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Involvement of Cyclic AMP Generation in the Inhibition of Respiratory Burst by 2-Phenyl-4-quinolone (YT-1) in Rat Neutrophils

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ABSTRACT. The inhibitory effect of 2-phenyl-4-quinolone (YT-1) on respiratory burst in rat neutrophils was investigated, and the underlying mechanism of action was assessed. YT-1 caused a concentration-dependent inhibition of the rate of $O_2^{\cdot-}$ release from rat neutrophils in response to formylmethionyl-leucyl-phenylalanine (fMLP), but not to phorbol 12-myristate 13-acetate (PMA), with an IC_{50} value of 60.7 ± 8.2 μM. A comparable effect was also demonstrated in the inhibition of O_2 consumption. Unlike superoxide dismutase, YT-1 had no effect on $O_2^{\cdot-}$ generation in the xanthine-xanthine oxidase system and during dihydroxyfumaric acid autoxidation. The fMLP-induced inositol trisphosphate (IP_3) formation was unaffected by YT-1. In addition, YT-1 did not affect the initial spike of $[Ca^{2+}]_i$, but it accelerated the rate of $[Ca^{2+}]_i$ decline in cells in response to fMLP. YT-1 was found to have little effect on the activity of neutrophil cytosolic protein kinase C (PKC). YT-1 increased the cellular cyclic AMP level, while having no effect on the cyclic GMP level. In addition, YT-1 increased neutrophil cytosolic protein kinase A (PKA) activity, but had no direct effect on the enzyme activity of pure porcine heart PKA. When neutrophils were treated with (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta-[*cde*]trinden-1-one, (KT 5720), a PKA inhibitor, the inhibition of $O_2^{\cdot-}$ generation by YT-1, as well as by prostaglandin E_1 (PGE_1) and dibutyryl cyclic AMP, was attenuated effectively. YT-1 did not activate the adenylate cyclase associated with neutrophil particulate fraction but inhibited the cytosolic phosphodiesterase (PDE) activity in a concentration-dependent manner. Neutrophils treated with YT-1 had a more pronounced increase in cellular cyclic AMP level by PGE_1. Moreover, the ability of PGE_1 to inhibit the respiratory burst in neutrophils was greatly enhanced by YT-1. These results suggest that the increase in cellular cyclic AMP levels by YT-1 through the inhibition of PDE (probably PDE4 isoenzyme) activity is involved in its inhibition of fMLP-induced respiratory burst in rat neutrophils. BIOCHEM PHARMACOL **56**;11:1505–1514, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. YT-1; neutrophil; respiratory burst; cyclic AMP; adenylate cyclase; phosphodiesterase

During ingestion of microbes or upon stimulation with various soluble molecules, neutrophils produce $O_2^{\cdot-}$. The ability of neutrophils to consume O_2 and generate $O_2^{\cdot-}$, which subsequently leads to the formation of other toxic O_2 metabolites, is of critical importance for host defense [1]. Production of these reactive O_2 species during the respiratory burst process involves activation of NADPH oxidase, dormant in resting cells, which catalyzes the reduction of O_2 to $O_2^{\cdot-}$ in conjunction with the oxidation of NADPH [2]. The active NADPH oxidase is found on the neutrophil membranes as an enzyme complex consisting of both membrane (cytochrome b_{558}) and cytosolic (mainly $p47^{phox}$, $p67^{phox}$, and Rac) components [2]. The heterodimeric cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$) contains all of the redox components (flavin and heme) of the oxidase and is the key catalytic component responsible for the transfer of electrons from NADPH to O_2. The function of the cytosolic components of oxidase in regulating electron flow from NADPH to O_2 is poorly understood. It has been proposed that $p67^{phox}$ regulates the transfer of electrons from NADPH for the reduction of flavin, while $p47^{phox}$ controls electron flow between the flavin and heme [3].

The significance of the phagocyte NADPH oxidase in host defense is made evident by the susceptibility of patients with chronic granulomatous disease to develop recurrent bacterial infections [4]. However, the generation of excess reactive O_2 species by uncontrolled neutrophils may deleteriously affect adjacent cells or structural matrix components of tissue. This is probably involved in the pathogenesis of many diseases [5]. Therefore, one approach

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for the treatment of these diseases is to develop agents that would prevent the generation of toxic oxygen radicals from these cells.

The mechanism for the activation of phagocytic NADPH oxidase has not been fully elucidated. It has been proposed that the stimulation of neutrophils by receptor-binding ligands results in an intracellular signalling cascade, including the activation of phospholipase C, which releases IP_3* and diacylglycerol, which, in turn, increase intracellular Ca^{2+} concentration and activate PKC, respectively [6]. The two pathways function synergistically for $O_2^{\cdot-}$ generation. Activations of phospholipase D, mitogen-activated protein kinase, phosphoinositide 3-kinase, and probably phospholipase A_2 are also functionally linked to $O_2^{\cdot-}$ generation [7–10]. Upon neutrophil activation, the cytosolic components of oxidase translocate and associate with the membrane components, forming a functional NADPH oxidase complex responsible for the production of $O_2^{\cdot-}$ through a univalent reduction of O_2 [2].

2-Phenyl-4-quinolone (YT-1) has been found to possess cytotoxicity against several human cancer cell lines [11], to exert a positive inotropic effect in both atrial and ventricular muscle [12], to inhibit the release of mediators from rat peritoneal mast cells *in vitro*, and to suppress the mouse cutaneous plasma extravasation caused by inflammatory mediators *in vivo* [13]. YT-1 was found to inhibit neutrophil $O_2^{\cdot-}$ generation in our recent preliminary study. In the present study, we examined the inhibitory effect of YT-1 on the respiratory burst in rat peripheral neutrophils and investigated the underlying mechanisms.

