

# Soluble Guanylyl Cyclase Activator YC-1 Inhibits Human Neutrophil Functions through a cGMP-Independent but cAMP-Dependent Pathway

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## ABSTRACT

3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1), a novel type of soluble guanylyl cyclase (sGC) activator, is useful in investigating the signaling of cGMP and may provide a new approach for treating cardiovascular diseases. Herein, YC-1 was demonstrated to inhibit the generation of superoxide anion ( $O_2^-$ ) and the release of  $\beta$ -glucuronidase release, to diminish the membrane-associated  $p47^{phox}$  and to accelerate resequestration of cytosolic calcium in formyl-L-methionyl-L-leucyl-L-phenylalanine-activated human neutrophils. YC-1 not only directly promoted sGC activity and cGMP formation but also dramatically potentiated sodium nitroprusside-induced sGC activity and cGMP formation in human neutrophils. However, the synergistic increase in the amount of cGMP was inconsistent with its cellular response. Moreover, neither an sGC inhibitor nor protein kinase G inhibitors reversed the inhibitory effect of

YC-1. Interestingly, YC-1 also increased the cAMP concentration and protein kinase (PK)A activity. The inhibitory effect of YC-1 was significantly enhanced by prostaglandin ( $PG$ ) $E_1$  and isoproterenol, and almost abolished by PKA inhibitors. These results show that cAMP, but not cGMP, mediates the YC-1-induced inhibition of human neutrophils. YC-1 increased the  $PG$  $E_1$ - and forskolin-induced but not 3-isobutyl-1-methylxanthine-produced cAMP formation, suggesting inhibition of phosphodiesterase. These findings thus reveal novel mechanism-mediated anti-inflammatory properties of YC-1 in human neutrophils, which can influence the progression of cardiovascular disease. cAMP, but not cGMP, plays an important role in the regulation of respiratory burst and degranulation in human neutrophils.

Soluble guanylyl cyclase (sGC) plays a pivotal role in the transduction of cellular signals conveyed by the signal molecules nitric oxide (NO) and carbon monoxide (CO). By the formation of cGMP, this enzyme mediates NO-elicited actions, such as vascular smooth muscle relaxation, as well as the inhibition of platelet aggregation and synaptic transmission (Moncada et al., 1991). The inappropriate activation of sGC has been associated with the pathogenesis of various disease states, especially of the cardiovascular system

(Hobbs, 2002). 3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1), a synthetic chemical compound, has been identified as an NO-independent and direct activator of sGC (Ko et al., 1994; Wu et al., 1995; Friebe et al., 1996). In addition to NO and CO, YC-1 represents the first activating pharmacopoeia of intracellular sGC in a biological milieu. YC-1 mimics many known functions of NO, such as inhibiting the aggregation and adhesion of platelets (Wu et al., 1997), and reducing the proliferation and contraction of vascular smooth muscle (Yu et al., 1995; Mülsch et al., 1997). YC-1 not only directly stimulates sGC but also increases the responsiveness of this enzyme toward NO and CO (Friebe et al., 1996;

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**ABBREVIATIONS:** sGC, soluble guanylyl cyclase; PDE, phosphodiesterase; PK, protein kinase; SNP, sodium nitroprusside; DMSO, dimethyl sulfoxide; HBSS, Hanks' balanced salt solution; H89, *N*-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole; KT5720, (9*S*,10*S*,12*R*)-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'-*k*)pyrrolo(3,4-*i*)(1,6)benzodiazocine-10-carboxylic acid hexyl ester; KT5823, (8*R*,9*S*,11*S*)-(–)-2-methyl-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo(*a,g*)cycloocta(*cde*)trinen-1-one; Ro318220, 3-(1-(3-(amidinothio)propyl-1*H*-indol-3-yl))-3-(1-methyl-1*H*-indol-3-yl)maleimide; AM, acetoxymethyl ester; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine;  $O_2^-$ , superoxide anion; CB, cytochalasin B; IBMX, 3-isobutyl-1-methylxanthine; LDH, lactate dehydrogenase; ODQ, 1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one; Rp-8-pCPT-cGMPs, 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, *Rp*-isomer.

Stone and Marletta, 1998). In accordance with the results obtained with purified sGC, this synergistic response of YC-1 has been reported in terms of smooth muscle relaxation and platelet aggregation (Mülsch et al., 1997; Friebe et al., 1998; Hwang et al., 1999). Hence, YC-1 is regarded as a useful tool for investigating the action of the sGC/cGMP pathway in various biological processes. It may also support a new approach to treating cardiovascular disease. For example, YC-1 is directly implicated in suppressing postangioplasty stenosis through endogenous CO- and/or NO-mediated, cGMP-dependent processes (Tulis et al., 2000). Also, i.v. injections of YC-1 acutely reduce the blood pressure in normotensive and hypertensive rats (Rothermund et al., 2000).

Neutrophils are important in a host's defenses against invasion by microorganisms and are extensively involved in inflammatory processes. In response to diverse stimuli, activated neutrophils exhibit adhesion, chemotaxis, degranulation, and superoxide anion ( $O_2^-$ ) production (Borregaard, 1988). The cyclic nucleotides cAMP and cGMP generally play important roles in regulating neutrophil functions. An increase in intracellular cAMP levels is believed to suppress the activation of neutrophils. Elevating intracellular cAMP by using physiological cAMP agonists, including E-type prostaglandins,  $\beta$ -adrenergic agents, and phosphodiesterase (PDE) inhibitors, has been widely recognized to diminish respiratory burst and degranulation from stimulated neutrophils (Coffey, 1992). The clinical potential of cAMP-elevating agents as inhibitors of neutrophil activities is supported by the suppression of endotoxin-induced acute lung injury in mice by the PDE4 inhibitor rolipram (Miotla et al., 1998), and the anti-inflammatory activity of the second-generation PDE4 inhibitor Ariflo (SB 207499), in experimental asthma in guinea pigs (Underwood et al., 1998). Neutrophils have been reported to contain sGC and PKG (Lad et al., 1985; Pryzwansky et al., 1990). Despite this fact, the physiological role of cGMP in regulating neutrophil function remains poorly understood. NO can either suppress or promote neutrophil activation depending on experimental conditions; in both cases, it acts probably through cGMP. However, NO has been reported to inhibit directly the activity of  $O_2^-$ -producing NADPH oxidase, 5-lipoxygenase, and ADP-ribosylate actin in human neutrophils (Armstrong, 2001). Clearly, the role of NO/cGMP in neutrophil functions must be clarified before the therapeutic potential of NO donors or inhibitors in inflammation can be realized.

