Mechanism of YC-1-Induced Activation of Soluble Guanylyl Cyclase

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ABSTRACT
The signaling molecule nitric oxide (NO) mediates many of its effects by the stimulation of soluble guanylyl cyclase (sGC). The activation process is initiated by high-affinity binding of NO to the enzyme’s prosthetic heme group. Despite its poor sGC-activating properties, carbon monoxide (CO) has also been suggested as a physiological activator of sGC. Recently, we have shown that the substance YC-1, a benzyl indazole derivative, stimulates sGC by 10-fold (independently of NO) potentiates the stimulatory effect of NO, and turns CO into a potent activator of sGC. In the present study, we show that activation of sGC by protoporphyrin IX, a ligand-independent activator, was potentiated by YC-1, yet a shift of the concentration-response curve as seen with NO and CO was not observed. YC-1 slowed down the dissociation rates for NO and CO from the activated enzyme as monitored by cGMP accumulation after addition of the NO and CO scavenger oxyhemoglobin. A direct interaction of YC-1 with the heme group can be ruled out because YC-1 did not change the Soret absorption of basal or stimulated sGC and, in addition, still bond to the heme-depleted enzyme. Together, our results indicate that YC-1 increases the maximal catalytic rate and sensitizes the enzyme toward its gaseous activators by binding to an allosteric site on sGC molecules, thereby reducing the ligand dissociation rate from the heme group.

The intra- and intercellular signal molecule NO has been implicated in a wide range of physiological functions, including neurotransmission, vasorelaxation, and inhibition of platelet aggregation (Ignarro et al., 1987; Garthwaite et al., 1988; Moncada and Higgs, 1995). Many of the effects of NO are mediated by the enzyme sGC (Waldman and Murad, 1987; Garbers and Lowe, 1994). By synthesis of cGMP, sGC produces an intracellular messenger molecule that is able to exert a variety of effects depending on the cGMP-dependent effector system(s) (e.g., cGMP-dependent kinases, cGMP-regulated phosphodiesterases, cGMP-gated channels) present in a given cell type. The formation of cGMP can also be catalyzed by membrane-bound GCs that belong to the group of receptor-linked enzymes and are regulated by different peptide hormones.

In contrast to the membrane-bound GC possessing a homomorphic structure, sGC consists of two different subunits and contains a prosthetic heme group that mediates the up-to-400-fold activation by NO (Humbert et al., 1990; Stone and Marletta, 1996). NO-induced activation is thought to proceed via binding of NO to the heme iron, breaking of the His-Fe bond, and subsequent conformational change of the enzyme. In accordance with this mechanism of activation, PP-IX, the iron-free precursor of heme, stimulates sGC independently of NO (Ignarro et al., 1982; Ignarro et al., 1984).

Another gaseous molecule, CO, has been discussed as a putative activator of sGC. CO has been assumed to participate in long-term potentiation (Zhuo et al., 1993; Stevens and Wang, 1993), olfactory signal transduction (Verma et al., 1993; Leinders-Zufall et al., 1995; Ingi and Ronnett, 1995), and vasorelaxation (Utz and Ullrich, 1991; Morita et al., 1995; Zakhary et al., 1996). Yet the proposal of CO as a physiological activator of sGC is opposed by the rather poor sGC-stimulatory properties of CO (Brüne and Ullrich, 1987; Stone and Marletta, 1994).

Recently, we were able to show that the new substance YC-1, a benzyl indazole derivative, turns CO into a potent activator of sGC (Friebe et al., 1996). In the presence of this substance, CO led to a 100-fold increase in enzyme activity, which is comparable to the stimulatory effect induced by NO. YC-1, which had been identified as an inhibitor of platelet aggregation (Ko et al., 1994; Wu et al., 1995), led to a ~10-fold activation of the nonstimulated, purified enzyme and potentiated NO- and CO-induced stimulation. In addition to an increase in maximal activity, YC-1 led to a leftward shift of the concentration-response curve. Recently, cGMP-increasing effects of YC-1 have been reported in smooth muscle cells and an increase in responsiveness toward NO has been dem-

ABBREVIATIONS: NO, nitric oxide; sGC, soluble guanylyl cyclase; CO, carbon monoxide; PP-IX, protoporphyrin IX; DEA-NO, 2,2-diethyl-1-nitroso-oxyhydrzone; oxyHb, oxyhemoglobin.
onstrated (Mülsch et al., 1997). In general, modulation of sGC sensitivity toward NO and CO implies important pharmacological and physiological functions.

This study was performed to further elucidate the mechanism of YC-1 action. YC-1 did not alter the Soret absorption of sGC, which adds to the argument against a direct interaction of YC-1 with the enzyme’s prosthetic heme group. Our data demonstrate that YC-1 binds independently of the activation state of the enzyme and even to the heme-deficient enzyme, which indicates an allosteric site. We show that although YC-1 increases PP-IX-induced sGC activity, it does not shift the concentration-response curve as it does for the gaseous ligands. Additional results suggest a reduction of the dissociation rate of the heme ligand as the underlying mechanism of the YC-1-induced sensitization of sGC.