MATERIALS AND METHODS

Reagents

YT-1 was synthesized as previously described [11]. All chemicals were purchased from the Sigma Chemical Co. except for the following: dextran T-500 (Pharmacia Biotech Ltd.); Hanks' balanced salt solution and PKA assay kit (Life Technologies Gibco BRL Co.); U73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), Ro 20-1724 (4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone, and KT 5720 (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one (Biomol Res. Lab. Inc.); fluo 3-AM (Molecular Probes Inc.); *myo*-[^{3}H]inositol, [γ-^{32}P]ATP, [^{3}H]cyclic AMP, cyclic AMP, and cyclic GMP enzyme immunoassay kits, and a PKC assay kit (Amersham International plc.); M&B 22948 (2-*O*-propoxyphenyl-8-azapurin-6-one) (Rhône–Poulenc Rorer Ltd.); AG 1-X8 resin (formate) (Bio-Rad Laboratories); and DE-52 cellulose (Whatman Pte Ltd.).

* *Abbreviations:* CB, dihydrocytochalasin B; dcAMP, dibutyryl cyclic AMP; fMLP, formylmethionyl-leucyl-phenylalanine; IBMX, 3-isobutyl-1-methylxanthine; IP_3, inositol trisphosphate; PDE, phosphodiesterase; PMA, phorbol 12-myristate 13-acetate; PKA, protein kinase A; and PKC, protein kinase C.

Isolation of Neutrophils

Rat blood was collected from the abdominal aorta, and the neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation through Ficoll–Hypaque [14]. Purified neutrophils containing >95% viable cells were normally resuspended in Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$ (HBSS), and kept on ice before use.

Measurement of $O_2^{\cdot-}$ Generation and O_2 Consumption

$O_2^{\cdot-}$ generation in neutrophil suspension or in a xanthine-xanthine oxidase system was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome *c*, as described previously [14]. Briefly, assay mixtures contained 10^6 cells and 0.5 mg/mL of ferricytochrome *c* in a final volume of 1.5 mL. In the xanthine-xanthine oxidase system, reaction was started by the addition of 0.15 mM xanthine to the reaction mixture that contained 2.5 mU/mL of xanthine oxidase and 0.5 mg/mL of ferricytochrome *c*. The reference cuvette also received 6.6 μg/mL of superoxide dismutase. The $O_2^{\cdot-}$ generation during dihydroxyfumaric acid autoxidation was determined in a reaction mixture that contained 0.89 mM dihydroxyfumaric acid, 0.27 mM nitroblue tetrazolium chloride, and drugs at 37° as previously described [15]. Absorbance changes of the reduction of ferricytochrome *c* and nitroblue tetrazolium chloride were monitored continuously at 550 and 560 nm, respectively, in a double-beam spectrophotometer. Whole cell O_2 consumption was measured continuously with a Clark-type oxygen electrode connected to a YSI biological oxygen monitor [16].

Determination of Inositol Phosphate Levels

Neutrophils (3×10^7 cells/mL) were loaded with *myo*-[^{3}H]inositol (83 Ci/mmol) at 37° for 2 hr [17]. Ten seconds after the stimulation with fMLP, reactions were stopped by adding a $CHCl_3$:CH_3OH (1:1, v/v) mixture and 2.4 M HCl. The aqueous phase was removed and neutralized by 0.4 M NaOH, and then applied to an AG 1-X8 resin (formate) column. IP, IP_2 and IP_3 were eluted sequentially by using 0.2, 0.4, and 1.0 M ammonium formate, respectively, in 0.1 M formic acid as eluents, and then counted as previously described [17].

Measurement of $[Ca^{2+}]_i$

Neutrophils (1×10^7 cells/mL) were suspended in HEPES buffer A (124 mM NaCl, 4 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 15.2 mM $NaHCO_3$, 5.56 mM dextrose, and 10 mM HEPES, pH 7.4) and loaded with 5 μM fluo 3-AM at 37° for 45 min. After washing, cells were resuspended in HEPES buffer A with 0.05% (w/v) BSA. The fluorescence was monitored by a fluorescence spectrophotometer (PTI, Deltascan 4000) at 525 nm with excitation

488 nm. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d \cdot [(F - F_{min})/(F_{max} - F)]$, where F is the observed fluorescence intensity [18]. The F_{max} and F_{min} values were obtained at the end of each experiment by sequentially adding 0.33% Triton X-100 and 50 mM EGTA. The K_d was taken as 400 nM [19].

Measurement of PKC Activity

For the preparation of cytosolic PKC, neutrophils were disrupted in Tris buffer A [50 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 10 mM EGTA, 0.01% (w/v) leupeptin and 10 mM benzamidine] by sonication. After centrifugation, the supernatant was subjected to a DE-52 cellulose column to obtain partially purified PKC [14]. The enzyme activity of neutrophil cytosolic PKC was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate by use of a PKC assay kit, based on the mixed micelle method [20]. Briefly, the reaction mixture contained 1 mM $CaCl_2$, 15 mM magnesium acetate, 2.5 mM dithiothreitol, 6 mM phosphatidylserine, 2 μg/mL of PMA, 50 μM ATP (0.2 μCi [γ-^{32}P]ATP/tube), 75 μM PKC substrate and PKC sample in 50 mM Tris–HCl buffer, pH 7.5. After the addition of stop reagent, a sample of the mixture was spotted onto the phosphocellulose disc. Phosphorylated substrate, which bound to binding paper, was washed and then counted.

Determination of Cellular Cyclic AMP and Cyclic GMP Levels

Cyclic AMP and cyclic GMP contents were determined as previously described [21] with modifications. Neutrophils in HBSS were incubated with test drugs for 10 min at 37°, and then added to 0.05 M acetate buffer, pH 6.2, containing 0.05 mM IBMX for cyclic AMP assay or 0.05 mM M&B 22948 for cyclic GMP assay. 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 cyclic AMP and cyclic GMP contents of the aliquots were assayed by using enzyme immunoassay kits.

