-containing or (B) Ca^{2+}-free HBSS were stimulated (arrow) with 0.3 μM fMLP in combination with the indicated concentrations (μM) of YC-1. Results presented are representative of 3 independent experiments with similar results.

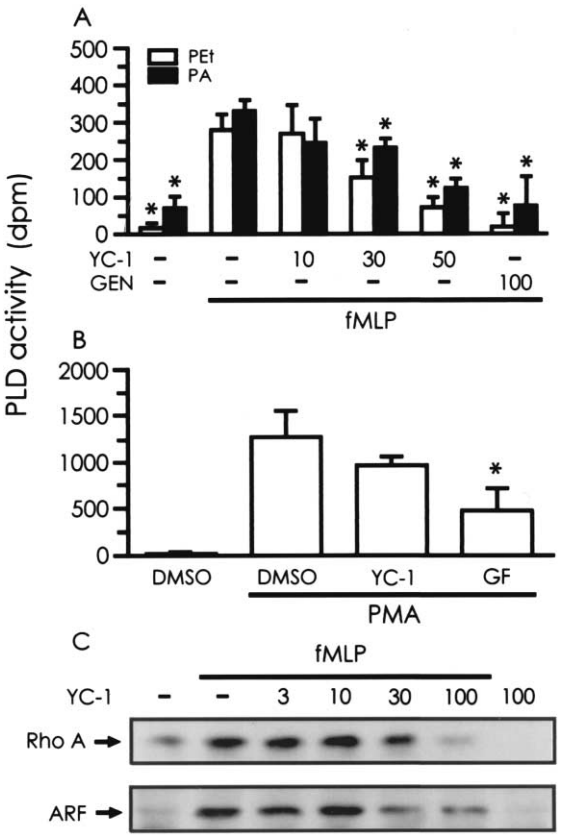

Fig. 4. Effect of YC-1 on PLD activity. (A) 1-*O*-[^{3}H]Octadecyl-*sn*-glycero-3-phosphocholine-loaded cells were preincubated with DMSO, 10–50 μM YC-1, or 100 μM genistein (GEN) in the presence of 0.5% ethanol and 5 μg/mL of dhCB for 3 min at 37° before the addition of DMSO or 1 μM fMLP for 0.5 min. Lipids in the reaction mixture were extracted and separated. The radioactivities of phosphatidylethanol (PEt) and phosphatidic acid (PA) were counted. Values are means ± SD of 3–7 separate experiments. (*) $P < 0.01$, compared with the corresponding control values (second group of columns). (B) 1-*O*-[^{3}H]Octadecyl-*sn*-glycero-3-phosphocholine-loaded cells were preincubated with DMSO, 50 μM YC-1, or 10 μM GF109203X (GF) in the presence of 0.5% ethanol for 3 min at 37° before the addition of DMSO or 0.2 μM PMA for 30 min. Lipids in the reaction mixture were extracted and separated. The radioactivities of PEt were counted. Values are means ± SD of 3–6 separate experiments. (*) $P < 0.01$, compared with the control value (second column). (C) Cells were preincubated with DMSO or 3–100 μM YC-1 in the presence of 5 μg/mL of dhCB for 3 min at 37° before addition of DMSO or 0.1 μM fMLP for 1 min. Membrane fractions were prepared and then subjected to immunoblot analysis using anti-ARF or anti-Rho A antibodies. Results are representative of 3 independent experiments with similar results.

of ethanol, phosphatidic acid yields phosphatidylethanol *via* a transphosphatidylation reaction. Addition of 1 μM fMLP for 0.5 min to 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine-loaded cells, pretreated with 5 μg/mL of dhCB, increased the formation of phosphatidic acid and phosphatidylethanol significantly. This effect was abolished by the general tyrosine kinase inhibitor genistein (100 μM) and attenuated by YC-1 in a concentration-dependent manner with IC_{50} values of 27.0 ± 9.2 and 28.9 ± 6.1 μM for phosphatidic acid and phosphatidylethanol, respectively (Fig. 4A). Inhibition by 50 μM YC-1 was partially reversed by 30 μM Rp-8-pCPT-cGMPS (from 89.4 ± 8.9 to 67.5 ± 5.7% inhibition of phosphatidylethanol). Cells treated with 0.2 μM PMA for 30 min in the presence of ethanol greatly increased the formation of phosphatidylethanol. GF109203X (10 μM), but not 50 μM YC-1, inhibited the PMA-induced response (Fig. 4B).

To determine the subcellular distribution of ARF and Rho A, immunoblot analysis was carried out. Only a small amount of ARF and Rho A was detected in the unstimulated cell membrane fraction. Both ARF and Rho A became significantly associated with the membrane fraction of neutrophils in response to 0.1 μM fMLP plus dhCB (5 μg/mL). These effects were similarly attenuated by YC-1 in a concentration-dependent manner with an IC_{50} value of about 30 μM in both cases (Fig. 4C).