Materials and Methods

Purification of soluble guanylyl cyclase and determination of guanylyl cyclase activity. sGC was purified from bovine lung to apparent homogeneity by an immunoaffinity purification procedure as described previously (Humbert et al., 1990). Cyclase activity was measured by the conversion of \( [\alpha-\text{32P}] \)GTP to \( [\text{32P}] \)cGMP at 37° for 10 min. Reaction mixtures contained 3 mM Mg\(^{2+}\) as divalent metal ion, 3 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 1 mM cGMP, 300 \( \mu \)M GTP and 50 mM triethanolamine hydrochloride, pH 7.4, in a total volume of 0.1 ml. Reactions were stopped by ZnCO\(_3\) precipitation, and \( [\text{32P}] \)cGMP was isolated as described (Schultz and Böhme, 1984). All measurements were performed in duplicates and repeated at least three times.

YC-1 was dissolved in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in all samples did not exceed 2% (v/v), a concentration that, by itself, did not influence sGC activity.

Synthesis of oxyhemoglobin. Oxyhemoglobin was prepared in 50 mM triethanolamine-HCl, pH 7.0, by reducing bovine methemoglobin with sodium dithionite. Subsequently, reduced hemoglobin was desalted by passing over a Sephadex G-25 (PD-10) column (Pharmacia, Freiburg, Germany). The concentration of oxyhemoglobin was determined photometrically.

Materials. YC-1 [3-(5-*β*-hydroxymethyl-2-*β*-furyl)-1-benzyl indazole] was a generous gift from Bayer (Wuppertal, Germany). DEA-NO was purchased from Research Biochemicals (Natick, MA). Hemoglobin and PP-IX were obtained from Sigma, and Tween 20 was purchased from Boehringer Mannheim (Mannheim, Germany). \( [\alpha-\text{32P}] \)GTP (800 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA). CO gas (100% as well as 1000 parts per million in \( \text{N}_2 \)) was from AGA Gas, Berlin, Germany.

Results

We previously demonstrated that YC-1 shifts the concentration-response curve for NO and CO to the left. This shift of the \( EC_{50} \) indicates an increase in affinity of the gaseous ligands to the enzyme’s prosthetic heme group. Here, we investigated whether YC-1 was able to also shift the concentration-response curve for PP-IX. The iron-free heme precursor stimulates sGC independent of a gaseous ligand. Fig. 1 shows that the concentration-dependent activation of sGC by PP-IX was potentiated by YC-1, yielding a maximal increase in enzyme activity of 360%. However, a leftward shift of the \( EC_{50} \) could not be detected (Fig. 1, inset).

As shown earlier (Friebe et al., 1996), YC-1 activates sGC by an NO-independent but heme-dependent mechanism. To determine whether YC-1 binds to the prosthetic heme group, we recorded UV-visual spectra of sGC under nonstimulated conditions.

![Fig. 1. Potentiation of PP-IX-induced sGC stimulation by YC-1. Increasing concentrations of PP-IX were applied in the absence (○) or presence (●) of 200 \( \mu \)M YC-1. For better illustration of the unchanged \( EC_{50} \), the inset shows the percentage of maximal stimulation. Data are mean ± standard deviation from three independent experiments.](https://doi.org/10.1002/mol.11900)

![Fig. 2. UV-visual spectra of sGC in the presence and absence of YC-1. The absorbance of the Soret band of sGC (8.4 \( \mu \)g) was recorded using a Cary 1E spectrophotometer. Enzyme was diluted in 50 mM triethanolamine-HCl, pH 7.4; the YC-1 concentration was 500 \( \mu \)M. The CO-stimulated state of sGC was achieved by bubbling with 100% CO gas. Similarly, YC-1 did not change the Soret absorption of NO-stimulated sGC (not shown).](https://doi.org/10.1002/mol.11900)
and stimulated conditions (Fig. 2). The presence of YC-1 resulted in no change of the Soret band of either the non-stimulated (431 nm), the CO-stimulated (423 nm), or the NO-stimulated enzyme (398 nm; not shown). Hence, it is unlikely that YC-1 binds to the prosthetic heme group of the enzyme.

Next, we intended to explore whether YC-1 binding requires the presence of the heme group or the activated state of sGC or, alternatively, whether YC-1 binds to the nonactivated or even heme-depleted form of the enzyme. Because of the low affinity of YC-1 for sGC, we had to investigate binding of YC-1 by monitoring enzyme activity, taking advantage of YC-1’s slow dissociation under NO-stimulated conditions (Table 1). Preincubation of sGC with 100 μM YC-1 under stimulated conditions (1 μM DEA-NO) in the absence of substrate and subsequent 5-fold dilution showed an increase in enzyme activity caused by the preincubation. This increasing effect of YC-1 preincubation can only be explained by the slow dissociation of YC-1 from sGC upon dilution.