Measurement of PKA Activity

Neutrophils (1×10^7 cells/mL, at 37°) in HEPES buffer A were preincubated with test drug for 10 min, washed twice, and then sonicated in Tris buffer A. After centrifugation at 8000 *g* for 10 min at 4°, supernatant was pooled as the PKA source. PKA activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into kemptide by using the PKA assay kit, based on the method described previously [22]. The assay mixture contained 10 mM $MgCl_2$, 100 μM ATP (0.3 μCi [γ-^{32}P]ATP/tube), 0.25 mg/mL of BSA, 50 μM kemptide, and PKA sample in 50 mM Tris–HCl buffer, pH 7.5, for 5 min at 30°. Phosphorylated substrate, which bound to phosphocellulose paper, was washed and counted. Total PKA activity of the control neutrophil supernatant and porcine heart PKA activity were determined in the presence of 10 μM cyclic AMP. In some experiments, porcine heart PKA activity was determined in the assay mixture without the addition of cyclic AMP.

Adenylate Cyclase Activity

Neutrophils (5×10^7 cells/mL) were sonicated in Tris buffer B (25 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and pepstatin) with 5 mM dithiothreitol, and then sedimented to pool membrane pellets as the adenylate cyclase source [23]. Adenylate cyclase activity was assayed by measuring the production of cyclic AMP during the hydrolysis of ATP, based on the method described previously [24]. Briefly, the neutrophil particulate fraction was incubated with drugs at 37° for 10 min in the presence of 10 mM $MgCl_2$, 7.5 mM creatine phosphate, 30 U/mL of creatine phosphokinase, 1 mM dithiothreitol, and 0.2 mM ATP. Reaction was terminated by boiling for 3 min. The cyclic AMP contents of the aliquots were assayed by using enzyme immunoassay kits.

Measurement of PDE Activity

Neutrophils (5×10^7 cells/mL) were sonicated in Tris buffer B with 5 mM $MgCl_2$ and 2 mM EDTA, and then sedimented to pool the supernatant fraction as the PDE source [25]. PDE activity was determined as described previously [26] with modifications. The assay mixture contained 40 mM Tris–HCl buffer, pH 8.0, 5 mM $MgCl_2$, 1 μM cyclic AMP (0.05 μCi [^3H]cyclic AMP/tube), 0.1 mg/mL of BSA, and PDE sample (~100 μg protein) in a final volume of 0.2 mL for 30 min at 37°. Reaction was stopped by the addition of 50 μL of 0.2 M HCl. After cooling, 50 μL of *Crotalus atrox* snake venom (1 mg/mL in 0.2 M Tris–HCl, pH 8.0) was added to the reaction mixture and incubated at 37° for another 15 min, and then subjected to an AG 1-X8 resin (formate) column. Nucleoside product was eluted by using 30 mM ammonium formate adjusted to pH 6.0 with formic acid as eluent, and then counted.

Statistical Analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. A P value of less than 0.05 was considered significant for all tests. Analysis of the regression line was used to calculate the IC_{50} values. Data are expressed as means ± SEM.

RESULTS

Effect of YT-1 on Respiratory Burst

Rat neutrophils release $O_2^{\cdot-}$ at a rate of 26.8 ± 1.1 and 5.2 ± 0.3 nmol/10 min per 10^6 cells when exposed to 3 nM PMA and 0.3 μM fMLP plus 5 μg/mL of CB, respectively.

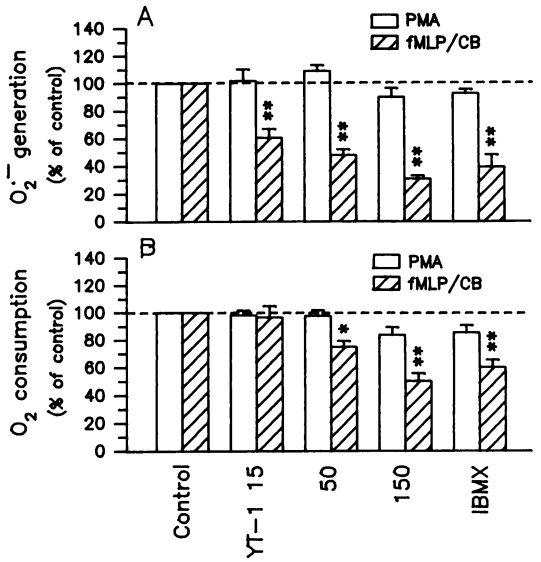

FIG. 1. Effect of YT-1 on $O_2^{\cdot-}$ generation and O_2 consumption in PMA- and fMLP-activated neutrophils. The conditions of the assay are described in Materials and Methods. Neutrophils were preincubated with DMSO (as control), 15–150 μM YT-1, or 300 μM IBMX for 3 min. (A) $O_2^{\cdot-}$ generation in cells stimulated with 3 nM PMA or 0.3 μM fMLP plus 5 μg/mL of CB and (B) O_2 consumption in cells stimulated with 10 nM PMA or 0.1 μM fMLP plus 5 μg/mL of CB are expressed as a percentage of the control value (26.8 ± 1.1 and 5.2 ± 0.3 nmol/10 min, respectively, for $O_2^{\cdot-}$ generation, and 53.3 ± 2.8 and 20.7 ± 1.0 nmol/5 min, respectively, for O_2 consumption). Values are means ± SEM of 5–6 separate experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the corresponding control values.