YC-1 was also recently used to elucidate the function of cGMP on lipopolysaccharide-activated p38 mitogen-activated protein kinase and in spontaneous apoptosis in human neutrophils (Browning et al., 1999; Brunetti et al., 2002). Additionally, YC-1 has been observed to inhibit  $O_2^-$  production in rat neutrophils (Wang et al., 2002). However, no study has yet clearly elucidated the mechanism of the action of YC-1 on neutrophil functions. This study investigates the effects of YC-1 on respiratory burst and degranulation, as well as the synergistic action of YC-1 and NO, in human neutrophils. Various pharmacological agonists and inhibitors were used to elucidate the mechanisms in detail. YC-1 was found not only to increase sGC activity and cGMP formation directly, but also to potentiate dramatically sodium nitroprusside (SNP)-induced sGC stimulation and cGMP formation in human neutrophils. Surprisingly, the present data provide evidence that the inhibitory effect of YC-1 on chemotactic pep-

tide FMLP-induced respiratory burst and degranulation occurs through a cGMP-independent but cAMP-dependent pathway.

## Materials and Methods

**Materials.** YC-1 was chemically synthesized as described previously and was dissolved in dimethyl sulfoxide (DMSO) for a stock solution (Yoshina and Kuo, 1978). Hanks' balanced salt solution (HBSS) was purchased from Invitrogen (Carlsbad, CA). Aprotinin, H89, KT5720, KT5823, leupeptin, phenylmethylsulfonyl fluoride, the protein kinase assay kits, Ro318220, rolipram, and zaprinast were obtained from Calbiochem (La Jolla, CA). Rp-8-pCPT-cGMPs was obtained from Biolog Life Science (Bremen, Germany). Fluo-3/AM was purchased from Molecular Probes (Eugene, OR). Monoclonal antibody of p47<sup>phox</sup> was obtained from R&D Systems (Minneapolis, MN). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). When drugs were dissolved in DMSO, the final concentration of DMSO in cell experiments did not exceed 0.5% and did not affect the parameters measured.

**Preparation of Human Neutrophils.** Whole blood was obtained from healthy donors (18–32 years old) by venepuncture. Heparinized blood was mixed with 3% dextran and kept for 30 min at room temperature. Neutrophils were isolated by density gradient centrifugation (400g for 40 min at 20°C) of leukocyte-rich plasma in Ficoll-Paque ( $d = 1.077$  g/ml). After centrifugation, the pellet was suspended in ice-cold 0.2% hypotonic saline buffer for 30 s to remove the remaining red blood cells. Purified neutrophils that contained >98% viable cells, as determined by trypan blue exclusion, were resuspended in a calcium-free HBSS buffer at pH 7.4, and kept at 4°C before use.

**Superoxide Anion ( $O_2^-$ ) Generation.** The measurement of the generation of  $O_2^-$  was based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*. The changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (U-3010; Hitachi, Tokyo, Japan). After supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM calcium chloride,  $10^6$ /ml neutrophils were equilibrated at 37°C for 2 min and incubated with drugs for 5 min. Cells were activated by FMLP or phorbol 12-myristate 13-acetate (PMA) for 10 min. When FMLP was used as a stimulant, 1  $\mu$ g/ml cytochalasin B (FMLP/CB) was incubated for 3 min before activation by peptide. Calculation is based on the difference of the reactions with and without 100 U/ml SOD divided by the extinction coefficient for the reduction of ferricytochrome *c* ( $\epsilon = 21.1$  mM/10 mm).

The  $O_2^-$ -scavenging potential of YC-1 was measured using the xanthine/xanthine oxidase system (SOD assay kit-WST; Dojindo Laboratories, Kumamoto, Japan), based on a method described previously (Tan and Berridge, 2000). After the enzyme working solution (xanthine oxidase) was added for 20 min at 37°C, the absorbance associated with  $O_2^-$ -induced WST-1 reduction was measured at 450 nm by using a microplate reader. The effect of YC-1 on SOD activity was also determined by this colorimetric method.

**$\beta$ -Glucuronidase Release.** Neutrophil degranulation was determined from the extent of release of the primary granule,  $\beta$ -glucuronidase. Cells ( $5 \times 10^6$ /ml) were incubated with drugs for 5 min at 37°C. The stimulant 0.1  $\mu$ M FMLP/5  $\mu$ g/ml CB was then added, and the reaction mixture was incubated for another 10 min.  $\beta$ -Glucuronidase activity was determined by measuring the extent of formation of phenolphthalein from phenolphthalein glucuronic acid as the substrate at 56°C for 1 h. Total  $\beta$ -glucuronidase activity was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37°C, and this value was taken as 100%.

**p47<sup>phox</sup> Membrane Translocation.** Neutrophils were incubated with drugs for 5 min at 37°C before being stimulated by FMLP. After 3 min, reactions were stopped by cooling to 4°C. Cells were pelleted

and resuspended in ice-cold relaxation buffer (100 mM HEPES, pH 7.3, 100 mM KCl, 3 mM NaCl, 3 mM MgCl<sub>2</sub>, 1.25 mM EGTA, and the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml each leupeptin and aprotinin, and 150  $\mu$ g/ml benzamide). Cells were then disrupted by sonication and further ultracentrifuged at 100,000g for 10 min at 4°C to pelletize the fractions of membranes. The pellet was resuspended in a buffer that contained 120 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, 20% (v/v) glycerol, 40 mM octylglucoside, and the protease inhibitors mentioned above. Then, the sample was recentrifuged at 100,000g for 40 min at 4°C. The supernatant thus obtained, containing solubilized membranes, was used to analyze p47<sup>phox</sup> (El Bekay et al., 2002). Protein concentrations were determined by Bradford assay using bovine serum albumin as the standard. Solubilized membrane fractions were electrophoresed through a 10% SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Blots were incubated with a mouse monoclonal anti-p47<sup>phox</sup> antibody (BD Biosciences Transduction Laboratories, Lexington, KY). Horse anti-mouse antibody conjugated with horseradish peroxidase was used as the secondary antibody (Vector Laboratories, Burlingame, CA). The band was visualized using the ECL system (Amersham Biosciences UK, Ltd.).

**Cyclic Nucleotide Assay.** cGMP and cAMP contents were assayed using enzyme immunoassay kits (Amersham Biosciences UK, Ltd.). The reaction of neutrophils was terminated by adding ice-cold 6% trichloroacetic acid, and the supernatant was extracted four times by using water-saturated diethyl ether. The remaining aqueous extract was lyophilized and reconstituted in assay buffer. For the cGMP determinations, samples were acetylated. The assay was performed according to the manufacturer's instructions.

**Assay of sGC Activity.** Neutrophils were sonicated in an ice-cold buffer, containing 25 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 5 mM MgCl<sub>2</sub>, 10  $\mu$ M leupeptin, 100  $\mu$ M phenylmethylsulfonyl fluoride, and 10  $\mu$ M pepstatin. The membrane fraction was removed by centrifugation at 100,000g for 40 min at 4°C. The supernatant was used as a source for the sGC sample. The reaction mixture used to assay sGC activity contained 25 mM Tris-HCl, pH 7.5, 7.5 mM MgCl<sub>2</sub>, 2 mM 3-isobutyl-1-methylxanthine (IBMX), 7.5 mM creatine phosphate, 3 units of creatine phosphokinase, 1 mM GTP, and the sGC sample. The reaction was carried out for 10 min at 37°C and was terminated by boiling for 3 min. cGMP contents were assayed using enzyme immunoassay kits.