Next, we preincubated sGC with 100 μM YC-1 under nonstimulated conditions, diluted the enzyme, and, subsequently, detected bound YC-1 under stimulated conditions (0.2 μM DEA-NO) (Fig. 3A). Preincubation with YC-1 led to a 27% increase in NO-stimulated cGMP production compared with control (i.e., preincubation with buffer alone). Thus, YC-1 also binds to nonactivated sGC molecules.

To find out whether the heme group is required for YC-1 binding, we performed the same set of experiments using heme-depleted sGC (Fig. 3B). In previous reports, we showed that 0.5% Tween 20 leads to the removal of heme from the enzyme. Heme-deficient sGC still has basal activity and can be stimulated by PP-IX (Foerster et al., 1996; Friebe et al., 1997). Because YC-1 does not lead to activation of the heme-deficient sGC, we had to stimulate the enzyme with PP-IX and detected bound YC-1 by its potentiating effect. Fig. 3B shows that YC-1 preincubation of the heme-depleted enzyme resulted in an 33% increase in cGMP production. Taken together, YC-1 preincubation resulted in an increase in sGC activity regardless of the state of the enzyme (activated, nonactivated, or heme-depleted). These results reinforce the spectral data in Fig. 2, and indicate that YC-1 binds to an allosteric site on sGC.

Next, we performed experiments to explain the observed YC-1-induced increase in affinity toward NO and CO. We hypothesized a YC-1-mediated decrease in the dissociation rate for the gaseous ligands and investigated the effect of YC-1 on the dissociation of NO from the heme group of sGC by monitoring cGMP forming activity in the presence of oxyHb, a scavenger of both NO and CO. Fig. 4 shows the accumulation of cGMP over a time range of 10 min. After 3 min of incubation, either buffer, oxyHb, or the nonionic detergent Tween 20 were added and incubation was continued for another 7 min. As stated above, Tween 20 removes the heme from sGC (Foerster et al., 1996; Friebe et al., 1997). In the absence of YC-1, neither oxyHb nor Tween 20 had a significant influence on nonstimulated cGMP synthesis (Fig. 4A, open symbols, inset). NO-stimulated cGMP synthesis (10 μM DEA-NO; Fig. 4A, closed symbols) was reduced to basal levels by both oxyHb and Tween 20.

Presence of YC-1 (200 μM; Fig. 4B) led to a 10-fold increase in non-stimulated cGMP production that was not affected by oxyHb. As we have shown before (Friebe et al., 1996), removal of the prosthetic heme group by Tween 20 results in the abolishment of YC-1 stimulation. Under stimulated conditions (10 μM DEA-NO; Fig. 4B, closed symbols) and in the

| TABLE 1 |
| Effect of YC-1 preincubation on sGC. |

<table>
<thead>
<tr>
<th>YC-1 Preincubiation</th>
<th>Enzyme activity (nmol cGMP · min⁻¹ · mg⁻¹)</th>
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<td>0 20</td>
<td>2880</td>
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<td>0 100</td>
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Fig. 3. Increasing effect of YC-1 preincubation on sGC activity. Purified sGC (0.1 μg) was preincubated for 3 min at 37°C with either buffer (control; □) or YC-1 (100 μM; ■) in the absence or presence of activator. A, After 3 min preincubation in the absence or presence of DEA-NO (1 μM), sGC was diluted 5-fold with incubation buffer containing substrate. Additional YC-1 and/or activator was added, yielding final concentrations of 20 μM YC-1 and 0.2 μM DEA-NO. sGC activity was then determined during 10 min at 37°C. B, sGC was heme-depleted (0.5% Tween 20) and then treated as described in A using 0.5 μM PP-IX in the preincubination instead of DEA-NO. In contrast to A, sGC was diluted 10-fold with final concentrations of 10 μM YC-1 and 0.05 μM PP-IX during incubation. sGC activity without YC-1 preincubation (but with the indicated final concentrations of YC-1 and activator) was taken as 100%. ■, increases in enzyme activity induced by YC-1 preincubation. Data are mean ± standard deviation from three independent experiments.
presence of YC-1, oxyHb was not able to completely reverse NO stimulation as observed in the absence of YC-1 (Fig. 4A). Because oxyHb was used in large molar excess over NO, we conclude a decreased dissociation of NO from sGC as the reason for the diminished inhibition by oxyHb.

