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Investigation of the cellular mechanism of inhibition of formyl-methionyl-leucyl-phenylalanine-induced superoxide anion generation in rat neutrophils by 2-benzyloxybenzaldehyde

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Abstract

The inhibition of formyl-methionyl-leucyl-phenylalanine (fMLP)-induced superoxide anion ($O_2^{\bullet -}$) generation by 2-benzyloxybenzaldehyde (CCY1a) was investigated in rat neutrophils, and the underlying mechanism of this inhibition was assessed. CCY1a concentration-dependently inhibited $O_2^{\bullet -}$ generation ($IC_{50} = 18.5 \pm 4.3$ μM). In cell-free systems, CCY1a failed to alter $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation, in phorbol 12-myristate 13-acetate (PMA)-activated neutrophil particulate NADPH oxidase preparations, or during arachidonic acid-induced NADPH oxidase activation. CCY1a increased cellular cyclic AMP (cAMP) levels in a time- and concentration-dependent manner, and this cAMP-elevating effect was inhibited by the adenylyl cyclase inhibitor 9-(tetrahydro-2′-furyl)adenine (SQ22536), adenosine deaminase (ADA), and the adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline. In neutrophils, inhibition of $O_2^{\bullet -}$ generation by CCY1a was partially reversed by the protein kinase A inhibitor (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT5720). CCY1a did not affect fMLP-induced p38 mitogen-activated protein kinase phosphorylation, but concentration-dependently attenuated the phosphorylation of extracellular signal-regulated kinase (ERK) and Akt (IC_{50} about 31.3 and 19.4 μM, respectively). The plateau phase, but not the initial spike, of fMLP-induced $[Ca^{2+}]_i$ changes was inhibited by CCY1a in a concentration-dependent manner. CCY1a inhibition of Ca^{2+} entry, ERK, and Akt phosphorylation was not prevented by SQ22536 or ADA. fMLP-induced phospholipase D (PLD) activation was inhibited by CCY1a ($IC_{50} = 13.9 \pm 2.0$ μM). ADA and KT5720 did not prevent the inhibition of PLD activation by CCY1a. Collectively, these results indicate that the inhibition by CCY1a of fMLP-induced $O_2^{\bullet -}$ generation in rat neutrophils can probably be attributed to the increase in cAMP levels, and to the blockade of Ca^{2+} entry, suppression of Akt, and PLD activation via cAMP-independent mechanisms.

Keywords: CCY1a; Rat neutrophil; Superoxide anion; cAMP; Cellular free Ca^{2+}; Phospholipase D

1. Introduction

Neutrophils are important participants in the host defense against microbial infection, and produce $O_2^{\bullet -}$, a precursor of other reactive oxygen species, upon activation of NADPH oxidase. Inherited deficiencies of this enzyme result in chronic granulomatous disease, characterized by enhanced susceptibility to microbial infection. However, under certain circumstances, the extensive or inappropriate release of toxic oxygen species can result in undesirable tissue damage. This is probably involved in the pathogenesis of many diseases [1]. Inhibition of the generation of $O_2^{\bullet -}$ from neutrophils has been proposed to ameliorate this tissue damage. The multicomponent NADPH oxidase consists of both membrane-bound cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$), which contains FAD and heme

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Abbreviations: AC, adenylyl cyclase; cAMP, cyclic AMP; CCY1a, 2-benzyloxybenzaldehyde; 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; PI3K, phosphatidylinositol 3-kinase; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase.

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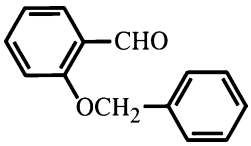

Fig. 1. Chemical structure of CCY1a.

redox centers, and cytosolic factors ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac) in the resting state. These cytosolic factors translocate and interact with cytochrome b_{558} to enter a functional state upon neutrophil activation [2]. Assembly of the oxidase component, probably through a conformational change, is essential for the activation of electron flow within cytochrome b_{558}. Thus, activated neutrophils evoke a respiratory burst in which the O_2 uptake from the extracellular medium is increased and large amounts of $O_2^{\bullet-}$ are generated.