**Assay of PKA Activity.** PKA activity in neutrophils was measured using a nonradioactive protein kinase assay kit (Calbiochem), as described previously (Osinski et al., 2001). Cells ( $2 \times 10^7$ /ml) were incubated with drugs for 5 min at 37°C. The stimulant FMLP was then added, and the reaction mixture was incubated for 3 min. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM  $\beta$ -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamide) and then briefly sonicated and centrifuged at 14,000g for 5 min at 4°C. Supernatants were assayed for PKA activity by an enzyme-linked immunosorbent assay, following the manufacturer's instructions.

**Measurement of [Ca<sup>2+</sup>]<sub>i</sub>.** Neutrophils were loaded with 2  $\mu$ M fluo-3/AM at 37°C for 45 min. After being washed, cells were resuspended in Ca<sup>2+</sup>-free HBSS to  $3 \times 10^6$  cells/ml. The change in fluorescence was monitored using an F-4500 spectrofluorometer (Hitachi) in a quartz cuvette with a thermostat (37°C), while being continuously stirred. The excitation wavelength was set at 488 nm, and the emission wavelength was 520 nm. FMLP was used to increase [Ca<sup>2+</sup>]<sub>i</sub> in the presence of either 1 mM Ca<sup>2+</sup> or 100  $\mu$ M EGTA. [Ca<sup>2+</sup>]<sub>i</sub> was calibrated by fluorescence intensity, as follows; [Ca<sup>2+</sup>]<sub>i</sub> =  $K_d \times [(F - F_{\min})/(F_{\max} - F)]$ , where  $F$  is the observed fluorescence intensity.  $F_{\max}$  and  $F_{\min}$  were obtained by the addition of 0.05% Triton X-100 and 10 mM EGTA.  $K_d$  was taken to be 400 nM.

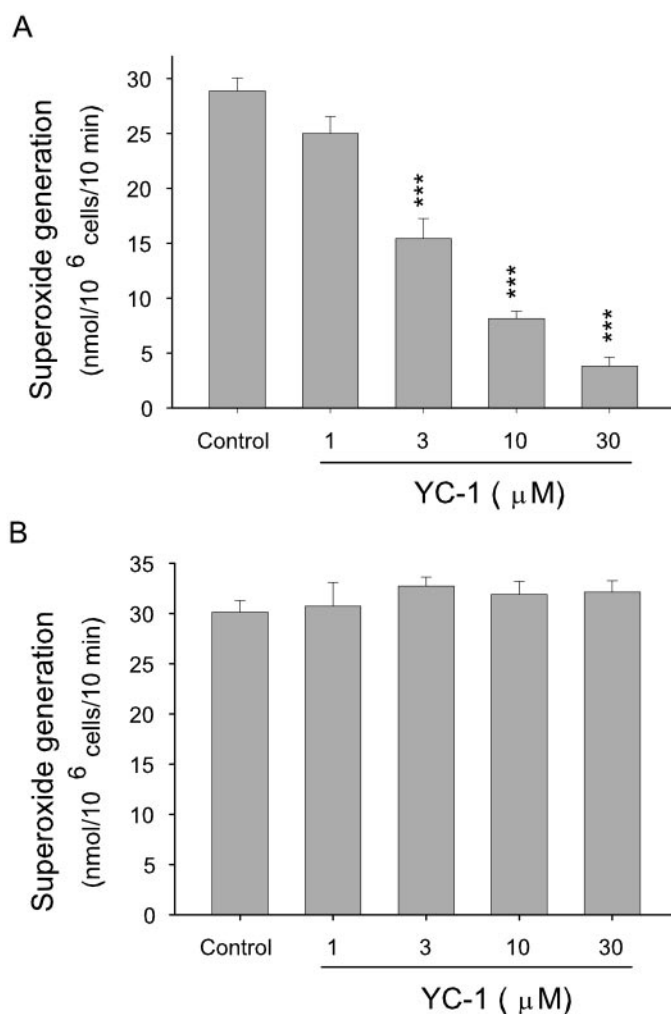
**Lactate Dehydrogenase Release.** LDH release was determined by a commercially available method (Sigma-Aldrich). Cytotoxicity

was LDH activity in the cell-free medium as a percentage of the total LDH activity. The total LDH activity was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37°C.

**Statistics.** Results are expressed as the mean  $\pm$  S.E.M., and comparisons were made using Student's  $t$  test. A probability of 0.05 or less was considered significant.

## Results

**Effects of YC-1 and cGMP on O<sub>2</sub><sup>-</sup> Generation.** YC-1 inhibited O<sub>2</sub><sup>-</sup> release by human neutrophils in response to FMLP/CB, but not to PMA, in a concentration-dependent manner with an IC<sub>50</sub> value of  $3.75 \pm 0.64$   $\mu$ M (Fig. 1). YC-1 (10  $\mu$ M) also inhibited the concentration-response curve of FMLP (0.01–1  $\mu$ M) in the absence of CB (data not shown). YC-1 did not alter the basal O<sub>2</sub><sup>-</sup> generation under resting conditions. Culturing with YC-1 (up to 30  $\mu$ M) did not affect cell viability, as assayed by LDH release. The effects of the NO donor SNP (1–1,000  $\mu$ M), the cGMP analog dibutyryl-cGMP (0.5 and 1 mM), and the PDE5 inhibitor zaprinast (10  $\mu$ M) on FMLP/CB-induced O<sub>2</sub><sup>-</sup> generation were examined to



**Fig. 1.** Effects of YC-1 on O<sub>2</sub><sup>-</sup> generation from human neutrophils in response to FMLP/CB or PMA. The O<sub>2</sub><sup>-</sup> generation was measured using SOD-inhibitable cytochrome *c* reduction, as described under *Materials and Methods*. Neutrophils were incubated with DMSO (control) or YC-1 (1–30  $\mu$ M) for 5 min and then activated by FMLP/CB ( $n = 8$ ) (A) or PMA ( $n = 4$ ) (B). All data are expressed as mean  $\pm$  S.E.M. \*\*\*,  $P < 0.001$  compared with the control.