YT-1 inhibited $O_2^{\cdot-}$ generation in response to fMLP/CB, but not to PMA, in a concentration-dependent manner with an IC_{50} value of 60.7 ± 8.2 μM (Fig. 1A). Upon exposure to 10 nM PMA or 0.1 μM fMLP plus 5 μg/mL of CB, the rate of O_2 utilization by neutrophils was 53.3 ± 2.8 and 20.7 ± 1.0 nmol/5 min per 2×10^6 cells, respectively. YT-1 caused a concentration-dependent inhibition of O_2 consumption in response to fMLP/CB, but not to PMA (Fig. 1B). These inhibitory profiles are consistent with those produced by a nonselective PDE inhibitor, IBMX (300 μM). More than 95% viability was observed with trypan blue exclusion in cells treated with 150 μM YT-1 for 10 min. Unlike superoxide dismutase (3 μg/mL), which greatly reduced the rate of $O_2^{\cdot-}$ generation in the xanthine-xanthine oxidase system and during dihydroxyfumaric acid autoxidation (1.2 ± 0.2 vs 4.1 ± 0.2 nmol $O_2^{\cdot-}$/10 min and 0.011 ± 0.002 vs 0.084 ± 0.006 ΔA_{560}, respectively, both $P < 0.01$), YT-1 (up to 150 μM) had no significant effect on either system (4.2 ± 0.2 nmol $O_2^{\cdot-}$/10 min and 0.083 ± 0.004 ΔA_{560}, respectively).

Effect of YT-1 on IP_3 Formation and $[Ca^{2+}]_i$

Addition of fMLP to the *myo*-[^{3}H]inositol-loaded neutrophils resulted in a significant increase in IP_3 formation ($P < 0.01$). Pretreatment with U73122, a phospholipase C inhibitor [27], greatly reduced the IP_3 formation by fMLP, while YT-1 (up to 150 μM) had no effect on fMLP-induced response (Fig. 2). To test the $[Ca^{2+}]_i$ of neutrophils, addition of fMLP to the fluo 3-loaded cells evoked an initial spike, followed by a plateau phase of $[Ca^{2+}]_i$ changes in the presence of extracellular Ca^{2+}. However, the fMLP-evoked initial spike and plateau phase were reduced and abolished, respectively, if the extracellular Ca^{2+} was removed by EDTA. Pretreatment of cells with U73122 abolished the fMLP-induced $[Ca^{2+}]_i$ elevation whether the extracellular Ca^{2+} was present or not, whereas YT-1 (at 150 μM) only attenuated the plateau phase but left alone the initial spike (Fig. 3, A and B).

Effect of YT-1 on PKC Activity

In the presence of $CaCl_2$, phosphatidylserine, and PMA, the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate was demonstrated in neutrophil cytosolic PKC preparations. PKC activity was greatly inhibited by staurosporine, a protein kinase inhibitor [28], while only weakly suppressed by YT-1 (18.2 ± 4.2% inhibition at 150 μM) (Fig. 4). In the presence of EDTA, Ca^{2+}-independent PKC activity was also demonstrated in neutrophil cytosolic PKC preparations.

Effect of YT-1 on Levels of Cellular Cyclic Nucleotides and PKA Activity

Analysis of the levels of cyclic nucleotides in neutrophils showed that a significant increase in cyclic AMP level was

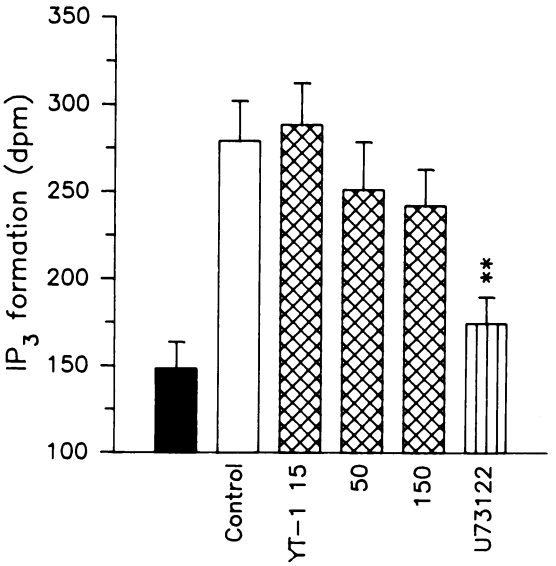

FIG. 2. Effects of YT-1 on fMLP-induced IP_3 formation in rat neutrophils. DMSO (as control), 15–150 μM YT-1, or 30 μM U73122 was added to a *myo*-[^{3}H]inositol-loaded cell suspension for 3 min before the addition of 0.3 μM fMLP to start the reaction. The IP_3 assay is described in Materials and Methods. Values are means ± SEM of 4–5 separate experiments. The resting level of IP_3 was measured from the cells exposed to DMSO without fMLP challenge (solid column). Key: (**) $P < 0.01$ compared with the control value.

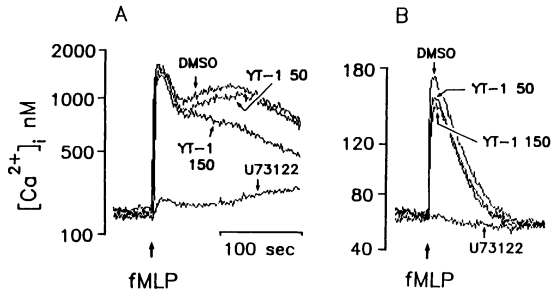

FIG. 3. Effects of YT-1 on fMLP-induced $[Ca^{2+}]_i$ elevation in rat neutrophils. (A) In the presence of 1 mM $CaCl_2$ or (B) 1 mM EDTA in medium, the fluo 3-loaded cell suspension was preincubated with DMSO (vehicle control), 50 and 150 μM YT-1, or 1 μM U73122 at 37° for 3 min before the addition of 0.1 μM fMLP. The $[Ca^{2+}]_i$ of neutrophils was measured as described in Materials and Methods. The results shown are representative of 4–5 separate experiments.

observed in cells treated with 50–150 μM YT-1, as well as with 10 μM forskolin, an adenylate cyclase activator [29], and 300 μM IBMX. Treatment of cells with 100 μM M&B 22948, a PDE V inhibitor [30], and 300 μM sodium nitroprusside increased the cellular cyclic GMP levels, whereas YT-1 had little effect in this respect (Table 1). Since YT-1 elevated the cellular cyclic AMP level, the PKA activity of neutrophils was determined thereafter.