The signaling mechanisms responsible for oxidase activation in neutrophils are complex and not clearly defined. Several signaling mechanisms, initiated by receptor–ligand interactions, have been reported to be involved in the regulation of NADPH oxidase. Stimulation of the G_i protein-coupled fMLP receptor leads to the activation of phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate and diacylglycerol, resulting in an increase in $[Ca^{2+}]_i$ and activation of protein kinase C, respectively [3]. These two second messengers act synergistically in $O_2^{\bullet-}$ generation. PLD and PI3K are activated by fMLP in neutrophils and appear to be functionally linked to $O_2^{\bullet-}$ generation [4,5]. Moreover, activation of MAPK by fMLP eventually leads to NADPH oxidase activation [6]. Increased cellular cAMP levels are also observed in fMLP-treated neutrophils. However, a constant high cAMP concentration has been shown to inhibit the production of oxygen radicals by activated neutrophils [7]. Therefore, a drug that interferes with these signaling pathways is expected to have an effect on $O_2^{\bullet-}$ generation in neutrophils.

In a preliminary study of the anti-inflammatory activities of synthetic benzyloxy derivatives, a novel compound, CCY1a (Fig. 1), was found to inhibit $O_2^{\bullet-}$ generation in neutrophils with cAMP-elevating activity [8]. The present study examined the possible cellular mechanism of inhibition of $O_2^{\bullet-}$ generation by CCY1a in rat neutrophils. The data provide evidence that the inhibition of fMLP-induced $O_2^{\bullet-}$ generation by CCY1a is mediated by cAMP-dependent and -independent mechanisms.

2. Materials and methods

2.1. Materials

CCY1a (purity > 99%) was synthesized by Chang *et al.* [8]. Dextran T-500, a cAMP enzyme immunoassay kit, enhanced chemiluminescence reagent, 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 was obtained from Research Biochemicals International Laboratories. 9-(Tetrahydro-2′-furyl)adenine (SQ22536), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), fluo-3 AM, and (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo-[3,4-l][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT5720) were purchased from the Calbiochem-Novabiochem Co. 1,4-Diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene (U0126) was obtained from the Promega Co. 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) was obtained from Biomol Research Laboratories. Rabbit polyclonal antibodies to phospho-p44/42 MAPK, phospho-p38 MAPK, p38 MAPK, and phospho-Akt(Ser^{473}) were purchased from New England Biolabs. Mouse monoclonal pan ERK antibody was purchased from BD Transduction Laboratories. Rabbit polyclonal antibodies to Akt1/2 were obtained from Santa Cruz Biotechnology. Polyvinylidene difluoride membranes were obtained from the Millipore Co. Other chemicals were purchased from the Sigma Chemical Co. The final percentage of DMSO in the reaction mixture was ≤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 [9]. The cell preparations consisting of >95% neutrophils (>95% viable cells as assessed by trypan blue exclusion) were resuspended in HBSS containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice bath until used.

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

The generation of $O_2^{\bullet-}$ in neutrophil suspensions was determined by the SOD-inhibitable reduction of ferricytochrome *c* [9]. For the determination of $O_2^{\bullet-}$ scavenging activity, $O_2^{\bullet-}$ generation in a cell-free system was assessed by measuring the reduction of nitroblue tetrazolium during dihydroxyfumaric acid (0.891 mM) autoxidation [10]. Absorbance changes were monitored continuously with a double-beam spectrophotometer at 550 and 560 nm to determine reductions of ferricytochrome *c* and nitroblue tetrazolium, respectively.

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

Neutrophils were treated with 2.5 mM diisopropyl fluorophosphate, then disrupted in Tris buffer by sonication, and

fractionated by centrifugation [11]. Supernatants were pooled as the cytosolic fractions. 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 the activity was determined as described previously [11]. 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 continuous detection of the absorbance changes of SOD-inhibitable ferricytochrome *c* reduction.

2.5. Determination of cellular cAMP levels

Neutrophils in HBSS were incubated with test drugs at 37° for 10 min, and then added to 0.05 M acetate buffer, pH 6.2, containing 50 μM 3-isobutyl-1-methylxanthine. After being boiled for 5 min, the suspension was sonicated, and then sedimented. Supernatants were acetylated by the addition of 0.025 vol. of triethylamine:acetic anhydride (2:1, v/v). The cAMP contents of the aliquots were assayed using an enzyme immunoassay kit.

2.6. Immunoblot analysis

Cells were preincubated with test drugs 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 (60 μg per lane) were resolved by SDS–PAGE and transferred to polyvinylidene difluoride membranes. 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, anti-phospho-p38 MAPK, or anti-phospho-Akt 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, anti-p38 MAPK, or anti-Akt1/2 antibody, and developed using an enhanced chemiluminescence reagent. Quantification was by densitometry.