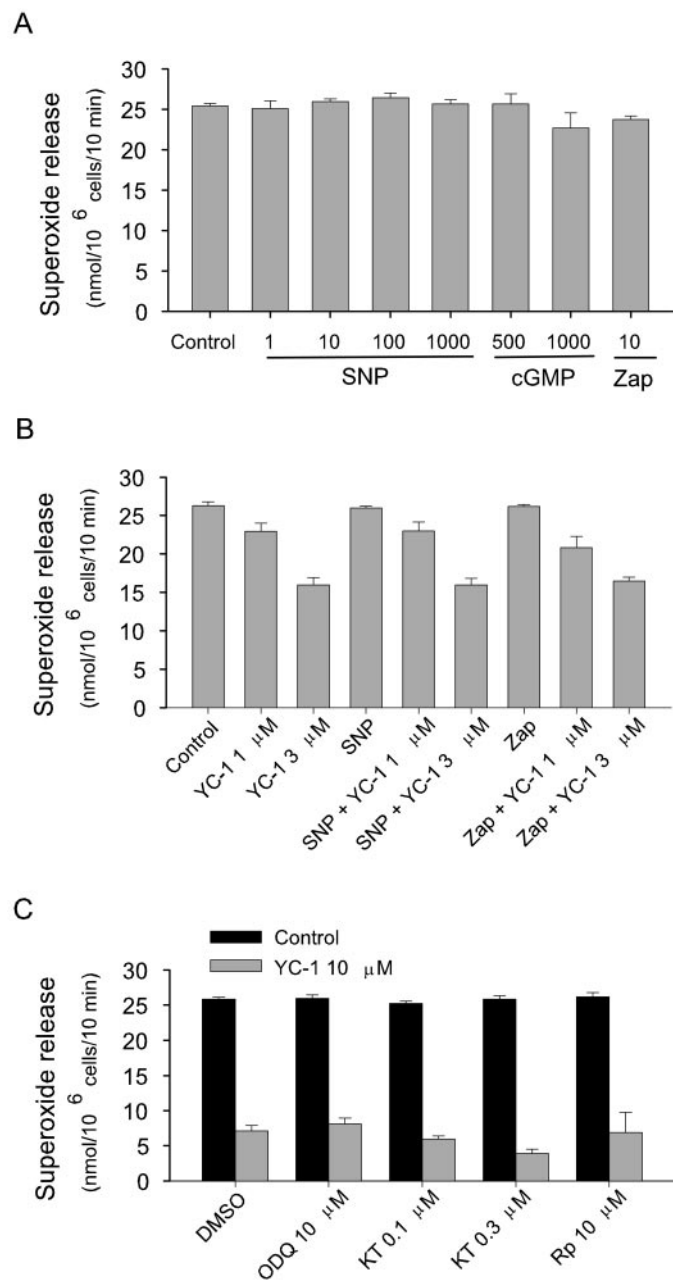
determine the effect of cGMP on  $O_2^-$  release. None of these cGMP-elevating agents altered neutrophil responses (Fig. 2A). Moreover, YC-1 (1 and 3  $\mu$ M) together with either SNP (300  $\mu$ M) or zaprinast (10  $\mu$ M) failed to modify the YC-1-induced response (Fig. 2B). These data indicate that cGMP does not mediate YC-1-produced inhibition. In agreement with this finding, the inhibitory effect of YC-1 was not re-

versed by the sGC inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), or by the PKG inhibitors KT5823 and Rp-8-pCPT-cGMPS (Fig. 2C). Additionally, YC-1 at concentrations up to 30  $\mu$ M did not scavenge  $O_2^-$  and also did not affect the removal of  $O_2^-$  by SOD in the xanthine/xanthine oxidase system (Fig. 3).

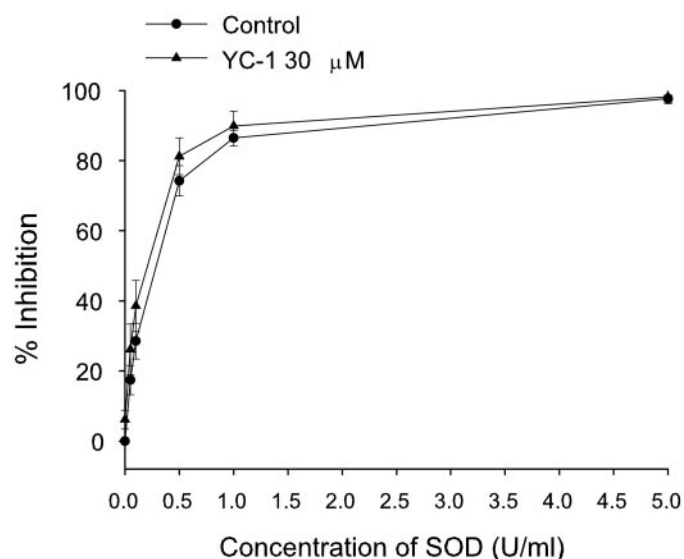
**Role of cAMP/PKA in YC-1-Induced Inhibition of  $O_2^-$  Generation.** The responses of the E-type prostaglandin  $PGE_1$ , the cAMP analog dibutyryl-cAMP (0.5 and 1 mM), and the PDE4 inhibitor rolipram to the FMLP/CB-induced  $O_2^-$  generation were examined to elucidate the effect of cAMP on  $O_2^-$  release. All such cAMP-elevating agents inhibited the  $O_2^-$  generation of human neutrophils induced by FMLP/CB (Fig. 4A), but not by PMA (Fig. 4B). Ro318220, a well documented inhibitor of PKC, was used as a positive control on PMA-caused  $O_2^-$  generation. YC-1 markedly potentiated  $PGE_1$ - and isoproterenol-induced inhibition (Fig. 4C). Furthermore, the PKA inhibitors H89 and KT5720 abolished the inhibition of  $O_2^-$  formation by YC-1 (Fig. 4D).

**Effect of YC-1 on  $\beta$ -Glucuronidase Release.** YC-1 (1–30  $\mu$ M) and dibutyryl-cAMP (0.5, 1, and 2 mM) significantly inhibited  $\beta$ -glucuronidase release in response to FMLP/CB in concentration-dependent manners (Fig. 5, A and B). In contrast, dibutyryl-cGMP, at concentrations of up to 2 mM, only caused minor effects (from  $26.60 \pm 1.58$  to  $22.26 \pm 2.73\%$ ;  $n = 4$ ,  $P > 0.05$ ) (Fig. 5C). The PKA inhibitor H89 was added to examine whether PKA is involved in the inhibitory effects of YC-1. As shown in Fig. 5D, H89 restored the inhibitory effects of YC-1 and dibutyryl-cAMP. These results suggest that cAMP also mediates the YC-1-induced inhibition of degranulation in human neutrophils. Additionally, H89 alone significantly increased FMLP/CB-induced  $\beta$ -glucuronidase release (from  $26.55 \pm 1.21$  to  $35.07 \pm 3.59\%$ ;  $n = 5$ ,  $P = 0.05$ ) (Fig. 5D), implying that cAMP feeds back to inhibit  $\beta$ -glucuronidase release in FMLP/CB-stimulated human neutrophils.

**Effects of YC-1 on sGC Activity and cGMP Formation.** Neutrophils have been documented to contain sGC



**Fig. 2.** Effects of cGMP on  $O_2^-$  generation from human neutrophils in response to FMLP/CB. The  $O_2^-$  generation was measured using SOD-inhibitable cytochrome *c* reduction, as described under *Materials and Methods*. A, neutrophils were incubated with SNP (1–1000  $\mu$ M), dibutyryl-cGMP (cGMP, 500 and 1,000  $\mu$ M), or zaprinast (Zap, 10  $\mu$ M) for 5 min and then activated by FMLP/CB ( $n = 6$ ). B, neutrophils were incubated with YC-1 (1 and 3  $\mu$ M) with or without SNP (300  $\mu$ M) or zaprinast (Zap, 10  $\mu$ M) for 5 min before activation ( $n = 6$ ). C, effect of sGC and PKG inhibitors on YC-1-induced inhibition of  $O_2^-$  generation. ODQ (10  $\mu$ M), KT5723 (KT, 0.1 and 0.3  $\mu$ M), or Rp-8-pCPT-cGMPS (Rp, 10  $\mu$ M) was preincubated before YC-1 (10  $\mu$ M) for 5 min ( $n = 4$ ). All data are expressed as mean  $\pm$  S.E.M.