The resting and the total PKA activities in rat neutrophil cytosolic fractions were estimated to be 0.21 ± 0.02 and 0.34 ± 0.03 nmol ^{32}P/min per mg protein, respectively, by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into kemptide in the absence or presence of 10 μM cyclic AMP,

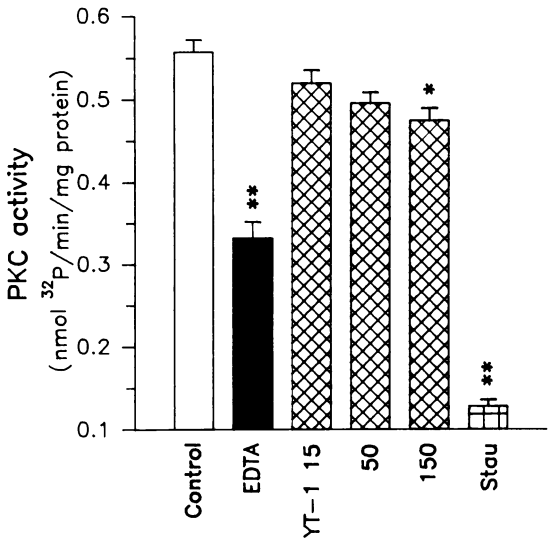

FIG. 4. Effect of YT-1 on PKC activity. Neutrophil cytosolic PKC was preincubated with DMSO (as control), 3 mM EDTA, 15–150 μM YT-1, or 1 nM staurosporine (Stau) at 25° for 3 min. The conditions of the PKC assay are described in Materials and Methods. Reactions were terminated after incubation for 15 min by the addition of stop reagent. Values are means ± SEM of 4–6 separate experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the control value.

TABLE 1. Effect of YT-1 on the cyclic AMP and cyclic GMP levels of neutrophils*

Drugs	Concentration (μM)	Cyclic AMP (pmol/2 × 10^6 cells)	Cyclic GMP (pmol/2 × 10^7 cells)
Control		0.33 ± 0.07	0.84 ± 0.13
YT-1	50	1.31 ± 0.28†	0.86 ± 0.12
	150	1.58 ± 0.26†	0.84 ± 0.11
IBMX	100	0.77 ± 0.13	ND‡
	300	1.27 ± 0.14§	ND
M&B 22948	100	ND	3.98 ± 0.82†
Forskolin	10	1.84 ± 0.19†	ND
Sodium nitroprusside	300	ND	2.74 ± 0.36†

* Cells were incubated with DMSO (as control) or drugs at 37° for 10 min before stopping the reactions. Values are expressed as means ± SEM, N = 5.
† Significantly different from the control group at $P < 0.01$.
‡ ND = not determined.
§ Significantly different from the control group at $P < 0.05$.

respectively, in the reaction mixture. As shown in Fig. 5, cytosolic PKA activity was greatly increased in cells treated with YT-1 and forskolin, and the effect of YT-1 was concentration dependent. To test whether YT-1 exerts a direct action on PKA, the effect of YT-1 on purified porcine heart PKA activity was determined. In the presence of ATP, porcine heart PKA induced the phosphorylation of kemptide, and this response was greatly enhanced by cyclic AMP (7.0 ± 0.4 vs 44.2 ± 3.5 pmol ^{32}P/min per μg protein). PKA activity, in the presence of cyclic AMP, was

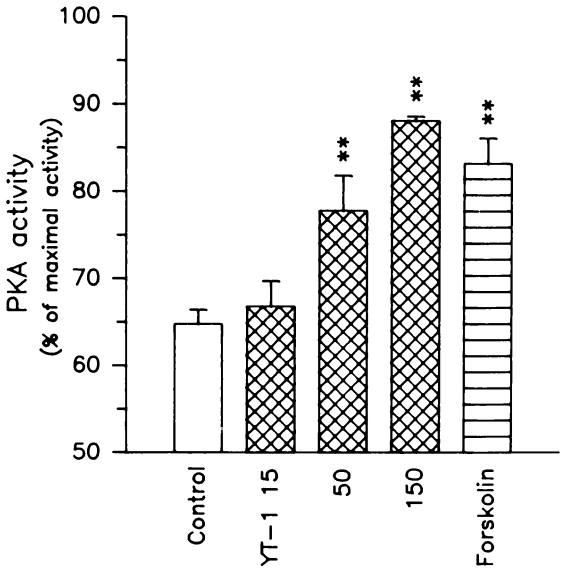

FIG. 5. Effect of YT-1 on PKA activity. The conditions of the assay are described in Materials and Methods. Neutrophils were preincubated with DMSO (as control), 15–150 μM YT-1, or 10 μM forskolin for 3 min before extraction of the cytosolic fraction as a PKA source. PKA activity is expressed as a percentage of the maximal activity (0.34 ± 0.03 nmol ^{32}P/min per mg protein) determined by the addition of 10 μM cyclic AMP to the control reaction mixture. Values are means ± SEM of 4 separate experiments. Key: (**) $P < 0.01$ compared with the control value.