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

Neutrophils were loaded with 5 μM fluo-3AM at 37° for 45 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/mL. Fluorescence was monitored with a fluorescence spectrophotometer at 535 nm with excitation at 488 nm. The $[Ca^{2+}]_i$ was calculated from the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where F is the observed fluorescence intensity [12]. 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. K_d was taken as 400 nM.

2.8. Measurement of PLD-mediated product

Neutrophils were loaded with 10 μCi 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine in HBSS at 37° for 75 min, then washed, and resuspended in HBSS to 5×10^7 cells/mL. Cells were incubated with test drugs in the presence of 1 mM $CaCl_2$ and 0.5% ethanol at 37° for 3 min before stimulation with fMLP. Lipids in the reaction mixture were extracted, dried, and separated on silica gel 60 [11]. The plates were developed halfway using the solvent 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]phosphatidylethanol were directly quantified with a PhosphorImager (Molecular Dynamics 445 SI) using ImageQuaNT software.

2.9. 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 calculate IC_{50} values. Data are expressed as means ± SD.

3. Results

3.1. Effect of CCY1a on $O_2^{\bullet -}$ generation

Addition of fMLP to the rat neutrophil suspension in the presence of dhCB rapidly evoked the transient generation of the $O_2^{\bullet -}$. This response was abolished by 1 μM trifluoperazine, an NADPH oxidase inhibitor [13]. CCY1a inhibited $O_2^{\bullet -}$ generation in a concentration-dependent manner with an IC_{50} value of 18.5 ± 4.3 μM (Fig. 2A). The viability was ≥96% when cells were incubated with 100 μM CCY1a at 37° for 10 min as assessed by trypan blue exclusion and lactate dehydrogenase release. To address the question of whether CCY1a acts as an $O_2^{\bullet -}$ scavenger, the effect of CCY1a on $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation in a cell-free system was examined. Unlike SOD (2.5 μg/mL), CCY1a (50 μM) showed no $O_2^{\bullet -}$ scavenging activity (0.059 ± 0.003 for

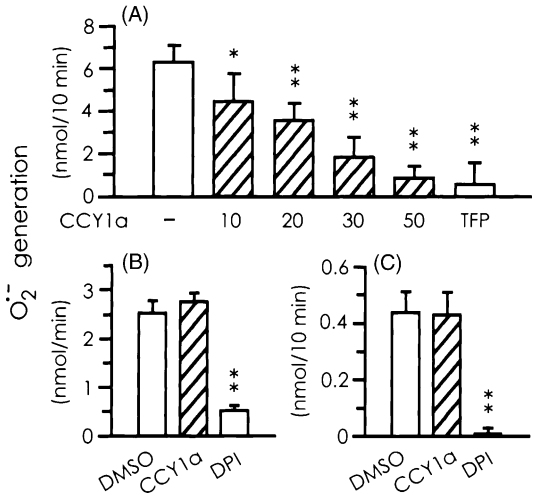

Fig. 2. Effect of CCY1a on $O_2^{\bullet-}$ generation in intact neutrophils and cell-free systems. (A) Cells (2×10^6) were preincubated with DMSO (as control), 10–50 μM CCY1a, or 1 μM trifluoperazine (TFP) at 37° for 3 min in the presence of dhCB (5 μg/mL) before stimulation with 0.3 μM fMLP. Values are means ± SD of 4–8 independent experiments. (B) A reaction mixture of neutrophil cytosolic and membrane fractions (1×10^7 cells) and (C) PMA-activated neutrophil particulate NADPH oxidase preparations (3×10^7 cells) were preincubated with DMSO (as control), 50 μM CCY1a, or 1 μM diphenylene iodonium (DPI) at 37° for 3 min, and then were supplemented with NADPH in the presence or absence of 100 μM arachidonic acid, respectively. Values are means ± SD of 4 independent experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$, compared with the corresponding control values (the first column of each panel).

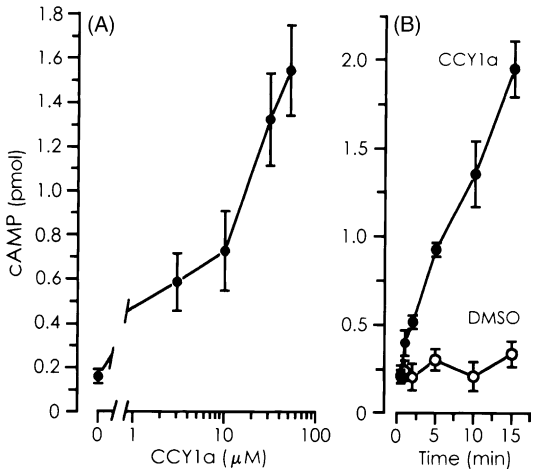

Fig. 3. Effect of CCY1a on cellular cAMP levels. (A) Cells (2×10^6) were incubated with DMSO or the indicated concentration of CCY1a for 10 min. (B) Cells (2×10^6) were incubated with DMSO or 30 μM CCY1a at 37° for the indicated time period. The cAMP contents were assayed using an enzyme immunoassay kit. Values are means ± SD of 3–5 independent experiments.