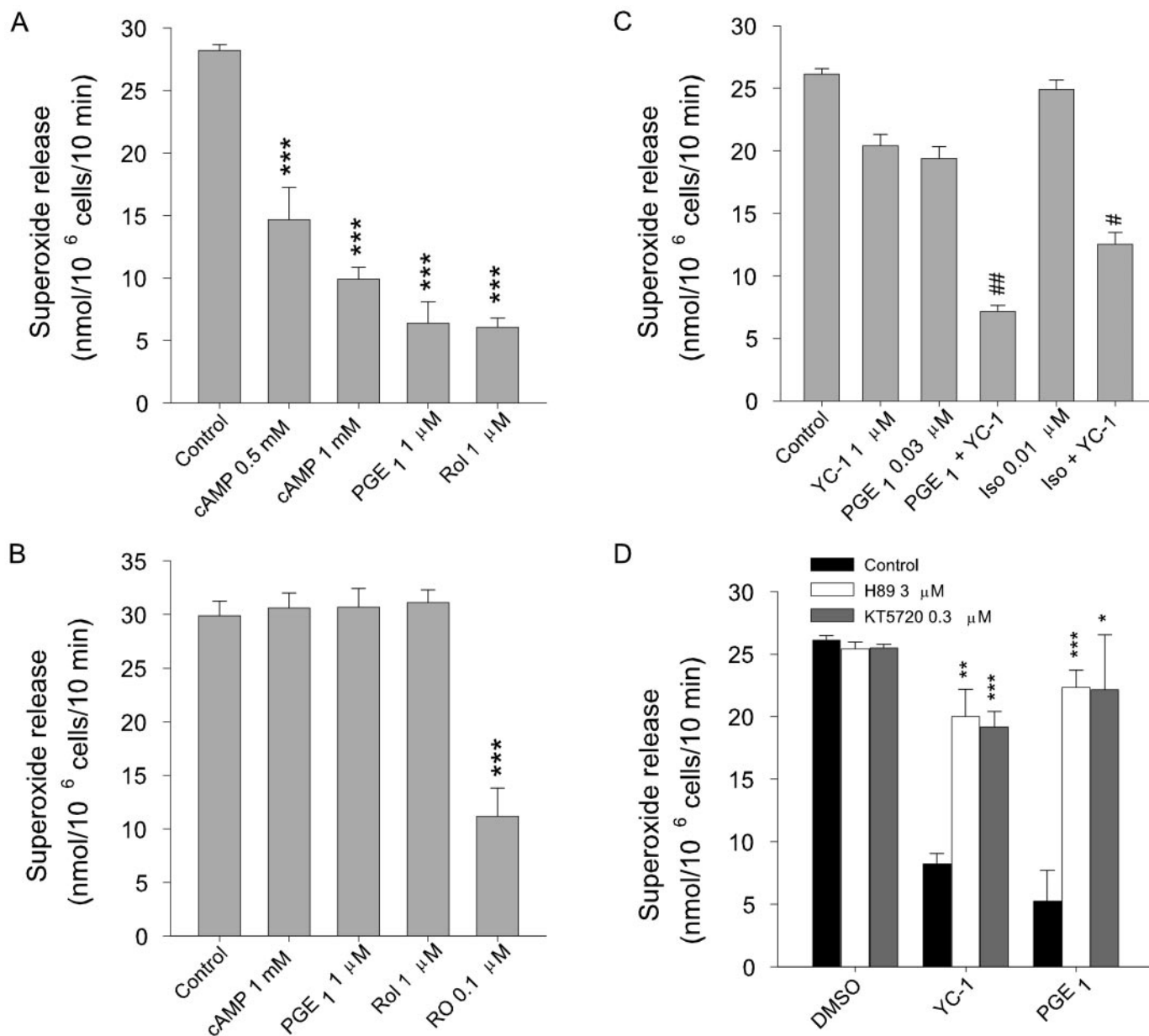


**Fig. 3.** Effect of YC-1 on  $O_2^-$  release by xanthine/xanthine oxidase in a cell-free system. The reduction of WST-1 was measured spectrophotometrically at 450 nm, as described under *Materials and Methods*. All data are expressed as mean  $\pm$  S.E.M. ( $n = 4$ ).

(Lad et al., 1985; Pryzwansky et al., 1990). However, whether YC-1 can activate sGC in human neutrophils is still unknown. As shown in Fig. 6A, YC-1 (10, 30, and 100  $\mu\text{M}$ ) was confirmed to activate sGC in a concentration-dependent manner. Furthermore, SNP at a concentration of 100  $\mu\text{M}$ , which elicited only a small effect by itself, dramatically potentiated the YC-1 (30  $\mu\text{M}$ )-increased sGC activity. This corresponded to a >60-fold increase in sGC activity, an elevation that to our knowledge has not been previously reported in neutrophils. Forskolin, a well documented activator of adenylyl cyclase, was used as a negative control on sGC activity. Moreover, YC-1 (3, 10, and 30  $\mu\text{M}$ ), IBMX (300  $\mu\text{M}$ ), and SNP (30 and 300  $\mu\text{M}$ ) increased cGMP contents in

FMLP/CB-activated human neutrophils. YC-1 (30  $\mu\text{M}$ ) together with IBMX (300  $\mu\text{M}$ ) slightly potentiated the cGMP concentration. However, YC-1 (10  $\mu\text{M}$ ) together with SNP (30 and 300  $\mu\text{M}$ ) markedly and synergistically increased the cGMP level (Fig. 6B).

**Effects of YC-1 on cAMP Formation and PKA Activity.** cAMP concentrations and PKA activity were measured to determine whether the inhibitory effects of YC-1 are indeed mediated by cAMP/PKA. YC-1 (3, 10, and 30  $\mu\text{M}$ ), PGE<sub>1</sub> (1 and 10  $\mu\text{M}$ ), and forskolin (30  $\mu\text{M}$ ) increased cAMP levels in FMLP/CB-stimulated human neutrophils. YC-1 together with PGE<sub>1</sub> or forskolin, but not with IBMX or SNP, produced a synergistic elevation in cAMP concentrations (Fig. 6C).