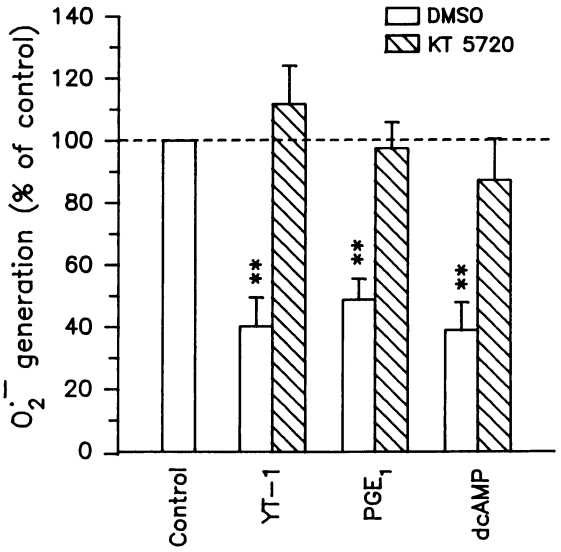

FIG. 6. Effect of KT 5720 on the inhibition of $O_2^{\cdot-}$ generation by YT-1. The conditions of $O_2^{\cdot-}$ generation are described in Materials and Methods. Neutrophils were preincubated with DMSO or 1 μM KT 5720 for 10 min; then DMSO (as control), 150 μM YT-1, 1 μM PGE_1, or 100 μM dcAMP was added for another 10 min before the addition of 0.3 μM fMLP plus 5 μg/mL of CB for $O_2^{\cdot-}$ generation. $O_2^{\cdot-}$ generation is expressed as a percentage of the control value (5.8 ± 0.5 nmol/10 min). Values are means ± SEM of 5–7 separate experiments. Key: (**) $P < 0.01$ compared with the control value.

reduced significantly by KT 5720, a PKA inhibitor [31], at 30 μM (to 27.8 ± 1.1 pmol ^{32}P/min per μg protein, $P < 0.01$). In contrast, YT-1 (up to 150 μM) had a negligible effect on PKA activity whether cyclic AMP was present or not (50.9 ± 2.5 and 7.2 ± 1.1 pmol ^{32}P/min per μg protein, respectively).

Effect of KT 5720 on the Inhibition of $O_2^{\cdot-}$ Generation by YT-1

The effect of KT 5720 on $O_2^{\cdot-}$ generation in neutrophils is illustrated in Fig. 6. Cells release $O_2^{\cdot-}$ at a rate of 5.8 ± 0.5 nmol/10 min per 10^6 cells in response to 0.3 μM fMLP plus 5 μg/mL of CB. Treatment of cells with 150 μM YT-1, 1 μM PGE_1, or 100 μM dcAMP for 10 min prior to stimulation significantly inhibited $O_2^{\cdot-}$ release. Pretreatment with 1 μM KT 5720 substantially enhanced the rate of $O_2^{\cdot-}$ production by fMLP (8.1 ± 0.7 nmol/10 min per 10^6 cells) and prevented the inhibition of $O_2^{\cdot-}$ release from cells by all three cyclic AMP-elevating agents.

Effect of YT-1 on Adenylate Cyclase and PDE Activities

Cellular cyclic AMP content can be increased either by enhancing the rate of cyclic AMP synthesis or by decreasing the rate of its metabolism. We therefore attempted to investigate the effect of YT-1 on adenylate cyclase activity in neutrophil particulate fraction and on PDE activity in the cytosolic fraction. Forskolin enhanced adenylate cyclase activity in a concentration-dependent manner, whereas YT-1 and IBMX were incapable of activating adenylate cyclase under the same experimental conditions (Fig. 7A). The cyclic AMP hydrolytic activity of the cytosolic fraction was determined to be at a rate of 61.5 ± 7.9 pmol/min per mg protein. Incubation of cytosolic fraction with various concentrations of YT-1, IBMX, or Ro 20-1724, a selective PDE4 inhibitor [32], prior to the addition of cyclic AMP resulted in a concentration-dependent inhibition of PDE activity (Fig. 7B). Ro 20-1724 and IBMX were more potent (IC_{50} values of 5.3 ± 1.5 and 15.1 ± 2.1 μM, respectively) than YT-1 as an inhibitor of cytosolic PDE activity.

Effect of Simultaneous Addition of YT-1 and PGE_1 on Cellular Cyclic AMP Level and fMLP-Induced Respiratory Burst

Neutrophils responded to the addition of 15 μM YT-1 and 0.3 μM PGE_1 with a significant increase in cellular cyclic AMP levels (1.00 ± 0.12 and 2.17 ± 0.23 pmol/2 × 10^6 cells, respectively, vs 0.34 ± 0.11 as control value, both $P < 0.01$). The cyclic AMP level in response to PGE_1 was apparently further elevated in cells that were also treated with YT-1 (6.01 ± 0.51 pmol/2 × 10^6 cells, $P < 0.01$ compared with the PGE_1 alone value). A comparable effect