3.6. Effects of YC-1 on PI3K activity and MAPK phosphorylation

Addition of 1 μM fMLP for 0.5 min to cells pretreated with 5 μg/mL of dhCB for 3 min and labeled with $^{32}P_i$, resulted in a 3-fold elevation of radioactivity in phosphatidylinositol trisphosphate ($11{,}683 \pm 2622$ for control vs. $35{,}419 \pm 7663$ counts, $P < 0.01$). Pretreatment of cells with 3 μM wortmannin, a PI3K inhibitor [29], abolished the fMLP-induced response ($12{,}344 \pm 2679$ counts, $P < 0.01$). However, YC-1 up to 50 μM did not affect the formation of phosphatidylinositol trisphosphate ($32{,}725 \pm 11{,}939$ counts, $P > 0.05$).

Stimulation of rat neutrophils with fMLP results in a rapid phosphorylation of ERK and p38 MAPK. YC-1 up to 30 μM had no effect on fMLP-induced ERK phosphorylation. A marked inhibition was observed only at a high

concentration of YC-1 (about 50% inhibition at 100 μM). A similar lack of inhibition by YC-1, up to 100 μM, was seen in fMLP-stimulated p38 MAPK phosphorylation (data not shown).

4. Discussion

YC-1 inhibited fMLP-induced $O_2^{\bullet -}$ generation in rat neutrophils in a concentration-dependent manner. The inhibition of O_2 consumption in intact cells together with the negligible effect on $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation in a cell-free system, precluded the $O_2^{\bullet -}$ scavenging activity of YC-1. The inhibition of $O_2^{\bullet -}$ generation by YC-1 was not due to cytotoxicity since cell viability was not changed after incubation with YC-1. PKC, which is directly activated by PMA, plays a role in the phosphorylation of $p47^{phox}$ and the assembly of an active NADPH oxidase complex [30]. The membrane fraction isolated from PMA-activated cells contained active NADPH oxidase, which produced $O_2^{\bullet -}$ in the presence of NADPH. Addition of arachidonic acid to the cytosolic and membrane fractions, which mimics the effect of phosphorylation of $p47^{phox}$ that occurs during cell activation, produces assembly and activation of NADPH oxidase [31]. Because YC-1 did not inhibit $O_2^{\bullet -}$ generation in the cell-free NADPH oxidase systems used in this study, it is plausible that inhibition of the respiratory burst by YC-1 occurs through an interaction with certain signal transduction steps that follow fMLP-receptor activation, not through direct suppression of NADPH oxidase.

YC-1, an sGC activator, increases cellular cyclic GMP levels in platelets and vascular smooth muscle [7,9]. Previous reports demonstrated that neutrophils possess sGC, cyclic GMP-hydrolyzing PDE, and cyclic GMP-dependent protein kinase [32–34]. However, the role of cyclic GMP in the neutrophil respiratory burst is not clear. It has been shown that 8-bromo-cyclic GMP (8-Br-cGMP), up to 1 mM, has little effect on $O_2^{\bullet -}$ generation induced by 10 nM fMLP in human neutrophils [12]. In contrast, N^2-2′-*O*-dibutyryl-cyclic GMP (Bt_2-cGMP; 1 mM) abolishes the 50 nM fMLP- but partially inhibits the 0.3 μM fMLP-induced $O_2^{\bullet -}$ generation in human neutrophils [11]. In the present study, we used 8-pCPT-cGMP, which is superior to 8-Br-cGMP, as the selective activator of cyclic GMP-dependent protein kinase [21]. 8-pCPT-cGMP inhibited fMLP-induced $O_2^{\bullet -}$ generation significantly in rat neutrophils. This effect was reversed by the cell membrane permeant cyclic GMP-dependent protein kinase antagonist Rp-8-pCPT-cGMPS. These results suggest that cyclic GMP has a negative regulatory effect on fMLP-induced $O_2^{\bullet -}$ generation. In addition, the inhibitory effect of YC-1 on fMLP-induced $O_2^{\bullet -}$ generation, being partially reversed by Rp-8-pCPT-cGMPS and LY83583, an agent that lowers cellular cyclic GMP [23], suggests the involvement of cyclic GMP.