Similar experiments were performed using CO instead of NO. As CO stimulation of sGC in the absence of YC-1 is only marginal, these experiments were only carried out in the presence of YC-1. Moreover, the dissociation rate of CO from sGC has been reported to be very fast (Kharitonov et al., 1995), and therefore one would expect immediate inhibition of CO-induced stimulation by oxyHb. As can be seen in Fig. 5 in the presence of YC-1, submaximally effective CO (1000 parts per million) led to a linear increase in cGMP formed over 10 min. Addition of Tween 20 after 3 min abolished stimulation, resulting in basal increase in cGMP formation for the remaining 7 min. As seen with NO, administration of oxyHb led to a partial inhibition of CO-stimulated cGMP synthesis but did not abolish the stimulatory effect, which indicates a reduced off-rate not only for NO but also for CO.

**Discussion**

The novel compound YC-1 sensitizes sGC for its physiological activator NO and also turns CO into a potent activator (Friebe et al., 1996). Both findings have potential pharmacotherapeutic and physiological implications. Here, we further investigated the effect of YC-1 and its mechanism of action. By spectrophotometric analysis, we studied the possible interaction of YC-1 with the prosthetic heme group of sGC. As YC-1 exerts its influence, especially on the activated form of the enzyme, spectra were also recorded in the presence of NO and CO. Although both gases bind to the sixth position of the heme iron, only binding of NO results in the formation of a five-coordinated complex by breaking of the His-Fe bond. Binding of CO is thought to result in a six-coordinated heme with the His-Fe bond remaining intact. In the light of the highly stimulatory properties of CO in the presence of YC-1, it was tempting to speculate on the formation of a five-coordinated heme similar to that induced by NO, reflecting a single activated state of sGC molecules. Nevertheless, we were not able to detect any YC-1-induced changes in absorbance of the heme (431 nm) or CO-stimulated (423 nm) enzyme, a finding that argues against an interaction of YC-1 with the heme group.

We wanted to define further the binding requirements for YC-1. YC-1 dilution experiments, originally performed to study the reversibility of the YC-1 action, revealed a relatively slow dissociation of YC-1 from sGC. To find out whether YC-1 bound to the nonactivated enzyme, we incubated sGC with YC-1 and detected bound YC-1 after dilution by determination of enzyme activity under stimulated conditions. Preincubation with YC-1 and subsequent dilution resulted in an elevation of enzyme activity. Even when we used a heme-depleted enzyme, YC-1 preincubation induced an
increase in catalytic rate. These results indicate that YC-1 binds to sGC independent of its activation state and also independent of the heme group. These results are reinforced by the spectral data in Fig. 2.

Although YC-1 shifted the concentration-response curve for NO and CO to the left, it failed to do so for PP-IX. However, potentiation of PP-IX-stimulation (360%) was still very pronounced. As PP-IX activates the enzyme independently of a gaseous ligand, we conclude that YC-1 indeed exerts two effects on sGC: first, it potentiates the action of different activators, increasing $V_{\text{max}}$ by a still unknown mechanism. Second, YC-1 changes the affinity of the gaseous activators.

A leftward shift of the EC$_{50}$ could be caused by a reduction of the dissociation rate of NO from the heme. Therefore, we investigated the effect of YC-1 on the dissociation rate of NO with the help of the NO scavenger oxyHb. The results are summarized in Fig. 4. In the absence of YC-1, NO activation of sGC is immediately abolished by the addition of oxyHb. Because oxyHb is supposed to react only with free NO, our results suggest that during NO stimulation, NO shuttles on and off the enzyme. In contrast, in the presence of YC-1, addition of oxyHb decreased NO-stimulated enzyme activity only slowly. The immediate reduction of NO-stimulated cGMP accumulation to basal level after the removal of the prosthetic heme group by Tween 20 underlines the prerequisite of the heme group for the YC-1 effect.

Similar to NO, CO has a very high affinity for heme groups, yet the off-rate of CO from heme, in general considered to be much faster than that of NO, has been shown to be exceptionally high for sGC (Kharitonov et al., 1995; Stone and Marletta, 1996). Thus, the fast dissociation of CO from the heme could account for the low stimulatory properties of CO. Obviously, YC-1 dramatically reduces the CO dissociation rate, as the CO scavenger oxyHb only leads to partial reversals in enzyme stimulation. We conclude that reduction of the dissociation rate of both gaseous ligands, NO and CO, represents part of the underlying mechanism of YC-1 action.

Taken together, YC-1 enhances the activity of the stimulated enzyme independently of the type of activator used and increases the affinity for heme ligands by reduction of their dissociation rates. Because of the lack of change in absorbance spectra, a direct interaction of YC-1 with the heme group of sGC is unlikely and, because YC-1 also binds to the heme-deficient sGC, we conclude that YC-1 binds to an allosteric site on sGC. Further experiments have to show whether this allosteric site can be used by physiologically or pharmacologically relevant compounds.

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References


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