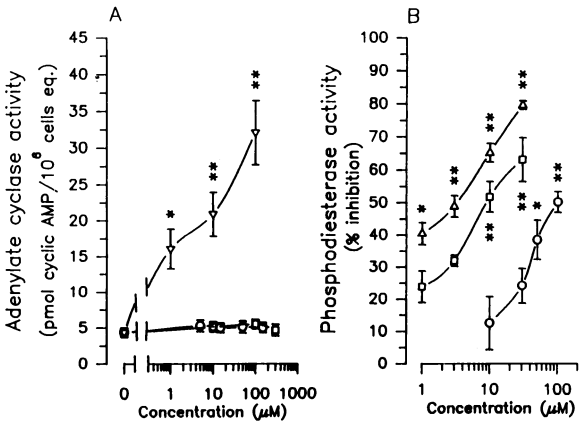

FIG. 7. Effects of YT-1 on adenylate cyclase and PDE activities. The conditions of the assay are described in Materials and Methods. (A) Neutrophil particulate fraction was incubated with DMSO (as control), or with various concentrations of YT-1 (○), IBMX (□), or forskolin (▽) at 37° for 10 min in the presence of 0.2 mM ATP. Reaction was terminated by boiling. Values are means ± SEM of 5–8 separate experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the control value. (B) Neutrophil cytosolic fraction was incubated with DMSO (as control), or with various concentrations of YT-1 (○), IBMX (□), or Ro 20-1724 (△) at 37° for 3 min, and then incubated with 1 μM cyclic AMP (0.05 μCi [^3H]cyclic AMP/tube) for 30 min before the addition of 0.04 M HCl to terminate the reaction. PDE activities are expressed as percent inhibition of the control value (61.5 ± 7.9 pmol/min per mg protein). Values are means ± SEM of 4–5 separate experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the control value.

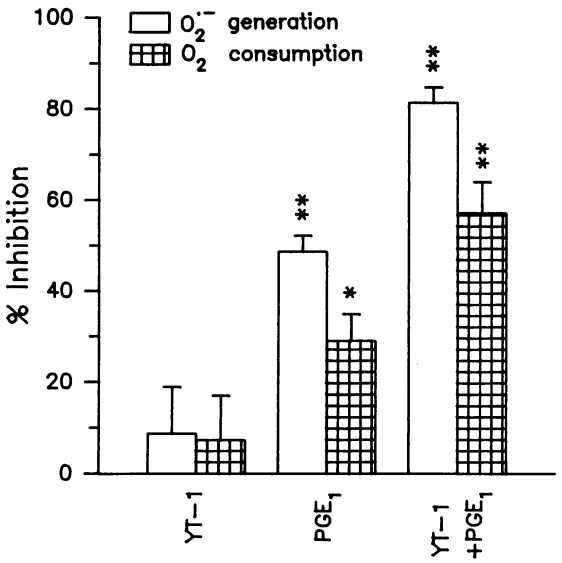

FIG. 8. Effect of YT-1 in combination with PGE_1 on $O_2^{\cdot-}$ generation and O_2 consumption in fMLP-activated neutrophils. The conditions of the assay are described in Materials and Methods. Neutrophils were preincubated with DMSO (as control), 10 μM YT-1, 0.3 μM PGE_1, or 10 μM YT-1 plus 0.3 μM PGE_1 for 3 min. $O_2^{\cdot-}$ generation in cells stimulated with 0.3 μM fMLP plus 5 μg/mL of CB and O_2 consumption in cells stimulated with 0.1 μM fMLP plus 5 μg/mL of CB are expressed as percent inhibition of the control values (7.0 ± 0.8 nmol/10 min for $O_2^{\cdot-}$ generation and 16.8 ± 0.9 nmol/5 min for O_2 consumption). Values are means ± SEM of 4 separate experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the corresponding control values.

was also observed in the respiratory burst elicited by fMLP, in which cells treated with 10 μM YT-1 significantly enhanced the inhibition of $O_2^{\cdot-}$ generation and O_2 consumption by 0.3 μM PGE_1 ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 8).

DISCUSSION

In the present study, we demonstrated that YT-1 inhibited the neutrophil respiratory burst in response to fMLP but not to PMA. fMLP activates by binding to its membrane receptor and then activating the signal transduction mediated by phospholipase C to give IP_3 and diacylglycerol, which, in turn, increase $[Ca^{2+}]_i$ and activate PKC, respectively [6]. In contrast, PMA bypasses the membrane receptor and directly activates PKC. Since YT-1 did not affect the PMA-induced responses and the $O_2^{\cdot-}$ generation in cell-free oxygen radical generation systems, precluding the possibility that it acted as an $O_2^{\cdot-}$ scavenger, it is plausible that YT-1 inhibits the respiratory burst through the interruption of the receptor-mediated signalling cascade.

Since the respiratory burst evoked by fMLP is a Ca^{2+}-dependent process, the effect of YT-1 on $[Ca^{2+}]_i$ was then examined in fluo 3-loaded cells. The $[Ca^{2+}]_i$ response elicited by fMLP is composed of an initial spike, supported primarily by IP_3-induced release of Ca^{2+} from internal stores, followed by a plateau phase, which is sustained by Ca^{2+} influx from the extracellular medium [33]. The phospholipase C inhibitor U73122 effectively attenuates fMLP-induced $[Ca^{2+}]_i$ changes and IP_3 formation [27]. The observations that YT-1 did not affect the initial spike of $[Ca^{2+}]_i$ changes and IP_3 formation in cells in response to fMLP preclude the involvement of the phospholipase C pathway in the inhibition by YT-1. However, YT-1 diminished the plateau phase of the $[Ca^{2+}]_i$ response. Since PKC plays an important role in PMA-induced response, the finding that YT-1 had little effect on the neutrophil cytosolic PKC activity is consistent with the observation that the PMA-activated respiratory burst was resistant to YT-1.

Cyclic nucleotides are well known cellular messengers for extracellular signals. The increase in cellular cyclic AMP levels in neutrophils is associated with a decrease in several neutrophil functions including respiratory burst [34]. Studies of the effects of cyclic AMP on neutrophil functions are complicated by the fact that total cellular cyclic AMP may not be representative of compartmentalized intracellular cyclic AMP concentrations. The relevant changes may presumably be localized near the membrane compartments [35] and may contribute only modestly to a change in the average cytosolic concentration. In the present study, YT-1 was found to increase the cellular cyclic AMP level, while failing to alter the cyclic GMP level. Our observation that the PMA-induced respiratory burst was hardly inhibited by YT-1 is, therefore, in line with the previous finding that cyclic AMP-elevating agents have no significant effect on PMA-stimulated respiratory burst [36]. Moreover, the increase in cyclic AMP by YT-1 may also account for its effect on IP_3 formation and $[Ca^{2+}]_i$ changes, since the cyclic AMP-elevating agents have little effect on IP_3 production [37] and do not affect Ca^{2+} release from internal stores, but inhibit the external Ca^{2+} influx [38], probably by closing the calcium channel. The precise role of cyclic GMP in regulating inflammatory cell functions is uncertain. It appears that cyclic GMP is of more importance for migration than for other neutrophil functions [39].