control vs. 0.004 ± 0.001 and 0.056 ± 0.003 ΔA_{560} for SOD and CCY1a, respectively). To determine whether CCY1a directly inhibits NADPH oxidase activity, experiments with NADPH oxidase in cell-free systems were performed. In the PMA-activated neutrophil particulate NADPH oxidase preparation, 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 then generation of $O_2^{\bullet-}$ in the presence of NADPH. Addition of the NADPH oxidase inhibitor diphenylene iodonium [14] greatly attenuated the $O_2^{\bullet-}$ generation in both systems. In contrast, CCY1a up to 50 μM had no effect on either system (Fig. 2B and C).

3.2. Effect of CCY1a on cellular cAMP levels and the role of cAMP in the inhibition of $O_2^{\bullet-}$ generation by CCY1a

Addition of CCY1a to a rat neutrophil suspension for 10 min elicited a concentration-dependent increase in cellular cAMP levels (up to 8-fold at 100 μM) (Fig. 3A). Pretreatment of cells with the AC inhibitor SQ22536 (30 μM), 0.4 unit/mL of adenosine deaminase, or 30 μM 8-(*p*-sulfophenyl)theophylline, an adenosine receptor antagonist [15], abolished the response caused by the addition of 30 μM CCY1a (data not shown). A time-dependent elevation of cellular cAMP levels was also observed in cells treated with 30 μM CCY1a (Fig. 3B). As described in a previous report [16], addition of prostaglandin E_1 (PGE_1) inhibited the fMLP-stimulated $O_2^{\bullet-}$ generation in rat neutrophils (6.1 ± 0.3 for control vs. 2.9 ± 0.3 nmol/10 min at 1 μM PGE_1, $P < 0.01$). Pretreatment of cells with 1 μM KT5720, a protein kinase A inhibitor [17], attenuated the inhibition of the fMLP-stimulated response by 1 μM PGE_1 or 30 μM CCY1a (6.2 ± 0.4 and 5.4 ± 0.3 nmol/10 min, respectively).

3.3. Effect of CCY1a on MAPK phosphorylation

Stimulation of rat neutrophils with fMLP results in a rapid phosphorylation of ERK (p44/42 MAPK) and p38 MAPK [18,19]. The phosphorylation of ERK in cells pretreated with CCY1a was inhibited in a concentration-dependent manner (IC_{50} about 31.3 μM) (Fig. 4A and C). The fMLP-induced response was abolished by 1 μM U0126, an MAPK kinase inhibitor [20]. Stripped membrane reprobing with anti-pan ERK indicated that the changes were not due to differences in protein loading. In contrast, there was a lack of inhibition of fMLP-stimulated p38 MAPK phosphorylation by CCY1a (up to 50 μM) (Fig. 4B and C). SB203580 (30 μM), a p38 MAPK inhibitor [21], attenuated the fMLP-induced response. Inhibition of ERK phosphorylation by CCY1a was not prevented by treatment with 30 μM SQ22536 or 0.4 unit/mL of adenosine deaminase (Fig. 4D).

3.4. Effect of CCY1a on $[Ca^{2+}]_i$

Addition of fMLP to fluo-3-loaded cells evoked an initial $[Ca^{2+}]_i$ spike, followed by a plateau phase, in the