**Fig. 4.** Effects of YC-1 and cAMP on O<sub>2</sub><sup>-</sup> generation from human neutrophils in response to FMLP/CB or PMA. The O<sub>2</sub><sup>-</sup> generation was measured using SOD-inhibitable cytochrome *c* reduction, as described under *Materials and Methods*. A, neutrophils were incubated with dibutyl- $\alpha$ -cAMP (cAMP, 0.5 and 1 mM), PGE<sub>1</sub> (1  $\mu\text{M}$ ), rolipram (Rol, 1  $\mu\text{M}$ ), or Ro318220 (0.1  $\mu\text{M}$ ) for 5 min and then activated by FMLP/CB ( $n = 5$ ) or PMA (B) ( $n = 4$ ). C, YC-1 (1  $\mu\text{M}$ ) was tested with or without PGE<sub>1</sub> (0.03  $\mu\text{M}$ ) or isoproterenol (Iso, 0.01  $\mu\text{M}$ ) for 5 min before activation with FMLP/CB ( $n = 4$ ). (D) Effect of PKA inhibitors on YC-1- and PGE<sub>1</sub>-induced inhibition of O<sub>2</sub><sup>-</sup> generation. H89 (3  $\mu\text{M}$ ) or KT5720 (0.3  $\mu\text{M}$ ) was preincubated for 5 min before the addition of YC-1 (10  $\mu\text{M}$ ) or PGE<sub>1</sub> (1  $\mu\text{M}$ ) ( $n = 4$ ). All data are expressed as mean  $\pm$  S.E.M. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with the control. #,  $P < 0.01$ ; ##,  $P < 0.001$  compared with YC-1.

Cytosolic PKA activity was greatly increased in cells treated with YC-1 (10 and 30  $\mu\text{M}$ ) and  $\text{PGE}_1$  (1  $\mu\text{M}$ ) in FMLP-activated human neutrophils, and the effect of YC-1 was concentration-dependent (Fig. 6D).

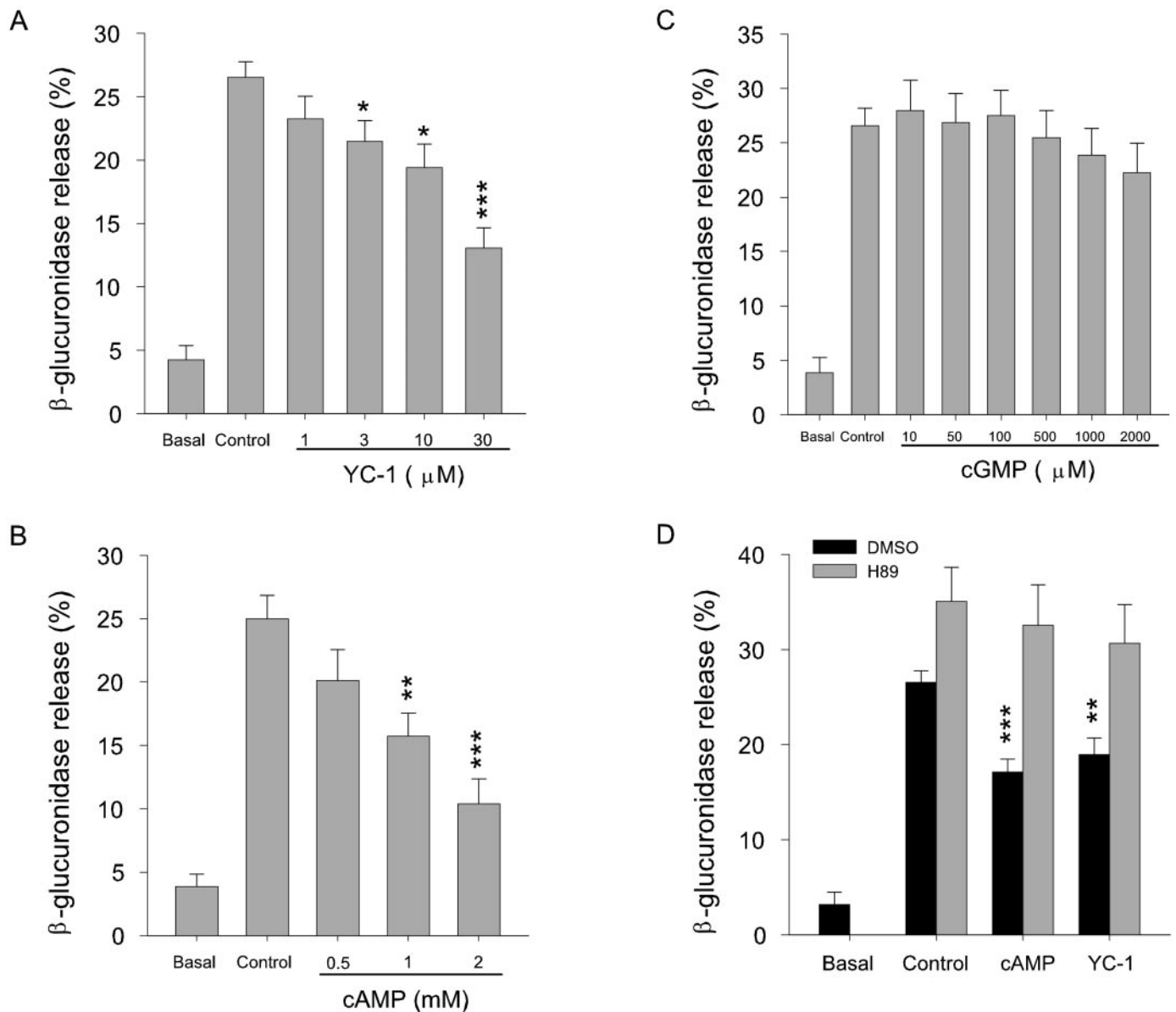
**Effect of YC-1 on  $[\text{Ca}^{2+}]_i$ .** The peak  $[\text{Ca}^{2+}]_i$  values were unaltered by YC-1 (3 and 10  $\mu\text{M}$ ),  $\text{PGE}_1$  (0.03 and 0.1  $\mu\text{M}$ ), or rolipram (1  $\mu\text{M}$ ) in FMLP-induced cells, but the time taken for  $[\text{Ca}^{2+}]_i$  to return to half of the peak values ( $t_{1/2}$ ) was significantly shortened (Table 1). The combination of  $\text{PGE}_1$  (0.03  $\mu\text{M}$ ) with either YC-1 (3  $\mu\text{M}$ ) or rolipram (1  $\mu\text{M}$ ) further reduced  $t_{1/2}$ . Additionally, in the absence of external  $\text{Ca}^{2+}$ , YC-1 only at the higher concentration of 30  $\mu\text{M}$  removed the kinetics of  $\text{Ca}^{2+}$  mobilization (data not shown).

**Effect of YC-1 on FMLP-Induced  $\text{p47}^{\text{phox}}$  Membrane Translocation.** The translocation of the subunit of  $\text{p47}^{\text{phox}}$  from the cytosol to the plasma membrane is a crucial step for

activating NADPH oxidase (Heyworth et al., 1989). As shown in Fig. 7, YC-1 (1, 3, and 10  $\mu\text{M}$ ) and dibutyryl-cAMP (1 mM) diminished the membrane-associated  $\text{p47}^{\text{phox}}$  caused by FMLP, and the effect of YC-1 was also concentration-dependent.