We next determined the cyclic GMP levels in cells treated with YC-1. YC-1 (100 μM) increased cellular cyclic GMP levels and activated sGC of the cytosolic fraction about 9- and 10-fold, respectively, compared with basal levels. At the same concentration, YC-1 has been found to cause a 5-fold increase in cellular cyclic GMP levels and a 4-fold stimulation of cytosol sGC activity in human platelets [7]. The PDE isoenzymes in the cytosol of neutrophils show the predominant presence of PDE4 (cyclic AMP specific) and PDE5 (cyclic GMP specific) [35]. The cyclic GMP-hydrolyzing activity in the cytosolic fraction of rat neutrophils was inhibited by the PDE5 inhibitor zaprinast and the high concentration of YC-1, but not by the PDE4 inhibitor Ro 201724. These results suggest that the elevation of cellular cyclic GMP levels by YC-1 is attributable to the activation of sGC and, at higher concentrations of YC-1, to the inhibition of PDE5 in rat neutrophils and, therefore, are consistent with previous reports in human platelets and vascular smooth muscle [36,37].

The fMLP-induced respiratory burst is a Ca^{2+}-dependent process. The fMLP-induced elevation in $[Ca^{2+}]_i$ is comprised of an initial spike phase, supported primarily by the inositol trisphosphate-induced release of Ca^{2+} from intracellular stores, followed by a plateau phase, which is sustained by Ca^{2+} entry from the extracellular medium [38]. YC-1 reduced the plateau phase but not the initial spike phase in the presence of extracellular Ca^{2+}. Only a high concentration of YC-1 (50 μM) in the absence of extracellular Ca^{2+}reduced the Ca^{2+} spike. These results suggest that YC-1, at concentrations capable of inhibiting the respiratory burst, did not affect the phospholipase C signaling pathway, but inhibited extracellular Ca^{2+} entry through certain mechanisms. Inhibition of Ca^{2+} entry might play an important role in the inhibition of the respiratory burst since both effects occur with similar IC_{50} values of YC-1. It has been shown that both 8-Br-cGMP and Bt_2-cGMP have little effect on the fMLP-induced elevation of $[Ca^{2+}]_i$ [12,39]. Therefore, the reduction of $[Ca^{2+}]_i$ by YC-1 probably takes place through a cyclic GMP-independent mechanism. This notion is supported by our recent report [40].

It is generally accepted that the fMLP-induced activation of oxidase occurs mainly *via* a PKC-independent signaling pathway. PKC activity is found primarily in the cytosol of unstimulated cells [41], but becomes firmly associated with the membrane fraction after PMA treatment. It seems likely that YC-1 did not affect the PKC signaling pathway since neither the PMA-stimulated cytosolic PKC activity prepared from unstimulated cells nor the membrane-associated PKC activity isolated from PMA-activated cells was inhibited by YC-1.

The observations that YC-1 inhibited fMLP- but not PMA-induced PLD activation in rat neutrophils suggest that YC-1 did not suppress PLD activity directly, but probably indirectly through the blockade of certain signal

transduction processes. The latter possibility is supported by the fact that YC-1 prevented the membrane association of Arf and Rho A in response to fMLP, because translocation of ARF and Rho A to membrane fractions is required for effective activation of PLD [42]. In neutrophils, PLD activation by fMLP has been shown to be Ca^{2+}- and tyrosine kinase-dependent, but PKC-independent [43], whereas PMA-induced PLD activation was through PKC but not Ca^{2+}. Our findings that YC-1 inhibited $[Ca^{2+}]_i$ elevation but not PKC activity as described above are compatible with the inhibition of PLD activation. It has been reported that 8-Br-cGMP partially blunts thrombin-induced PLD activity in human platelets [44]. Therefore, inhibition of PLD activation by YC-1 probably occurs *via* both cyclic GMP-dependent and -independent mechanisms since Rp-8-pCPT-cGMPS partially reversed the inhibition (cyclic GMP-dependent), and inhibition involved the elevation of $[Ca^{2+}]_i$ (cyclic GMP-independent).

PI3K phosphorylates phosphatidylinositols at the D3 position; therefore it converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, which is a critical component of the signaling pathway leading to NADPH oxidase activation by fMLP [20]. Unlike the PI3K inhibitor wortmannin, YC-1 did not affect the fMLP-induced PI3K activation. Cell stimulation induces a signaling cascade that leads to the activation of MAPK *via* phosphorylation on both tyrosine and threonine residues [45]. YC-1 had no effect on fMLP-induced p38 MAPK phosphorylation and weakly attenuated ERK phosphorylation.

In conclusion, the elevation of cellular cyclic GMP levels by YC-1 in rat neutrophils resulted from the activation of sGC and the inhibition of PDE5. YC-1 inhibits the fMLP-induced respiratory burst *via* cyclic GMP-dependent and -independent mechanisms. The latter mechanism may involve the blockade of Ca^{2+} entry. The results exclude a role for PKC, PI3K, and MAPK in the inhibition by YC-1.

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