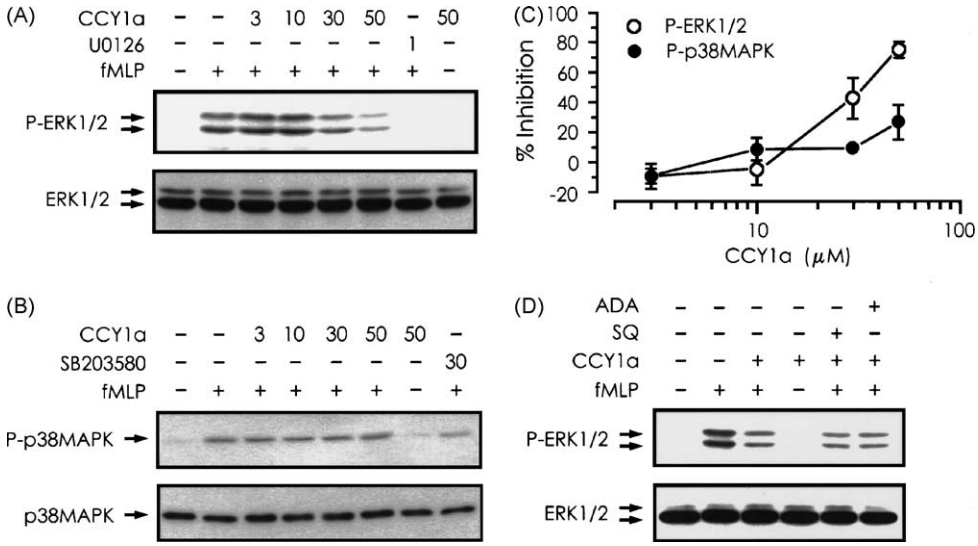

Fig. 4. Effect of CCY1a on fMLP-stimulated MAPK phosphorylation. Cells (1×10^7) were preincubated with DMSO, 1 μM U0126, or 30 μM SB203580 for 10 min, 3–50 μM CCY1a for 3 min, or 30 μM SQ22536 (SQ) or 0.4 unit/mL of adenosine deaminase (ADA) for 3 min prior to the addition of 50 μM CCY1a for another 3 min at 37° in the presence of dhCB (5 μg/mL) before stimulation with or without 0.3 μM fMLP for 1 min. Proteins were resolved by 10% SDS–PAGE. Phosphorylation of ERK (A and D) and p38 MAPK (B) was detected by immunoblot analysis using anti-phospho-p44/42 MAPK or anti-phospho-p38 MAPK antibodies, respectively. The blots above were then stripped and reprobed with anti-pan ERK and anti-p38 MAPK antibodies, respectively. The results presented are representative of 3 independent experiments with similar results, and (C) shows means ± SD of percent inhibition of MAPK phosphorylation.

presence of extracellular Ca^{2+}. The plateau phase was inhibited by CCY1a in a concentration-dependent manner (Fig. 5A). On the contrary, the initial $[Ca^{2+}]_i$ spike was unaffected by CCY1a in the same concentration range. The inhibition of $[Ca^{2+}]_i$ by CCY1a was not reversed in the presence of 30 μM SQ22536 or 0.4 unit/mL of adenosine deaminase (Fig. 5B).

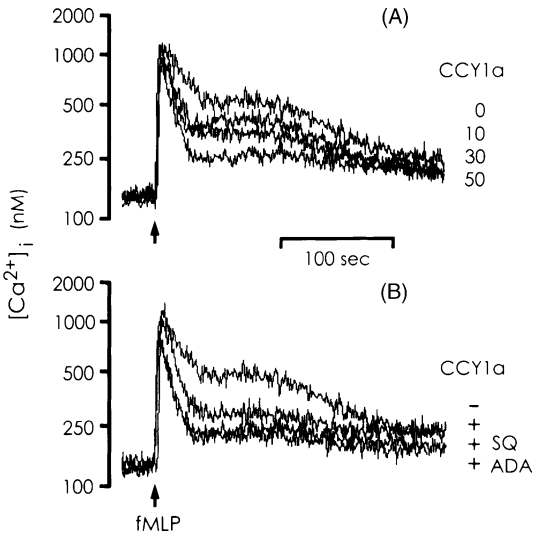

Fig. 5. Effect of CCY1a on $[Ca^{2+}]_i$. Fluo-3-loaded cells, in 1 mM Ca^{2+}-containing HBSS, were preincubated with (A) DMSO or 10–50 μM CCY1a for 10 min, or (B) DMSO, 30 μM SQ22536 (SQ) or 0.4 unit/mL of adenosine deaminase (ADA) at 37° for 3 min, before the addition of DMSO or 50 μM CCY1a for another 10 min. Then the cells were stimulated (arrow) with 0.3 μM fMLP. The results presented are representative of 3 independent experiments with similar results.

3.5. Effect of CCY1a on Akt phosphorylation

fMLP-stimulated optimal Akt phosphorylation in rat neutrophils at 1–2 min (data not shown). To investigate the effect of CCY1a on PI3K-dependent Akt activation, we measured fMLP-stimulated Akt phosphorylation in the presence or absence of CCY1a or LY294002, a selective PI3K inhibitor [22]. Fig. 6 shows that 3 μM LY294002 abolished the fMLP-stimulated Akt phosphorylation, and CCY1a concentration-dependently attenuated the fMLP-induced response (IC_{50} about 19.4 μM). CCY1a inhibition of Akt phosphorylation was not prevented by treatment with 30 μM SQ22536 or 0.4 unit/mL of adenosine deaminase (Fig. 6C).