## Discussion

In addition to NO and CO, YC-1 represents the first activating pharmacopoeia of intracellular sGC in a biological milieu. This study investigated the effect of YC-1 on respiratory burst and degranulation by human neutrophils, which are important in the pathogenesis of sepsis, myocardial ischemia-reperfusion injury, atherosclerosis, and other inflammatory processes. The results obtained reveal that YC-1 significantly inhibited the FMLP-induced  $\text{O}_2^-$  generation and

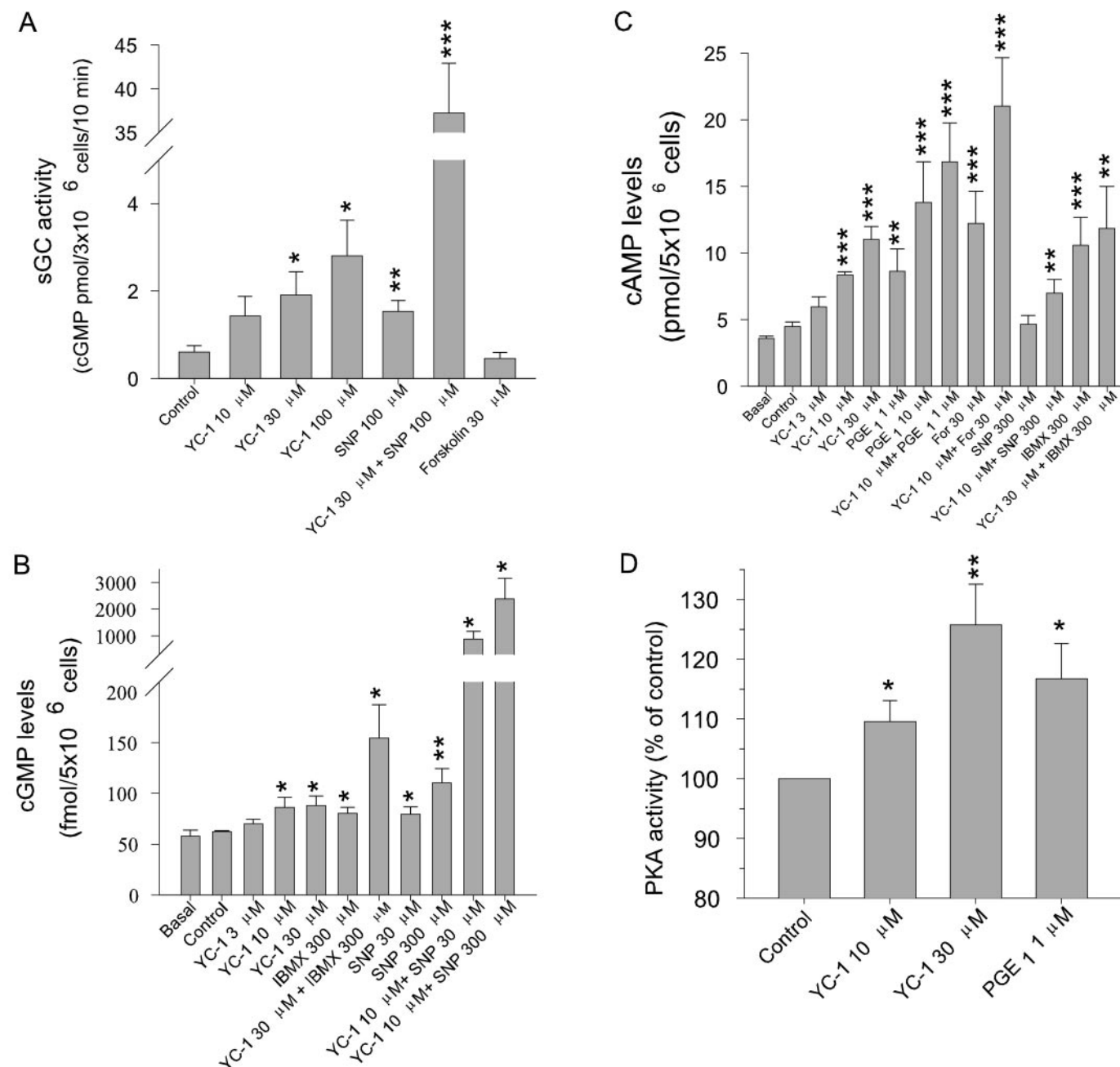


**Fig. 5.** Effects of YC-1, cAMP, and cGMP on  $\beta$ -glucuronidase release from human neutrophils.  $\beta$ -Glucuronidase release was assayed as described under *Materials and Methods*. Neutrophils were incubated with YC-1 (1–30  $\mu\text{M}$ ) ( $n = 5$ ) (A), dibutyryl-cAMP (cAMP, 0.5–2 mM) ( $n = 6$ ) (B), or dibutyryl-cGMP (cGMP, 10–2,000  $\mu\text{M}$ ) ( $n = 4$ ) (C) for 5 min and then activated by FMLP/CB. D, H89 (3  $\mu\text{M}$ ) was preincubated for 5 min before the addition of YC-1 (10  $\mu\text{M}$ ) or cAMP (1 mM) ( $n = 5$ ). All data are expressed as mean  $\pm$  S.E.M. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with the control.

$\beta$ -glucuronidase release from human neutrophils in a concentration-dependent manner. This inhibition is not associated with cytotoxicity, because YC-1 did not alter the viability of the cell. This result shows that this new type of sGC activator may protect against the progression of inflammation.

YC-1 at concentrations of up to 30  $\mu$ M did not scavenge  $O_2^-$  and did not affect the removal of  $O_2^-$  by SOD in a cell-free system, indicating that intracellular signaling pathways are mediated by the action of YC-1. Indeed, the ability of YC-1 to diminish membrane-associated  $p47^{phox}$  demonstrates that YC-1 exerts its inhibitory influence upstream of NADPH

oxidase. Despite previous reports on the regulation of neutrophil functions by cGMP, the importance of cGMP in the regulation of neutrophil functions still has been a matter of debate (Coffey, 1992). cGMP has been observed to have both an inhibitory effect and a negligible effect on FMLP-induced responses (Ervens et al., 1991; Morikawa et al., 1995). The controversial results in those studies may have followed from the use of different species and NO donors, as well as the lack of effective activators of sGC. Data from the present study indicate that YC-1 not only increases sGC activity and cGMP formation, but also drastically potentiates SNP-induced sGC



**Fig. 6.** Effects of YC-1 on cyclic nucleotide levels and activities of sGC and PKA. A, neutrophil cytosolic fractions were incubated with YC-1 (10, 30, and 100  $\mu$ M), SNP (100  $\mu$ M), forskolin (For, 30  $\mu$ M), or YC-1 (30  $\mu$ M) with SNP for 10 min in the presence of 1 mM GTP. Cellular cGMP (B) and cAMP (C) were measured as described under *Materials and Methods*. Cells were incubated with YC-1 (3, 10, and 30  $\mu$ M) with or without IBMX, SNP, PGE<sub>1</sub>, or forskolin for 5 min before stimulation with FMLP/CB. D, YC-1 (10 and 30  $\mu$ M) or PGE<sub>1</sub> (1  $\mu$ M) was added for 5 min before activation. The activity of PKA was measured by enzyme-linked immunosorbent assay at OD<sub>492</sub>. All data are expressed as mean  $\pm$  S.E.M. ( $n = 3-9$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with the control.