The molecular mechanism of cyclic AMP-mediated neutrophil inhibition is not understood. Since cyclic AMP acts by PKA in many cellular systems, its inhibitory action on neutrophils appears to depend on the activation of PKA. Rap1A is a membrane-localized regulator of NADPH oxidase [40], and this small G-protein is an abundant substrate for PKA in human neutrophils. Complex formation between Rap1A-GTP and cytochrome b_{558} is inhibited by phosphorylation of Rap1A with PKA [41]. PKA may also affect the phospholipase D pathway, probably at a site proximal to phospholipase D (the receptor or G protein) [42]. Moreover, cyclic AMP was reported to inhibit fMLP-induced arachidonic acid mobilization, and this effect may be important in the regulation of respiratory burst [43]. YT-1 showed a concentration-dependent increase in cellular PKA activity. The finding that the porcine heart PKA activity was unaffected by YT-1, whether cyclic AMP was present or not, indicates that

YT-1 has no ability to activate PKA directly. Since PKA inhibitor antagonizes the inhibition of $O_2^{\cdot-}$ production by cyclic AMP-elevating agents [36], we next sought to determine whether the inhibition of respiratory burst by YT-1 is through the elevation of the cellular cyclic AMP level, which, in turn, activates PKA. Inhibition of $O_2^{\cdot-}$ generation in fMLP-activated neutrophils by YT-1 as well as by other cyclic AMP-elevating agents, PGE_1 and dcAMP, was greatly attenuated by pretreatment of cells with the PKA inhibitor KT 5720. These results confirm the proposal that the increase in cellular cyclic AMP level by YT-1 accounts for its inhibitory effect on respiratory burst. It has been reported that fMLP causes a transient increase in cyclic AMP level in neutrophils, and this may participate in the negative-feedback control of cell activation [44]. This offers a plausible explanation to our finding that treatment with KT 5720 enhanced fMLP-induced $O_2^{\cdot-}$ generation. A similar result was also demonstrated in a recent study, which reported that fMLP-induced $O_2^{\cdot-}$ generation is enhanced significantly by treatment of cells with the PKA inhibitor H-89 [45].

Cellular cyclic AMP levels are regulated by the rate of cyclic AMP production by receptor-coupled adenylate cyclase and the rate of cyclic AMP degradation by PDE. The finding that YT-1 did not activate adenylate cyclase raises the possibility that PDE may be inhibited by YT-1. Based on genetic, biochemical, and pharmacological data, PDE isoenzymes have been classified into seven distinct families (PDE1–7) [46]. In neutrophils, PDE4 isoenzyme plays a predominant role [34, 38] and has a marked preference for cyclic AMP as a substrate. Like Ro 20-1724, YT-1 concentration-dependently suppressed neutrophil cytosolic PDE activity, suggesting that YT-1 may suppress PDE4 activity. In addition, the findings that YT-1 greatly enhanced the increase in cellular cyclic AMP level and the inhibition of $O_2^{\cdot-}$ generation from cells treated with PGE_1 further support this proposal. Several lines of evidence indicated that the neutrophil respiratory burst is inhibited by the selective PDE4 inhibitors rolipram and Ro 20-1724, and the nonselective PDE inhibitor IBMX, but not by the inhibitors of PDE1, 2, 3, and 5 [25, 47], confirming the proposal that YT-1 inhibited respiratory burst through the inhibition of PDE4, which, in turn, increases cellular cyclic AMP levels. However, YT-1 is better able to inhibit the respiratory burst than PDE activity in neutrophils. These results are compatible with the findings of others that, with the PDE4 inhibitor, the IC_{50} values for fMLP-induced functions are significantly lower than those determined for PDE enzyme inhibition and have been explained by the fact that less than 50% of PDE inhibition is sufficient for 50% functional inhibition [38]. IBMX is less effective at oxidative burst inhibition than PDE inhibition; this may be due to cell permeability problems, since higher concentrations of IBMX were required to yield levels of cyclic AMP similar to those produced by YT-1 in intact cells. IBMX is also an adenosine receptor antagonist and may be antagonizing endogenous adenosine-mediated inhibition of neutrophil activation [48]. These receptors are present on human and rat neutrophils [49, 50]. Recently, it has become apparent that PDE4 comprises a group of enzymes (PDE4A–D), in which PDE4B is prominently expressed in neutrophils [51]. Whether YT-1 may directly inhibit PDE4 activity or selectively affect the PDE4B subtype activity needs further investigation. Thus far, we do not have evidence to explain how YT-1 might be inhibiting PDE. The mechanism of suppressing PDE4 activity may also be involved in the pharmacological effects of YT-1 in our previous reports [12, 13], in which YT-1 was found to induce a positive inotropic effect on heart and to inhibit plasma extravasation in mice, since PDE4 is also expressed in heart and endothelium [51, 52].

In summary, we have shown that YT-1 is capable of inhibiting respiratory burst in rat neutrophils in response to fMLP but not to PMA. The inhibition of PDE, probably PDE4 isoenzyme, rather than the activation of adenylate cyclase by YT-1 contributes to an increase in the cellular cyclic AMP level, which, in turn, activates PKA and inhibits the respiratory burst in fMLP-activated neutrophils.

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