3.6. Effect of CCY1a on PLD activation

PLD catalyzes the hydrolysis primarily of phosphatidylcholine to produce phosphatidic acid [23]. In the presence of ethanol, phosphatidic acid yields phosphatidylethanol via a transphosphatidylation reaction. Activation of 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine-loaded cells with fMLP rapidly increased the cellular PLD activity as assessed by the formation of phosphatidylethanol in the presence of ethanol. This effect was attenuated by CCY1a in a concentration-dependent manner (Fig. 7), with an IC_{50} value of 13.9 ± 2.0 μM, and abolished by the general tyrosine kinase inhibitor genistein (100 μM). The finding that genistein inhibits PLD activation is consistent with that of a previous report [24]. Inhibition of PLD activation by 50 μM CCY1a was not reversed in the presence of 0.4 unit/mL of adenosine deaminase or 1 μM KT5720.

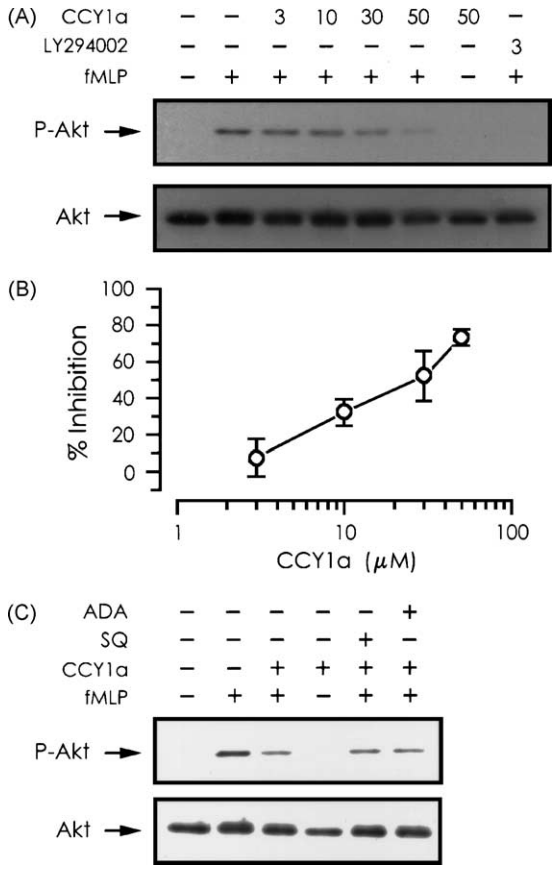

Fig. 6. Effect of CCY1a on fMLP-stimulated Akt phosphorylation. Cells (5×10^7) were preincubated with DMSO, 3 μM LY294002 or 3-50 μM CCY1a for 3 min, or 30 μM SQ22536 (SQ) or 0.4 unit/mL of adenosine deaminase (ADA) for 3 min prior to the addition of 50 μM CCY1a for another 3 min at 37° in the presence of dhCB (5 μg/mL) before stimulation with or without 0.3 μM fMLP for 1 min. Proteins were resolved by 7.5% SDS–PAGE. Phosphorylation of Akt was detected by immunoblot analysis using anti-phospho-Akt antibody. The blots above were then stripped and reprobed with anti-Akt1/2 antibody. Panels A and C represent the results of western blot analysis, and panel B shows the means ± SD of percent inhibition of Akt phosphorylation from 3 independent experiments.