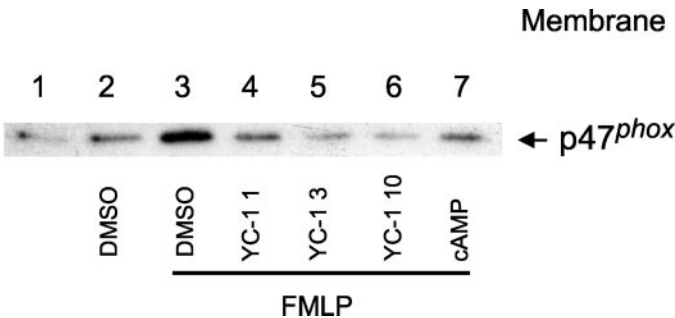
stimulation and cGMP levels in human neutrophils. Despite this, a role for cGMP in respiratory burst and degranulation of human neutrophils is excluded because 1) the sGC inhibitor ODQ and the PKG inhibitors KT 5723 and Rp-8-pCPT-cGMPS failed to reverse the inhibitory effect of YC-1; 2) SNP significantly potentiated the YC-1-increased sGC activity and cGMP formation, but was not consistent in its cellular responses; and 3) the cGMP-elevating agents zaprinast, SNP, and dibutyryl-cGMP did not alter the neutrophil functions. These findings are in contrast to the data of Wang et al. (2002), who reported that the inhibition of FMLP-induced  $O_2^-$  formation in rat neutrophils by YC-1 depends partially on cGMP. This discrepancy may be explained by species differences, because their experiments were performed on rat neutrophils. Clearly, although large amounts of cGMP could be produced by the combination of an sGC activator and an NO donor, the biological functions of cGMP in human neutrophils still need to be investigated.

Many reports have established that YC-1 exhibits some additional effects that do not involve the activation of sGC. For example, YC-1 led to an inotropic effect in ventricular myocardium (Wegener et al., 1997), a stimulation of NO synthesis and its release in endothelial cells (Wohlfart et al., 1999), and a rise in apoptosis in adrenomedullary endothelial cells (Ferrero and Torres, 2001), all of which processes are independent of the activation of sGC/cGMP pathways. Unfortunately, there additional actions of this compound have not yet been reliably explained.

**TABLE 1**  
Effects of YC-1 and rolipram, with and without PGE<sub>1</sub>, on the peak  $[Ca^{2+}]_i$  and the time taken for this concentration to decline to half of its peak value ( $t_{1/2}$ ) in FMLP-activated neutrophils  
Neutrophils labeled with fluo-3 AM as described under *Materials and Methods* were stimulated with 0.1  $\mu$ M FMLP in the presence of 1 mM  $Ca^{2+}$ , and fluorescence was monitored at 37°C with stirring. All data are expressed as mean  $\pm$  S.E.M.

Drug	Peak $[Ca^{2+}]_i$	$t_{1/2}$
	nM	s
Control	366.00 $\pm$ 19.66	30.17 $\pm$ 0.87
YC-1 3 $\mu$ M	348.67 $\pm$ 24.33	27.20 $\pm$ 1.07*
YC-1 10 $\mu$ M	359.00 $\pm$ 19.29	19.60 $\pm$ 0.99**
Rolipram 1 $\mu$ M	348.00 $\pm$ 21.36	26.40 $\pm$ 0.31*
PGE <sub>1</sub> 0.03 $\mu$ M	362.00 $\pm$ 20.13	29.80 $\pm$ 0.64
PGE <sub>1</sub> 0.1 $\mu$ M	352.00 $\pm$ 25.89	22.67 $\pm$ 1.54*
YC-1 3 $\mu$ M + PGE <sub>1</sub> 0.03 $\mu$ M	361.00 $\pm$ 13.43	18.77 $\pm$ 1.13**
Rolipram 1 $\mu$ M + PGE <sub>1</sub> 0.03 $\mu$ M	362.33 $\pm$ 25.64	18.23 $\pm$ 0.96**

\* $P < 0.05$ , and \*\* $P < 0.01$  compared with the control.



**Fig. 7.** Effects of YC-1 and cAMP on FMLP-induced p47<sup>phox</sup> membrane translocation. Neutrophils were incubated with DMSO (lanes 2 and 3), YC-1 (1, 3, and 10  $\mu$ M) (lanes 4–6), or dibutyryl-cAMP (cAMP, 1 mM) (lane 7) for 5 min and then stimulated with or without FMLP for another 3 min. Cells were also rested (lane 1) for a total 10-min reaction time at 4°C. Analysis was performed as described under *Materials and Methods*. The result shown is representative of three separate experiments.

The increase in cellular cAMP concentration is associated with a suppression of several neutrophil functions including respiratory burst and exocytosis (Coles et al., 2002). The results are in line with previous findings that various cAMP-elevating agents can suppress the  $O_2^-$  generation and the  $\beta$ -glucuronidase release stimulated by FMLP. YC-1 increased the cAMP level and PKA activity in human neutrophils. Furthermore, two structurally different PKA inhibitors, H89 and KT5720, significantly restored YC-1-induced inhibition. These results indicate that PKA mediates the inhibition of respiratory burst and degranulation by YC-1. YC-1 synergistically raised cAMP concentrations in the presence of PGE<sub>1</sub> or forskolin, but failed to change cAMP levels in the presence of IBMX, suggesting that YC-1 inhibits the breakdown of cAMP by IBMX-sensitive PDEs. Galle et al. (1999) showed that YC-1 inhibits the cAMP-hydrolyzing PDEs, cGMP-inhibited PDE (PDE3) and cAMP-specific PDE (PDE4). However, a role for PDE3 was excluded, because even the formation of large amounts of cGMP by the combination of YC-1 and SNP failed to change the cAMP level. Indeed, human neutrophils contain only two major cytosolic isoforms of PDE, subtypes PDE4 (cAMP-specific) and PDE5 (cGMP-specific) (Schudt et al., 1991).

YC-1 did not inhibit the PMA-activated  $O_2^-$  release by neutrophils, suggesting that YC-1 may inhibit signaling upstream of protein kinase C. cAMP inhibition of FMLP- but not PMA-induced  $O_2^-$  generation by neutrophils has been reported elsewhere and is confirmed here (Sedgwick et al., 1985). YC-1, rolipram, or PGE<sub>1</sub> did not alter the FMLP-induced peak  $Ca^{2+}$  but did accelerate the resequestration of cytosolic  $Ca^{2+}$ , consistent with previous findings that cAMP increases the clearance of  $Ca^{2+}$  from the cytosol (Tintinger et al., 2001).

In summary, this study shows that 1) YC-1 inhibits respiratory burst and degranulation by human neutrophils through a cAMP/PKA-dependent pathway; 2) cAMP/PKA, but not cGMP/PKG, is importantly involved in regulating respiratory burst and degranulation in FMLP-activated human neutrophils; and 3) the biological function of cGMP by the combination of YC-1 and an NO donor in human neutrophils remains to be investigated.

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