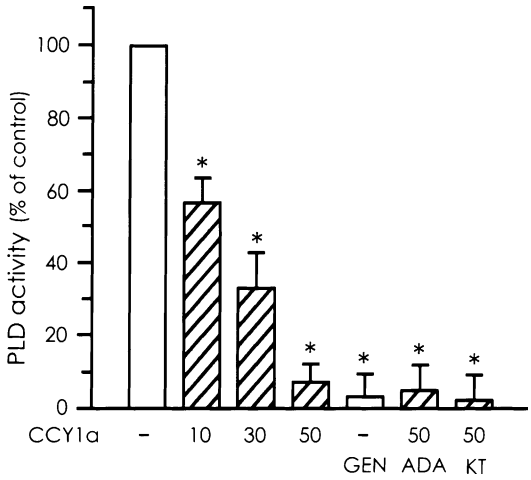

Fig. 7. Effect of CCY1a on PLD activation. 1-*O*-[^{3}H]Octadecyl-*sn*-glycero-3-phosphocholine-loaded cells were preincubated with DMSO, 10–50 μM CCY1a, or 100 μM genistein (GEN) at 37° for 3 min. In some experiments, cells were pretreated with 0.4 unit/mL of adenosine deaminase (ADA) or 1 μM KT5720 (KT) at 37° for 3 min before the addition of 50 μM CCY1a for another 3 min in the presence of dhCB (5 μg/mL). After stimulation with 1 μM fMLP for 0.5 min, lipids in the reaction mixture were extracted and separated. The radioactivities of phosphatidylethanol were counted with a PhosphorImager. Values are means ± SD of 3–5 independent experiments. Key: (*) $P < 0.01$, compared with the control value (326 ± 39 dpm, first column).

4. Discussion

CCY1a inhibition of fMLP-induced $O_2^{\bullet -}$ generation in rat neutrophils was assessed by determining the SOD-inhibitable reduction of ferricytochrome *c*. However, CCY1a had a negligible effect on $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation in a cell-free system, precluding $O_2^{\bullet -}$ scavenging activity of CCY1a. It is unlikely that the inhibition of $O_2^{\bullet -}$ generation by CCY1a was caused by a cytotoxic effect since cell viability was not changed during the reaction. Protein kinase C, which is directly activated by PMA, plays a role in the phosphorylation of oxidase components and the assembly of an active NADPH oxidase complex [25]. 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 mimics the effect of phosphorylation of p47^{phox} upon cell activation, and leads to assembly and activation of NADPH oxidase [26]. CCY1a failed to alter $O_2^{\bullet -}$ generation in both cell-free oxidase systems, excluding the possibility of direct suppression of NADPH oxidase. It is plausible that the inhibition of $O_2^{\bullet -}$ generation by CCY1a is caused by the interaction of certain signal transduction steps that follow fMLP-receptor activation.

In general, increased cellular cAMP levels in neutrophils are associated with a decrease in several neutrophil functions including $O_2^{\bullet -}$ generation [7]. Synthesis of cAMP from ATP is via the activation of AC. In the present study, CCY1a increased cellular cAMP levels in rat neutrophils in a time- and concentration-dependent manner. The cAMP-elevating effect of CCY1a was reversed by the AC inhibitor SQ22536, which interacts with the enzyme catalytic subunit. The ability of CCY1a to increase intracellular cAMP was eliminated by adenosine deaminase, an enzyme that hydrolyses adenosine to inosine, and 8-(*p*-sulfophenyl)-theophylline, an adenosine receptor antagonist [15]. It has been reported that adenosine acts via the A_2 adenosine receptor, which activates AC, on neutrophil membranes [7] and that the release of endogenous adenosine involves a transient increase in cAMP levels in fMLP-stimulated human neutrophils [27]. Moreover, our previous report showed that CCY1a has no effect on phosphodiesterase activity in neutrophil cytosolic fractions [8]. Taken together, these results suggest that occupancy of the adenosine

receptor by the released endogenous adenosine mediates the ability of CCY1a to stimulate AC activation.

We next determined the role of cAMP in the CCY1a-induced inhibition of $O_2^{\bullet-}$ generation. PGE_1 has been shown to elevate intracellular cAMP levels by the activation of AC through the G_s protein-coupled EP_2 receptor [28]. cAMP acts via protein kinase A in many cellular systems. Protein kinase A inhibitors antagonize the inhibition of $O_2^{\bullet-}$ generation by cAMP-elevating agents [29]. In fMLP-stimulated neutrophils, inhibition of $O_2^{\bullet-}$ generation by CCY1a and PGE_1 was reversed by pretreatment with the protein kinase A inhibitor KT5720 [17], suggesting the involvement of cAMP. However, the implication of other signaling pathways in the inhibition of $O_2^{\bullet-}$ generation by CCY1a cannot be excluded because of the lack of complete effectiveness of KT5720 in reversing the CCY1a-induced inhibition of $O_2^{\bullet-}$ generation.

Three distinct mammalian MAPKs have been identified: ERK (p44/42 MAPK), p38 MAPK, and c-Jun N-terminal kinase, each with different physiological roles. It has been reported that both ERK and p38 MAPK phosphorylate $p47^{phox}$ *in vitro* [30]. Cell stimulation induces a signaling cascade that leads to the activation of MAPK via the phosphorylation of both tyrosine and threonine residues [31]. CCY1a attenuated ERK phosphorylation with an IC_{50} value higher than that required for the inhibition of $O_2^{\bullet-}$ generation. In addition, CCY1a had no effect on fMLP-induced p38 MAPK phosphorylation. Therefore, a role for MAPK can be excluded.

fMLP-induced $O_2^{\bullet-}$ generation is a Ca^{2+}-dependent process. In the presence of extracellular Ca^{2+}, fMLP-induced $[Ca^{2+}]_i$ elevation is composed of an initial spike, supported primarily by an inositol trisphosphate-induced release of Ca^{2+} from intracellular stores, followed by a plateau phase, sustained by Ca^{2+} entry from the extracellular medium [32]. CCY1a reduced the plateau but not the initial spike phase, indicating that CCY1a does not affect the phospholipase C signaling pathway, but inhibits extracellular Ca^{2+} entry. It has been shown that cAMP-elevating agents inhibit Ca^{2+} entry [33], probably by closing the Ca^{2+} channel. In the present study, pretreatment of cells with SQ22536 and adenosine deaminase, at concentrations that abolished CCY1a-induced intracellular cAMP elevation, did not reverse CCY1a-induced inhibition of $[Ca^{2+}]_i$, suggesting a cAMP-independent mechanism. Protein kinase C plays an important role in the activation of NADPH oxidase. As described above, it is unlikely that CCY1a affects protein kinase C activation through the phospholipase C signaling pathway. However, the possibility of the direct inhibition of kinase activity by CCY1a awaits further investigation.

Parallel to the activation of phospholipase C by fMLP, a Ca^{2+}-independent pathway leads to the activation of PI3K, which is also required for the respiratory burst of neutrophils [5]. Activation of the serine/threonine kinase Akt, the major target of PI3K-dependent signaling, correlates with the well-documented kinetics of the respiratory burst in human neutrophils [34]. Activation of Akt was demonstrated to associate with the phosphorylation on Ser^{473} and Thr^{308} [35]. CCY1a inhibited Akt phosphorylation with an IC_{50} value similar to that required to inhibit the fMLP-induced respiratory burst, suggesting the involvement of a PI3K/Akt signaling pathway. A recent report has demonstrated that the cAMP-dependent signaling pathway inhibits Akt activity by blocking the coupling between Akt and its upstream regulators in the plasma membrane [36]. However, SQ22536 and adenosine deaminase at concentrations that abolished the cAMP elevating effect of CCY1a failed to prevent the CCY1a inhibition of Akt phosphorylation, implying a cAMP-independent mechanism.

Two mammalian PLD isoenzymes, PLD1 (PLD1a and PLD1b) and PLD2, have been cloned and the splice variants have been described [37]. PLD1 is under elaborate control, whereas PLD2 exhibits high basal activity and is less subject to control. PLD1b is the predominant PLD isoform expressed in rat tissue [38]. The PLD signaling pathway regulates neutrophil function through phosphatidic acid (PA), which activates a novel protein kinase that induces $p47^{phox}$ phosphorylation and NADPH oxidase activation [39]. CCY1a concentration-dependently inhibited fMLP-induced PLD activation in rat neutrophils. Inhibition of PLD may play an important role in the inhibition of $O_2^{\bullet-}$ generation, because both occur at similar IC_{50} values of CCY1a. It has been reported that cAMP may regulate the PLD signaling pathway, probably at a site proximal to PLD [40]. The failure of adenosine deaminase and KT5720 to prevent the CCY1a-induced inhibition of PLD activation suggests the involvement of a cAMP-independent mechanism. In neutrophils, PLD activation by fMLP has been shown to be Ca^{2+}- and tyrosine kinase-dependent [41]. In addition, the translocation of two small G proteins, ADP-ribosylation factor and Rho A, to membrane fractions is required for an effective activation of PLD [42]. The CCY1a-induced inhibition of $[Ca^{2+}]_i$ as described above is probably related to the inhibition of PLD activation. Further studies are needed to determine whether CCY1a affects protein tyrosine phosphorylation and the membrane translocation of ADP-ribosylation and Rho A.

In conclusion, CCY1a inhibits fMLP-stimulated $O_2^{\bullet-}$ generation in rat neutrophils. This effect is attributed to: (i) the elevation of cellular cAMP through the activation of membrane adenosine receptors following the release of endogenous adenosine from neutrophils; (ii) the inhibition of extracellular Ca^{2+} entry; and (iii) suppression of Akt and PLD activation via cAMP-independent mechanisms.

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